18SrDNA Phylogeny of the Green Algae with Evaluation of Morphological Characters

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CONTENTS

SUMMARY

Phylogenetic relationships of the green algae were inferred based on the comparisons of 18SrDNA sequences. The ultrastructure of the cell including the flagellar apparatus was also investigated for some selected algae. From the results of various analyses and evaluation of morphological characters, the following conclusions were obtained.

1. Phylogeny of the green plants in the eukaryotes

1) Green plants (green algae and land plants) form a monophyletic lineage in the eukaryotes. 2) A heterotrophic flagellate, *Cercomonas*, is closely related to the Chlorarachniophyta.

2. Phylogeny within the green plants

1) The green algae (Chlorophyta *sensu lato*) are not monophyletic, but consist of two major clades (Streptophyta and Chlorophyta *sensu stricto*). 2) The Prasinophyceae, which has been suggested as a "primitive" group of the green algae, is not monophyletic, but divided into five independent clades. 3) In the "Prasinophyceae", *Mesostigma* is a possible relative of the Streptophyta, and other members have affinities to the Chlorophyta *sensu stricto*. 4) The Tetraselmidales is probably a primitive member of the Chlorophyta *sensu stricto*, and the genus *Pyramimonas*, which is alternatively suggested to be another "ancestor" of this division, is not closely related. 5) Ancestral green algae would have had two laterally inserted flagella, a covering of square scales and asymmetrical flagellar apparatus with MLS.

3. Phylogeny within the Prasinophyceae

1) *Crustomastix didyma* gen. et. sp. nov. represents the most "primitive" mamiellalean alga. 2) The simple cell architecture seen in most members of the Mamiellales is not primitive but is derived condition characteristically evolved in this order. 3) In the Pyramimonadales, the genus *Pyramimonas* have gained the homoplasous characters to the Chlorophyta *sensu stricto* (convergence) such as cruciate flagellar apparatus and swimming with ciliary beat.

4. Phylogeny of the Chlorophyta.

1) In the Chlorophyta *sensu stricto*, the Tetraselmidales represents the first divergence,

and simultaneous radiation of the Trebouxiophyceae, Ulvophyceae and Chlorophyceae would have occurred. 2) *Oltmannsiellopsis* is one of independent lineages in the Chlorophyta *sensu stricto*. 3) *Hafniomonas* is not a primitive member of the Chlorophyceae, but is suggested to be assigned to the CW group. 4) In the Chlorophyceae, four independent clades, the Chaetopeltidales, Chaetophorales, Sphaeropleales and CW group, are recognized.

5. Phylogeny within the Ulvophyceae

1) The Ulvophyceae *sensu lato* is divided into two distinct lineages, the Ulvophyceae *sensu stricto* and Siphonocladales-Dasycladales clade. 2) *Halochlorococcum* has sister group relationship to the Ulvales. 9) The Prasiolales is probably a member of the Trebouxiophyceae.

6. Phylogeny of the Chlamydomonadales and Chlorococcales

1) Either the Chlamydomonadales or Chlorococcales *sensu stricto* is not monophyletic, and these two orders should be merged into a single order. 2) The presence or absence of walled motile cells, number of nucleus and vegetative organization are not informative characters for the classification at class or order level.

Based on these evidences, a classification system of the green algae including five new classes and four new orders is proposed.

GENERAL INTRODUCTION

What are the green algae and green plants?

The green algae is one of the major algal groups and displays a great diversity in organization and habitat. The organisms show a wide range in body construction - unicellular, colonial, filamentous, membranous and siphonous (see below). They live in almost all habitats: freshwater, blackish water, seawater, soil, subaerial, snow and desert.

The green algae and land plants share the chloroplasts surrounded by two membranes, chlorophyll *b* as major accessory photosynthetic pigment, stacked thylakoids, storage of a-1,4-linked glucans, mitochondria with flattened cristae and smooth flagella lacking hairs. These characters may not be synapomorphic, but the combination of these components has never been seen in any other eukaryotes. Furthermore, there are some characters unique only to green algae and land plants (synapomorphic characters); storage of true starch within the chloroplast and the presence of the stellate structure in the flagellar transitional region (Mattox & Stewart 1984; Bremer 1985; Mishler & Churchill 1985). Based on these features, it has been well accepted that they form a monophyletic lineage, and that land plants would have evolved from a green algal ancestor. Cavalier-Smith (1981) established a new subkingdom, Viridiplantae, in the kingdom Plantae for this monophyletic clade. In this thesis, therefore, the terms "green plants" or the Viridiplantae are applied to this monophyletic group, including the green algae and land plants.

Traditional classifications of the green algae

As mentioned above, green algae are thought to form a natural assemblage with land plants. However, green algae and land plants are usually treated as separate taxa, because they differ in organizational level, i.e., green algae have "simpler" organization, and only the land plants produce embryos in the life cycle. The green algae are generally classified into a single division, the Chlorophyta, and have traditionally been classified mainly based on morphology of the vegetative stage - such as flagellate, coccoid, filamentous and siphonous forms. As an example, the classification system by Fritsch (1935) is given bellow. Based on the level of organization, he classified all green algae into nine orders of a single class, the Chlorophyceae.

Class Chlorophyceae

Volvocales: unicellular or colonial, flagellate or palmelloid easily revert to a motile condition.

Chlorococcales: unicellular or colonial, motionless in the vegetative condition (coccoid).

Ulotrichales: simple unbranched filamentous or membranous fronds.

Cladophorales: simple or much branched filament with multinucleate cells.

Chaetophorales: heterotrichous, branched filament.

Oedogoniales: simple or branched filament; multiflagellate zoospore; oogamous.

Conjugales: unicellular, colonial or filamentous; sexual reproduction by conjugation of non-motile gametes.

Siphonales: siphonous; coenocytic thallus without septa.

Charales: thallus markedly differentiated, with whorled arrangement of laterals of limited growth and segregation into nodes and internodes; sexual reproduction with oogonia and antheridia.

The traditional classification systems proposed by other phycologists (e.g. Fott 1971, Bold & Wynne 1985) are basically identical with that of Fritsch (1931). These systems reflect a phylogenetic view that the organization level of plant has changed from simple to complex. Early phycologists considered that unicellular flagellate chlorophytes (such as *Chlamydomonas*) are primitive, and evolved to coccoid, sarcinoid, filamentous, siphonous forms and parenchymatous multicellular organisms (land plants) (Blackman 1900, Pascher 1914).

New classification systems of the green algae

Accumulation of electron microscopical studies on green algal cells since 1960's have led phycologists to reconsideration of traditional classification systems and phylogenetic hypotheses of the green algae. By the middle of 1970's, ultrastructural studies led to recognition of two distinct phylogenetic lineages of green algae, the Charophyceae and Chlorophyceae (*sensu* Stewart and Mattox 1975, not *sensu* Mattox and Stewart 1984, see below) (Pickett-Heaps and Marchant 1972; Pickett-Heaps 1975; Stewart and Mattox 1975).

The Charophyceae is characterized by producing biflagellate cells with lateral flagellar insertion, and asymmetrical flagellar apparatus consisting of two microtubular roots

(Sluiman 1983) which are associated with only one basal body (termed no.1 basal body; Melkonian *et al.* 1987). One microtubular root (termed 1d root; d = dextrosum, right in latin; Moestrup and Hori 1989) is comprised of many microtubules (spline) and is associated with multilayered structure (MLS; it is reduced to amorphous structure in the Charales) (Turner 1968; Pickett-Heaps and Marchant 1972; Marchant *et al.* 1973; Moestrup 1974; Graham and McBride 1979; Rogers *et al.* 1980; Sluiman 1983). The Charophyceae is also characterized by the open mitotic spindle, distantly separated telophase nuclei, the oxidation of glycolate by glycolate oxidase located in microbody (peroxysome), the Cu/Zn superoxide dismutase and rosette-like complex of cellulose synthase (Frederik *et al.* 1973; Pickett-Heaps 1975; De Jesus *et al.* 1989; Okuda and Brown 1992). The well-known green algal group, the Zygnematales, never produces flagellate cells, but has the same mitotic and biochemical characters as the Charophyceae such as those mentioned above (but some members have semiclosed spindle; see Bakker and Lokhorst 1987). Thus, the assignment of the Zygnematales in the charophycean line is widely accepted. The land plants also share characters of the flagellate cell and the mitotic and biochemical features with the Charophyceae. Furthermore, a unique cytokinetic structure of the land plants, the phragmoplast, is present in some charophycean algae (*Coleochaete* and Charales). So, a close phylogenetic relationship is supposed between the charophycean green algae and land plants, and an evolutionary route from the former to the latter is also considered.

In contrast to the Charophyceae, the Chlorophyceae *sensu* Stewart and Mattox (1975) have flagellate cells with apical flagellar insertion and a cruciate microtubular root system which shows 180° rotational symmetry (Moestrup 1978; Floyd et al 1980). After 1975, variations were found in the flagellar apparatus, mitosis and cytokinesis in the Chlorophyceae *sensu* Stewart and Mattox (1975). *Chlamydomonas* and most other freshwater green algae have a mitotic spindle which collapses at the telophase, and have a plate of microtubules lying in the plane of cell division. The latter is called the phycoplast, and thought to steer the cytokinesis. In contrast, most marine green algae have a persistent interzonal spindle and develop neither the phragmoplast nor the phycoplast during the cytokinesis (but see Sluiman 1991). Mattox and Stewart (1978) classified these two groups as separate classes, the Chlorophyceae (*sensu* Mattox and Stewart 1978 or Stewart and Mattox 1984; In this thesis, hereafter, I use the term Chlorophyceae in this sense) and the Ulvophyceae (originally spelled Ulvaphyceae), respectively. The differences were also found in the absolute orientation of basal bodies in the flagellate cells between these two

classes (Roberts *et al.* 1982; Melkonian and Berns 1983; O'Kelly and Floyd 1983, 1984a). The arrangement of the basal bodies in the Chlorophyceae, when viewed from above, represents a clockwise (or 1/7 o'clock; Melkonian and Berns 1983) rotation, whereas, in the Ulvophyceae, basal bodies are proximally overlapped and displaced in counterclockwise (or 11/5 o'clock) orientation. The different type of basal body orientation was recognized recently (e.g. Wilcox and Floyd 1988; Watanabe and Floyd 1989a); basal bodies are directly opposed (12/6 o'clock) without showing displacement. The green algae with this type of basal bodies in flagellate cell stage are classified in the Chlorophyceae (e.g. Deason *et al.* 1991).

Despite various differences mentioned above, the Chlorophyceae and Ulvophyceae are considered to share a common ancestor, because they produce flagellate cells displaying basically the same architecture, the cruciate root system and 180° rotational symmetry (Pickett-Heaps 1975; Stewart and Mattox 1975, 1978; Melkonian 1982; Mattox and Stewart 1984; O'Kelly and Floyd 1984a).

The flagellar and cytokinetic apparatuses, which are probably evolutionarily independent from each other, are uniform and distinct in each of the Charophyceae, Ulvophyceae and Chlorophyceae. Therefore, the classification based on ultrastructural features is considered to reflect phylogenetic relationships within the green algae more properly than that based on vegetative morphology (traditional classification). Each of three classes recognized by ultrastructural studies includes the members that possess various levels of organization. For example, unbranched filamentous green algae, traditionally classified as a single genus *Ulothrix*, are now divided into three genera (*Klebsormidium*, *Ulothrix* and *Uronema*) and these are classified in three different classes, the Charophyceae, Ulvophyceae and Chlorophyceae, respectively (e.g. Lokhorst 1985).

Mattox and Stewart (1984) reviewed new classification systems and phylogenetic hypotheses of the green algae and erected two more classes, the Pleurastrophyceae and Micromonadophyceae. The Pleurastrophyceae seemed to be intermediate group between the Ulvophyceae and Chlorophyceae, because the algae assigned to this class had, on one hand, flagellate cells with basal bodies displaced in counterclockwise direction as in the Ulvophyceae, and, on the other hand, had a collapsing telophase spindle and phycoplast as in the Chlorophyceae. Because the Pleurastrophyceae has flagellate cell with apical flagellar insertion and a cruciate microtubular root system, it would belong to the evolutionary line including the Ulvophyceae and Chlorophyceae, so that it would represents an intermediate

clade between these two classes (Mattox and Stewart 1984; Bremer 1985; Mishler and Churchill 1985). Mattox and Stewart (1984) included the Tetraselmidales in this class, which had been usually regarded as a member of the Prasinophyceae (see below). As the Prasinophyceae was named after a tetraselmidalean genus, *Prasinocladus* (now merged with *Tetraselmis*; Norris *et al.* 1980), Mattox and Stewart (1984) created a new class, the Micromonadophyceae, for the remainder of the Prasinophyceae (but see Moestrup and Throndsen 1988; Sym and Pienaar 1993). However, this classification was criticized, because the Tetraselmidales and the other members of the Pleurastrophyceae shared little resemblance (e.g. Melkonian 1984; Sym and Pienaar 1993). In recent articles, the Tetraselmidales is usually classified as a member of the Prasinophyceae, and other pleurastrophytes are placed in the Microthamniales (Melkonian 1982b; 1990c) or the Trebouxiophyceae (Friedl 1995) (see Chapter 4).

As mentioned above, comparative ultrastructural studies led to recognition of two principle evolutionary lineages in the green plants (Charophyceae/land plants and Ulvophyceae/Trebouxiophyceae/Chlorophyceae). Of course, phycologists have made effort to find out the ancestral members of the green plants, and they have reached to the Prasinophyceae as possible candidate of such organisms. The Prasinophyceae was originally established by Christensen (1962) for the green algae with anterior depression from which flagella emerged. Through many ultrastructural studies (e.g. Moestrup and Ettl 1979; Norris 1980; Salisbury *et al.* 1981; Moestrup 1983, 1984; Melkonian 1984, 1989; Inouye *et al.* 1985, 1990; Moestrup and Throndsen 1988; Sym and Pienaar 1993), the Prasinophyceae is now assigned to the green flagellates having characters such as follows: 1) cell body and flagella are covered by organic scales, 2) basal bodies are parallelly arranged, 3) tubular flagellar hairs (hair-scales) are present in two opposite rows along the flagella, 4) the flagella are inserted in a depression or groove, 5) location of Golgi bodies is parabasal (close to the basal bodies) (Melkonian 1990a). Because some members of the Charophyceae, Ulvophyceae and Chlorophyceae have flagellate cells covered by a layer of organic-scales, the ancestral green algae were considered to be scaly flagellates (e.g. Pickett-Heaps 1975; Stewart and Mattox 1975; Moestrup 1978; Melkonian 1982a). The Prasinophyceae agrees with this hypothetical ancestor of green algae, and has been regarded as the most "primitive" group of the green algae and as "ancestral stock" from which other green algal (and land plants') lineages may have diversified (e.g. Moestrup 1982; Mattox and Stewart 1984; Melkonian 1984; O'Kelly and Floyd 1984; Melkonian 1989a; Sym and Pienaar 1993).

Molecular phylogenetic approach

Ultrastructural studies on green algal cells have not only given us more natural view on their classification but also suggested a revolutional phylogenetic view. However, various systematic and phylogenetic problems of the green algae still remain to be resolved. For example, because it is difficult to determine the primitive extreme in set of homologous characters, reconstruction of the phylogeny based only on morphological characters is far from confidence. In addition, morphological characters, in some cases, evolved independently (homoplasies). As morphological characters of green algae are usually simple, we easily fail in distinguishing homoplasies from true homologues. Therefore, other approaches are needed for constructing more natural classification system and reliable phylogenetic tree. One of such approaches would be molecular phylogenetic approach.

The molecular phylogenetic approach, especially of DNA sequence analysis, has been developed rapidly for the last decade. Molecular characters have many advantages to infer the phylogenetic relationships. These are, 1) the pattern of character change is relatively uniform so that quantitative and statistical analyses are easily applicable, 2) a large number of characters is easily obtained, 3) recognition of a pair of homologues (homologous site) is easy, and 4) character changes are independent from each other. I think that the most important point of application of the molecular characters for phylogenetic studies is that molecular and morphological characters evolve independently without mutual affection. Therefore, if the same trees are obtained from these two independent approaches, it probably imply that phylogenetic significance of these distinct characters are mutually supported. I agree to the opinion that it is the best approach to analyze these two (or more) types of characters as system of reciprocal illumination. "....truth is approached asymptotically, that is, by testing and retesting in a system of reciprocal illumination" (p.139, Wiley 1981).

Purposes of this study

Among a large number of genes, nuclear-encoded small subunit ribosomal RNA gene (18SrDNA) is widely used to analyze phylogenetic relationships of the eukaryotes, especially protists (e.g. Sogin *et al.* 1986, 1989; Gounderson *et al.* 1987; Schlegel 1991). The 18SrDNA is regarded as a useful molecule in inferring relationships between distantly related taxa, because 1) this gene is sequenced in a large number of species, 2) it has very slow evolutionary rate, 3) it is a relatively large molecule (about 1800 base pairs), 4) it

shows little variation between repeated sequences (Dover and Flavell 1984), and 5) it has highly conserved regions which are usable to design the amplification and sequencing primers. As all eukaryotes have 18SrDNA (homologous gene is also present in all prokaryotes and symbiotic organelles as 16SrDNA) that work for the same function, the phylogeny of the 18SrDNA is fundamentally regarded as that of organisms.

Some phylogenetic studies about green algae based on 18SrDNA (or 18SrRNA) sequences were published in the last few years (Huss and Sogin 1990; Lewis *et al.* 1992; Wilcox *et al.* 1992a,b, 1993; Friedl and Zeltner 1994; Steinkötter *et al.* 1994; Surek *et al.* 1994; Friedl 1995). However, available 18SrDNA sequences data are not enough and restricted to analyse phylogenetic relationships of whole green algae.

In this study, I analyzed 18SrDNA sequences from many green algae to infer the phylogenetic relationships and to test the phylogenetic hypothesis based on morphological characters. I also investigated ultrastructural morphology of some green algae to obtain better interpretation of morphological characters. From these analyses, I proposed the classification system of the green algae that reflects phylogenetic relationships (the genealogical descent) and verified the evolution of morphological characters of green algae.

Monophyly, paraphyly and polyphyly

I use in the present study the phylogenetic terms, monophyly, paraphyly and polyphyly. There are some different definitions for these terms (Wiley 1981). I use Farris (1974) definitions for discussion of phylogenetic relationships of green plants. In some cases, it is difficult to distinguish paraphyly from polyphyly, as both are non-monophyletic, namely, "non-natural" taxon, which is important from taxonomical or phylogenetical view point. Farris's (1974) deffinitions are referred below.

> **Monophyly**: 1, a group that includes a common ancestor and all of its descendants. 2, a group with unique and unreversed group membership characters.
> **Paraphyly**: 1, a group that includes a common ancestor and some but not all of its descendants. 2, a group with unique but reversed group membership characters.
> **Polyphyly**: 1, a group in which the most recent common ancestor is assigned to some other group and not to the group itself. 2, a group whose membership characters are not uniquely derived.

Recognition of monophyly, paraphyly and polyphyly is important for phylogenetic classification, because supraspecific taxon should basically be monophyletic group (but there are some arguments about this matter) (e.g. Wiley 1981). I agree with the opinion of the phylogenetic taxonomy (see Wiley 1981) that supraspecific clasification system should reflect only the best hypothesis concerning the genealogical descent (phylogeny) of organisms, and not include any other "evolutionary phenomena".

GENERAL MATERIALS AND METHODS

MATERIALS

Materials used in this study are listed in Table 1.

METHODS

DNA isolation

I used two methods for extraction of total DNA from materials.

For wall-less organisms (prasinophytes, swamers of some ulvophytes, some chlorophytes etc.), I used extraction buffer containing HTE buffer (50 mM Tris-HCl, 20 mM EDTA, pH 8), N-lauroylsarcosine (20 mg/ml) and proteinase K (200 μg/ml) to extract DNA. Cells harvested by centrifugation were suspended in 400 μl extraction buffer and incubated at room temperature for 1 h. Then, equal volume of phenol saturated with 1M Tris-HCl (pH 8) was added and mixed gently for 10 min. After centrifugation (10000 rpm, 10 min), the upper aqueous phase was removed to a new tube, and equal volume of phenol / CIA (chroloform:isoamyl alchol = 24:1) mixture (1:1) was added and mixed gently for 10 min. The mixture was centrifuged and removed the upper aqueous phase again. Equal volum of CIA was added and mixed, and then centrifuged as before. The upper phase was removed to a new tube, and 15 μl 4M NaCl was added. Total DNA was precipitated with 2.5 vol cold ethanol at -20℃ (1 h to overnight) followed by centrifugation at 14000 rpm for 10 min. The pellet was rinsed in 70% ethanol, air-dried and dissolved in 0.1 - 1 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA).

For walled organisms, UNSET buffer (Garriga *et al.* 1984, Lewis *et al.* 1992) was used for extraction of total DNA. Thalli grounded in liquid nitrogen or cells harvested by centrifugation were lysed in 400 μl UNSET buffer (8 M urea, 2% SDS, 0.15% NaCl, 1 mM EDTA, 100 mM Tris-HCl, pH 7.5). The lysate was mixed vigorously followed by the addition of equal volume of phenol / CIA mixture (see above). The tube was mixed gently for 10 min, and centrifuged at 10000 rpm for 10 min. Equal volume of CIA was added to the upper aqueous phase which was removed to a new tube, and then mixed for 10 min. After centrifugation, the upper phase was removed to a new tube and added 2.5 vol of cold ethanol. Total DNA was precipitated at -20℃ for 1 h to overnight followed by centrifugation

at 14000 rpm for 10 min. The DNA was rinsed and dissolved as mentioned above.

Amplification of 18SrDNA

To obtain complete 18SrDNA, I used polymerase chain reaction (PCR) protocols (Saiki *et al.* 1988) with amplification primers SR1 and SR12 (Table 2). Each 50 μl of PCR reaction mixture containing 35.25 μl sterile water, 5μl PCR buffer (Promega Co., Madison, Wisconsin), 1.5 mM $MgCl_2$ solution, 20 mM each dNTP, 0.2 μM each primer, 2.5 units Taq polymerase (Promega) and 0.5 μl (ca. 0.01-1 μg) genomic DNA was overlaid mineral oil. PCR amplifications were performed in a QTP-1 thermal cycler (Nippon Genetics Corp., Tokyo, Japan) with 28 cycles of 93℃ for 1 min, 50℃ for 2 min, and 72℃ for 3 min. 0.5 μl of PCR product were used directly for the second PCR. The reaction content and temperature profile of the second PCR was the same as for the first PCR but contained PCR product instead of genomic DNA and another pair of amplification primers. The sequence and annealing positions of primers using for second PCR are shown in Table 2. For the second PCR, I used six pairs of primers, SR1-SR3, SR2-SR5, SR4-SR7, SR6-SR9, SR8-SR11 and SR10-SR12. Each second PCR product was ca. 300-400 nucleotide fragments.

Sequencing

PCR products were checked on 1.2% TAE-agarose gels with ethidium bromide staining according to standard methods (Sambrook *et al.* 1989). Excess primers and dNTP were removed from PCR products using polyethylene glycol (PEG) precipitate method. Precipitate solution of 0.6 vol (20% PEG [MW. 7500] and 2.5 M NaCl) was added before incubation at 0℃ for 1 h. After centrifugation at 14000 rpm for 10 min, the pellet was washed with cold 70% ethanol, air-dried and then redissolved in sterilized water. Double stranded PCR products were sequenced directly using a DNA autosequencer (model 373A; Applied Biosystems Inc., Foster City, California) with dyeterminator method according to the manufacture's instructions. Primers used sequencing were the same as for the second PCR. Sequences were determined over both strands of 18SrDNA except for 5' and 3' regions corresponding first PCR primers.

Sequence analysis

The sequences determined in this study were aligned manually with other previously known 18SrDNA sequences. The alignments are referred to secondary structure model of

rRNA (e.g. Huss and Sogin 1989; Rausch *et al.* 1989; De Rijk 1992). The published sequences included in this study are listed in Table 3. I used many data sets for the analyses, which will be explained in the "Materials and methods" of each chapter.

To construct phylogenetic trees from sequence data, I used three different methods, distance matrix, maximum parsimony and maximum likelihood methods. Because each of these methods has advantage and disadvantage (Hillis and Moritz 1990; Miyamoto and Cracraft 1991; Hillis *et al.* 1994) For the distance matrix method, I used Kimura's two-parameter method (Kimura 1980) to calculate the distance matrix, and used neighbor-joining (NJ) method (Saitou and Nei 1987) to construct trees. These procedures were performed using the CLUSTAL W computer program (ver. 1.5; Thompson *et al.* 1994). Maximum parsimony (MP) analyses were implemented with PAUP computer program (ver. 3.1.1; Swofford 1993) mainly using heuristic search with a branch-swapping algolism (tree bisection-reconnection [TBR]) under the equal weighting criterion. I also performed MP method using PAUP under the weighted scheme (reweight characters option, maximum value for rescaled consistency index, base weight = 1000), because the MP method is improved when sites that have relatively higher rates of change are given less weight in phylogenetic analysis (Hillis *et al.* 1994). As it is difficult to apply the of PAUP computer program for the large data set (e.g. 100 OTU), I also used the DNAPARS program in PHYLIP (ver. 3.5; Felsenstein 1993) to calculate the MP tree. For the maximum likelihood (ML) method, I used fastDNAml program (ver. 1.0; Olsen *et al.* 1994) with global search option.

Bootstrap analyses (Felsenstein 1985) were used with both distance matrix and maximum parsimony methods to evaluate statistical reliability of monophyly of each clade. I also used likelihood ratio test (LRT) of Kishino and Hasegawa (1989) to verify the alternative phylogenetic hypotheses. For the LRT, I created the topologies that reflect hypotheses with the RETREE program in PHYLIP (ver. 3.5; Felsenstein 1993). The log-likelihood of these alternate trees and that of the "best" tree (i.e. found with the grobal search) were compared by the DNAML program in PHYLIP. The LRT uses the mean and variance of log-likelihood differences between trees, taken across sites, and when this mean is >1.96 standard are declared significantly different (Felsenstein 1993).

CHAPTER 1.
PHYLOGENY OF THE GREEN PLANTS IN THE EUKARYOTES

INTRODUCTION

Green algae and land plants are considered to have originated from a common ancestor, because they share many common characters such as; 1) chlorophylls *a* and *b*, 2) chloroplast enclosed by two membranes, 3) storage of true starch in the chloroplast, and 4) "stellate" structure in the flagellar transition region. Therefore, green plants are regarded as a monophyletic clade in the eukaryotes, and treated as a single taxon (subkingdom Viridiplantae [Cavalier-Smith 1981] or subkingdom Chlorobionta [Bremer 1985]). All chloroplasts including those of green plants are considered to originate from (a) photosynthetic prokaryotic endosymbiont(s) (Mereschkowsky 1910; Margulis 1970; Raven 1970). If this "endosymbiotic theory" is correct, the ancestor of the green plants would be a colorless phagotrophic flagellate which engulfed the photosynthetic prokaryote. However, no colorless flagellate that resemble the green plants has been known. So, the evolutionary history of the eukaryotic "host" is still unclear.

The Chlorarachniophyta and some members of the Euglenophyta also have chloroplasts with chlorophylls *a* and *b* like in the green plants. However, their chloroplasts are surrounded by three (Euglenophyta) or four (Chlorarachniophyta) membranes, and the cellular architecture (e.g. the flagellar apparatus, shape of mitochondrial cristae) of these algae are basically different from that of the green plants. So, the chloroplasts of these algae are thought to have arisen through the secondary endosymbioses, that is, their chloroplasts have been originated from the green plants engulfed by colorless eukaryotic flagellates (Gibbs 1978; Hibberd and Norris 1984). As most members of the Euglenophyta are colorless (phagotrophic or osmotrphic), endosymbiotic event would have occurred within this lineage. On the other hand, colorless flagellate that resemble the chlorarachniophytes is not known. The "host" and evolutionary history of the chlorarachniophytes are still uncertain.

Based on the phylogenetic analyses using 18SrDNA sequence data, I tested phylogenetic hypotheses that the green plants is monophyletic clade in the eukaryotes. The ancestors of the green plants and other "green algae" (Euglenophyta and Chlorarachniophyta) were also investigated using molecular data.

MATERIALS AND METHODS

I created a data set including 69 sequences from eukaryotes, ten of which were determined in this study. The data set represents the Viridiplantae, Metazoa, Fungi, alveolates, stramenopiles, Rhodophyta, Cryptophyta, Haptophyta, Chlorarachniophyta, Euglenozoa, lobose amoebae, testate filose amoebae, slime molds, Heterolobosea and Parabasalia. The Parabasalia are amitochondriate protists and are thought to be an early divergence in the Eukaryota (e.g. Cavalier-Smith 1983), and this idea is supported by the 18SrDNA sequence comparison (Reipe *et al.* 1993; Gunderson *et al.* 1995). Thus, I dealt *Tritrichomonas foetus*, a member of the Parabasalia, as an outgroup taxon. This data set included 1618 bp. The MP and NJ analyses performed by the DNAPARS program in PHYLIP and clustal W program, respectively (see geneal Materials and Methods). To evaluate statistical reliability, I used the bootstrap analysis (100 replications) in both MP nad NJ methods.

RESULTS

The 18SrDNA sequence from all green algae examined in this study and some other eukaryotes (Chromophyta, Haptophyta, Chlorarachniophyta, Rhodophyta and some heterotrophic flagellates) were successfully amplified and sequenced using twelve primers and PCR conditions mentioned above (this study; Nakayama unpubl. data; Yokoyama, Ishida and Honda pers. comm.).

The MP analysis generated six most parsimonious trees from data set including 18SrDNA sequences from 69 eukaryotes. The topology of these six MP trees and the tree obtained from the NJ method were almost identical except the branching order that could not be resolved by the bootstrap analyses. Therefore, I presented only one MP tree in Fig. 1. Bootstrap values were cited above and under the internal clades (using MP method and NJ method, respectively). These analyses clearly demonstrated with high bootstrap supports (88% in MP method, 98% in NJ method) that *Euglena gracilis* (Euglenophyta), *Bodo caudatus* (Kinetoplastida), *Naegleria gruberi* (Heterolobosea) and *Dictyostelium discoideum* (cellular slime mold) were early divergences in the eukaryotes. In these protists, *Euglena gracilis* and *Bodo caudatus* form a clade (100% bootstrap support). After the divergence of these protists, the radiation of major eukaryotic groups occurred. Those included Metazoa/choanoflagellate/Fungi clade, cryptophytes, lobose amoebae, red algae

(Rhodophyta), stramenopiles (bicosoecids, thraustochytrids, oomycetes and chromophytes), alveolates (ciliates, apicomplexan sporozoa and dinoflagellates), glaucocystophytes, haptophytes, chlorarachnid/cercomonad/euglyphinid clade and green plants (Viridiplantae). However, the branching order of these major eukaryotic groups was not resolved. The twenty-two representatives from major groups of the green plants, including the Prasinophyceae, Ulvophyceae, Trebouxiophyceae, Charophyceae and land plants, formed a monophyletic clade supported by high bootstrap values (95% in MP method, 88% in NJ method) (Figs. 1,2). Monophyly of most other eukaryotic clades were also supported by bootstrap analyses, but those of lobose amoebae (*Acanthamoeba castellanii* and *Hartmannella vermiformis*) and the rhodophytes were weakly resolved. The 18SrDNA sequences of *Cercomonas* sp. form a clade with *Euglypha rotunda* and *Chlorarachnion reptans* 18SrDNAs. This relationship was supported by high bootstrap values.

DISCUSSION

The primers designed for this study made possible to amplify nuclear-encoded small subunit ribosomal RNA gene (18SrDNA) from many eukaryotes. Therefore, these would be usable as universal primers for phylogenetic studies of eukaryotes.

Present study demonstrated monophyletic origin of the green algae and land plants, which is in accordance with the results of recent papers (Schlegel 1991; Cavalier-Smith 1993; Bhattacharya *et al.* 1995a, b). It should be emphasized that the present result was obtained from data set including sequences from almost all groups of the green algae and land plants, so that it gives more reliable proof for the monophyly of the Viridiplantae (i.e. green algae and land plants). Distant relationships between the Viridiplantae and other green-colored algae, Euglenophyta and Chlorarachniophyta, support secondary symbiotic origins of the chloroplast in these two algal groups (Gibbs 1978; Hibberd and Norris 1984). Sister-group relationships of the Euglenophyta and Chlorarachniophyta to colorless flagellates (*Bodo* and *Cercomonas*, respectively) also agree to this hypothesis (see below).

Recent molecular phylogenetic studies (sequence comparisons, gene arrangement) indicate that all chloroplasts have a common cyanobacterium origin (see Douglas 1994; Reith 1994). This evidence leads to the hypothesis that the eukaryotes possessing the chloroplast gained by the primary endosymbiosis (colorless eukaryote + photosynthetic prokaryote), which is thought to comprise the Glaucocystophyta, Rhodophyta and Viridiplantae, are monophyletic.

Cavalier-Smith (1981) treated these eukaryotes as a single taxon, Kingdom Plantae, giving new circumscription for it. If these three taxa are descendants of "primary endosymbiosis", nuclear encoded gene (e.g. 18SrDNA) comparison would show monophyletic relationships of the Kingdom Plantae *sensu* Cavalier-Smith (of course, organisms secondarily lost chloroplast would also be included in this Kingdom), and the colorless ancestor of the Viridiplantae should be assigned to the common ancestor of the Kingdom Plantae *sensu* Cavalier-Smith. However, present 18SrDNA analyses could not reveal phylogenetic position of the Viridiplantae in the eukaryotic "crown" group (after Knoll 1992), which may have radiated in very short period of geological time during evolution of eukaryotes. In the 18SrDNA tree, major eukaryotic groups including the Viridiplantae, Rhodophyta and Glaucocystophyta diverge almost simultaneously, and these branching patterns are not resolved by the bootstrap analyses. However, the monophyly of the Plantae cannot be discarded in the present analyses. Early molecular phylogenetic study based on 5SrRNA sequences suggested that the Rhodophyta is an early divergence in the eukaryotes (e.g. Hori and Osawa 1987). But recent 18SrDNA studies including this study suggest that the Rhodophyta is a member of the crown group. I think that the unresolved radiation in the 18SrDNA and other molecule trees is due the rapid and simultaneous divergence of major eukaryotic groups. Thus the relationships of major eukaryotic groups including phylogenetic position of the Viridiplantae may be resolved using more long sequences (many genes) and qualitative characters (e.g. gene arrangement, morphological characters).

One of most interesting results from present analyses is the position of *Cercomonas* sp.. *Cercomonas* is an amoeboid organism with two flagella that lack both hairs and paraxial rod, and has mitochondria with tubular cristae and characteristic paranuclear body (e.g. Mignot and Brugerolle 1975). This amoeboflagellate usually glides but sometimes swims, and takes bacteria by pseudopodial engulfment. The genus *Cercomonas* is classified into the order Cercomonadida with other two genera, *Heteromita* and *Massisteria* (Patterson and Zölffel 1991), but phylogenetic position of the Cercomonadida has been uncertain. Traditionally, the Cercomonadida was classified into the phylum Protozoa, class Zoomastigophorea, but it is apparently not a natural taxon. Ultrastructural studies of *Cercomonas* suggested affinities to the chrysomonads (Chrysophyceae) or mycetozoa (Mignot and Brugerolle 1975; Schuster and Pollak 1978). However, present analyses clearly showed the close relationships between *Cercomons*, testate filose amoeba (*Euglypha*) and chlorarachniophyte (*Chlorarachnion*). This phylogenetic position of cercomonads was unexpected and has not been suggested before.

This result suggests that colorless flagellates show great phylogenetic divergence in the eukaryotes.

This result also gives some implications about the origin of the Chlorarachniophyta, a chlorophylls *a* and *b* containing amoeba. The distinctness of the cell architecture and similarity of photosynthetic pigments between the Viridiplantae and Chlorarachniophyta suggest secondary symbiotic origin of the chlorarachniophytes' chloroplast (Hibberd and Norris 1984). The presence of nucleomorph in the chlorarachniophytes strongly supports this idea. Based on 18SrDNA sequence comparison, Bhattacharya *et al.* (1995b) showed close relationship between chlorarachniophytes and testate filose amoeba (*Euglypha* and *Paulinella*). Present study reveals the affinity between the chlorarachniophytes and *Cercomonas*. The Chlorarachniophyta, euglyphinids and cercomonads are amoeboid organisms and have mitochondria with tubular cristae. They share capability to form walled-coccoid cells (cyst) and "plasmodial" colonies (Hedley and Ogden 1973; Hibberd and Norris 1984; Mylnikov 1986b; Patterson and Fenchel 1990; Ishida and Hara 1994). So, euglyphinids or cercomonads are possible candidates that have close relationship to the "host" of chlorarachniophytes. However, euglyphinids have some specialized characters such as a shell constructed with siliceous scales and lack of flagella in any stages of life history. In contrast, the cercomonads is simple amoeboflagellates and seem to retain more primitive conditions of this lineage. Furthermore, cercomonads and chlorarachniophytes share the microtubular root extending under the flagellum and ejectosome-like organelle (Mignot and Brugerolle 1975; Hibberd and Norris 1984; Mylnikov 1986a; Ishida pers. com.). These features suggest that hypothetical common ancestor of cercomonads, testate filose amoebae and chlorarachniophytes resembled the cercomonad flagellate, which evolved to the chlorarachniophytes by engulfing green alga.

I should point out that the 18SrDNA (and, I think, any other gene) is not perfect "ideal molecule" to infer the phylogenetic relationships of organisms. Reconstruction of phylogeny from sequence comparison is sometimes influenced by great divergence of evolutionary rate (e.g. "long branch") and/or base compositional bias (e.g. GC content). These effects (individually or conjointly) lead the phylogenetic tree to be misleading (Felsenstein 1978; Lockhart *et al.* 1992, 1994). For example, the position of *Dictyostelium* (cellular slime mold) in Fig. 1 is probably mistaken. The 18SrDNA trees indicate that *Dictyostelium* diverged before the crown group radiation (e.g. Cavalier-Smith 1993). However, morphological characters suggest close affinity between the Mycetozoa (Eumycetozoa, Protostelids and

cellular slime mold) and *Acanthamoeba* which is a member of crown eukaryotes in the 18SrDNA tree (Olive 1975; Spiegel 1990). Molecular phylogenetic studies using protein sequences showed that *Dictyostelium* is situated in the crown group (Loomis and Smith 1990; Hasegawa *et al.* 1992). Furthermore, close relationship between *Dictyostelium* and *Acanthamoeba* was suggested by phylogenetic trees based on the myosin heavy chain and actin sequences comparisons (Loomis and Smith 1990; Bhattacharya and Ehlting 1995). Early divergence of *Dictyostelium* in the 18SrDNA tree may be due to base compositional bias (unusual AT richness). Recently, Spiegel *et al.* (1995) reported the 18SrDNA tree, including sequence from protosterids (primitive mycetozoa), that *Dictyostelium* forms a clade with eumycetozoa (this situation is not seen in the typical 18SrDNA tree) and is positioned in the crown group. These data suggest that limited taxon sampling influence to the topology in the molecular tree (taxon sampling effect: Felsenstein 1978; Buchheim and Chapman 1992), and that addition of taxa in the data set sometimes reduce the topological mistakes caused by the effect from nature of sequences. These evidences indicate that we should carefully estimate the nature of sequences (e.g. evolutionary rate, base composition) and the taxon sampling in constructing phylogenetic relationships.

CHAPTER 2. PHYLOGENY WITHIN THE GREEN PLANTS

INTRODUCTION

The green algae are traditionally classified based on morphology of the vegetative phase. However, electron microscopical studies since 1960s' have led to serious reconsideration of classification and phylogenetic hypothesis of the green algae (see General discussion). Now three major classes, the Charophyceae, Ulvophyceae and Chlorophyceae, are widely accepted, and they are considered to be divided into two major evolutionary lines. The Ulvophyceae and Chlorophyceae (and Treboxiophyceae; see General discussion) are regarded to form a monophyletic assemblage in the green plants, while the Charophyceae are considered to be closely related to the land plants, and evolution from the former to the latter is believed. This means that the green algae (division Chlorophyta) is not a monophyletic but paraphyletic group. Thus, in the cladistic classification system such as that proposed by Bremer (1985), the Charophyceae is excluded from the division Chlorophyta and treated as a single taxon with land plants (division Streptophyta) (see also Bremer and Wanntorp 1981; Jeffrey 1982; Sluiman 1985; Bremer *et al.* 1987).

The Prasinophyceae is considered as the most primitive group of the green algae and as ancestral stock from which other green algal (and land plants) lineages have diversified (see General introduction). In the other classes (Charophyceae, Ulvophyceae, Chlorophyceae), features of the flagellar apparatus and mitosis/cytokinesis are uniform in each class, but the Prasinophyceae displays the great diversities in these features. There is no character which is unique only to the Prasinophyceae or has been found in all prasinophytes, so, we cannot find synapomorphic character in this class. If prasinophytes are descendants of these green algal "ancestral stock", the heterogeneity of the Prasinophyceae is understandable as remainders, i.e., the prasinophytes may reflects ancestral diversity of the green algae. These phylogenetic views imply that the Prasinophyceae is not a monophyletic but paraphyletic taxon, and that the phylogeny of this group is important to consider the phylogeny of the green plants.

The Prasinophyceae is very diverged group as mentioned above, and several groups are recognized within the class, though the classification is not stable. Moestrup and Throndsen (1988) classified the Prasinophyceae into two orders, the Mamiellales and Chlorodendrales. The former is characterized by the cell covered by a single layer of spider-web scales, and

the latter has a layer of square scales. Based on differences of the scales covering the square scales and other ultrastructural characters, Melkonian (1990a) divided the Chlorodendrales *sensu* Moestrup and Throndsen into three orders, the Pyramimonadales, Pseudoscourfieldiales and Chlorodendrales *sensu stricto* (= Tetraselmidales in Ettl [1983] or Stewart and Mattox [1984]; I use the name Tetraselmidales for this thecate prasinophytes).

In the Prasinophyceae, the Tetraselmidales is often considered to be more closely related to the Ulvophyceae/Chlorophyceae lineage than to the other prasinophytes (e.g. Moestrup 1982; Melkonian 1990a; van den Hoek 1995), because the tetraselmidalean algae have a cytokinetic apparatus with development of phycoplast (e.g. Stewart *et al.* 1974) and a cruciate microtubular root system (e.g. Salisbury *et al.* 1981). Furthermore, the Tetraselmidales is sometimes excluded from the Prasinophyceae and placed in the Pleurastrophyceae based on its similarity of mitosis/cytokinesis to the more "advanced" pleurastrophytes (Mattox and Stewart 1984).

The genus *Pyramimonas* (Pyramimonadales), which has a nearly symmetrical flagellar apparatus, is also regarded as a relative of the Ulvophyceae/Chlorophyceae lineage (O'Kelly and Floyd 1984a; Floyd and O'Kelly 1990; Segaar 1991; O'Kelly 1992). Relatedness of *Mesostigma* (Pyramimonadales) or *Nephroselmis* (Pseudoscourfieldiales) to the Charophyceae/land plants lineage is proposed (e.g. Moestrup 1982; Melkonian 1984, 1989; van den Hoek et al 1995). Based on 18S/26S rRNA sequence data, Kants *et al.* (1990) analyzed phylogenetic relationships between prasinophytes and other green algae, and found close affinities of *Pyramimonas* and *Tetraselmis* with the Ulvophyceae/Chlorophyceae lineage. The study of 18SrDNA sequence comparison by Steinkötter *et al.* (1994) indicated the sister-group relationship between the Tetraselmidales and the Ulvophyceae/Chlorophyceae lineage and early divergence of the Pseudoscourfieldiales. However, since the taxa analyzed in these studies were limited, analysis including many more prasinophytes needs to clarify the phylogeny of green plants.

One of the most important and interesting questions on the phylogeny of green plants is what the nature of the "ancestral green flagellate" is. Although controversial views have been presented for a long time, the answer for this question is still unknown (reviwed by Sym and Pienaar 1993). Some phycologists suggested that simple cell with one or two flagella is most primitive green plant (Norris 1980; Melkonian 1982a, 1984; Moestrup 1982, 1990, 1991; Moestrup and Throndsen 1988). In contrast, others proposed the hypothesis that complicated quadriflagellate which have an asymmetrical flagellar apparatus is more primitive

(Stewart and Mattox 1978; Mattox and Stewart 1984; O'Kelly and Floyd 1984a; O'Kelly 1992).

I analyzed global phylogeny of green plants based on the comparison of 18SrDNA sequences. From the results obtained, I tested and discussed the following points about the phylogeny of green plants:

1) Is the Chlorophyta (green algae) monophyly or paraphyly?
2) How should groups be recognized within the Prasinophyceae?
2) What are phylogenetic relationships between prasinophyte groups and other green algal lineages?

MATERIALS AND METHODS

Two data sets were made to analyze global phylogeny within the Viridiplantae. First data set (data set A) comprised 18SrDNA sequences from 104 taxa (33 of which were determined in this study) including those of the Glaucocystophyta (*Cyanophora*, *Glaucocystis*), Rhodophyta (*Porphyridium*, *Rhodella*) and Fungi (*Saccharomyces*) as outgroups. This data set included 1687 bp after exclusion of ambiguous sites in alignment. To avoid the "long-branch effect" (see discussion), I also made the smaller data set (data set B; including 38 taxa) which excluded sequences showing long branch in the tree generated from data set A.

The MP and NJ analyses performed by the DNAPARS program in PHYLIP or the PAUP and the clustal W program, respectively (see General Materials and Methods). To evaluate statistical reliability, I used the bootstrap analysis (100 replications) in both MP and NJ methods.

RESULTS

The NJ trees obtained from the data set A and B are shown in Figs. 3 and 5, respectively. In these trees, the Viridiplantae was divided into two large clades. The one included the Charophyceae, land plants and *Mesostigma*, and the other comprised the representatives from the Chlorophyceae, Trebouxiophyceae, Ulvophyceae and Prasinophyceae except *Mesostigma* (Prasinophyceae) (Fig. 3). The bootstrap values supporting these two clades are 90% and 73%, respectively, in the analysis using data set A (Fig. 3). In the analysis using data set B, the monophyly of the *Mesostigma* and Charophyceae/land plants assemblage was

weakly supported (54%), but another clade was supported at the same level (75%) (Fig. 5). The monophyletic relationship of Charophyceae/land plants was better resolved in the data set B than in the data set A (95%, 71% respectively).

Six MP trees were found by the MP analysis using data set A, and the strict consensus tree of these was shown in Fig. 3. In contrast to the NJ trees, *Mesostigma* was the first divergence within the all Viridiplantae, and the Charophyceae/land plants clade was not monophyletic in the MP tree (Charales was the second divergence within the all Viridiplantae) (Fig. 2). However, this relationship was not supported in the bootstrap analyses. In the MP analysis using data set B, the Charophyceae/land plants clade was monophyletic (63% support) (Fig. 4). The monophyletic origin of another clade (including the Chlorophyceae, Trebouxiophyceae, Ulvophyceae and Prasinophyceae except *Mesostigma*) was well supported in the MP analyses (87% and 83%) (Figs. 2, 4).

The branching orders between the major groups of the Charophyceae and land plants were not resolved in both NJ and MP methods (Figs. 2-5).

In all analyses, prasinophycean algae were divided into five different groups, *Mesostigma*, Pyramimonadales, Mamiellales, Pseudoscourfieldiales and Tetraselmidales. The monophyly of each prasinophycean clade is well supported in bootstrap analyses (Figs. 2-5)

In the clade comprising the Chlorophyceae, Trebouxiophyceae, Ulvophyceae and Prasinophyceae (excepting *Mesostigma*), the Pyramimonadales and Mamiellales formed a clade and diverged first in the NJ trees (Figs 3, 5). However, in the MP trees, the Pyramimonadales diverged first and Mamiellales diverged next (Figs. 2, 5). After the divergence of the Pyramimonadales and Mamiellales, the Pseudoscourfieldiales diverged from the major clade in both NJ and MP trees. The monophyletic relationship of the other green algae (Chlorophyceae, Trebouxiophyceae, Ulvophyceae and Tetraselmidales) was well supported (92-100%), but the branching orders between the major groups of this clade was not resolved.

DISCUSSION

Paraphyletic nature of the Chlorophyta

The NJ analyses using both data set A and B clearly indicate paraphyly of the green algae, because all charophytes and the genus *Mesostigma* are more closely related to the land plants than to other green algae (Figs. 3,5). The Chlorophyta *sensu lato* is also paraphyletic in the

MP analyses from data set A (Fig. 2), but the Charales (*Chara*, *Nitella*) and *Mesostigma* do not form a clade with other members of the Charophyceae/land plants clade. The morphological and biochemical characters clearly suggest that the Charales belong to the lineage of land plants (see General Introduction). The plasmodesmata generated as a result of phragmoplast formation and the group II introns situated in the *trn*I of the chloroplast DNA are unique characters shared by *Coleochaete*, Charales and land plants (Pickett-Heaps 1975; Manhart and Palmer 1990). Furthermore, specialized sperm with backwardly-directed flagellar insertion suggests that the Charales is a candidate which is most closely related to the land plants (but see Bremer 1985). Unusual position of the Charales in the MP analysis would be due to fast evolutionary rate of some sequences of charophytes/land plants (including Charales), because phylogenetic inference from sequence data (especially by MP method) is sensitive to unequal rates of substitutions (Felsenstein 1978, 1981). This may be one of the reasons why the 18SrDNA analyses sometimes lead to non-monophyly of the Charophyceae/land plants clade (Wilcox *et al.* 1993; Ragan *et al.* 1994). Analyses using data set B, in which sequences with long blanch in the trees (presumably because of rapid evolutionary rates) were excluded, showed monophyletic origin of the Charophyceae/land pants clade with higher bootstrap supports (especially, 95% in the NJ analysis) than those using data set A. The common origin of the Charophyceae/land plants is also suggested by comparison of *rbc*L sequences (Manhart 1994). Furthermore, *tuf*A transferred to the nucleus and group II intron located in the *trn*A of the chloroplast DNA strongly support the monophyly of the Charophyceae/land plants clade (Baldauf *et al.* 1990; Manhart and Palmer 1990). So, morphological and molecular data support the existence of large monophyletic group comprised of the Charophyceae and land plants in the Viridiplantae. This clade is termed the Streptophyta (Bremer and Wanntrop 1981; Jeffrey 1982; Bremer 1985) or the Anthoceratophyta (Sluiman 1985), and hereafter I will use the term Streptophyta for this clade.

In the Streptophyta, *Coleochaete* and the Charales are regarded as the most closest relative of the land plants based on morphological characters and the distribution of introns situated in the chloroplast tRNA genes (see above). However, phylogenetic relationships within the Streptophyta were not resolved by the present analyses (Figs. 2-5). Previous 18SrDNA studies also failed to indicate robust phylogeny within the Streptophyta (Wilcox *et al.* 1993; Battacharya *et al.* 1994; Surek *et al.* 1994; Ragan *et al.* 1994; see also McCourt 1995), and some authors noted that rapid ancient divergences would make it difficult to resolve their

relationships (e.g. Surek *et al.* 1994).

In contrast to the situation of the Charophyceae/land plants clade, 18SrDNA analyses clearly revealed the monophyly of another large clade comprised of the Ulvophyceae, Trebouxiophyceae, Chlorophyceae and some prasinophytes (i.e. Tetraselmidales, see below). This grouping nearly agrees with the Chlorophyceae of the original sense (Stewart and Mattox 1975) or the Chlorophyta *sensu stricto* (Bremer and Wanntrop 1981; Jeffrey 1982; Bremer 1985). I will use hereafter the term Chlorophyta *sensu stricto* for this evolutionary lineage. Bremer (1985) noted that the cruciate flagellar root system, the system II fiber (Melkonian 1980) and reduction of the MLS are probable synapomorphic characters which support the monophyly of the Chlorophyta *sensu stricto*. However, these characters have been found in some members of enigmatic green algal group, the Prasinophyceae. So it is difficult to evaluate the state of these characters (see below). The 18SrDNA analyses, however, strongly support the monophyly of the Chlorophyta *sensu stricto* ($\geqq$92% bootstrap values). This result further supports the result suggested from previous 18SrDNA studies (Steinkötter *et al.* 1994; Surek *et al.* 1994)

In conclusion, molecular and morphological characters indicates that the Chlorophyta in traditional sense (i.e. green algae) is not monophyletic but paraphyletic taxon, and two large clades, the Streptophyta and Chlorophyta *sensu stricto*, are distinct entities of the Viridiplantae.

Phylogeny and taxonomy of the Prasinophyceae

Eighteen genera have been recognized in the Prasinophyceae. In this study, 18SrDNA were determined for ten species from seven genera. The analyses clearly demonstrated that there are five independent lineages in the Prasinophyceae. These are the Chlorodendrales *sensu* Melkonian (*Scherffelia, Tetraselmis*), Pseudoscourfieldiales (*Pseudoscourfieldia, Nephroselmis*), Mamiellales (*Mamiella, Mantoniella, Micromonas*), Pyramimonadales except *Mesostigma* (*Pyramimonas, Halosphaera, Cymbomonas, Pterosperma*) and *Mesostogma*. The monophyletic origin of each lineage is also well supported. With the excepion of the *Mesostigma* lineage, all prasinophytes are more closely related to the Chlorophyta *sensu stricto* than to the Streptophyta. These four lineages diverged in turn from the Chlorophyta-clade in the 18SrDNA tree, and these relationships are supported by bootstrap analyses. So, present study indicates that the Prasinophyceae is not a monophyletic but may be paraphyletic taxon. These results further supports the results presented in studies using more

restricted species based on partial 18S/26S rRNA sequences (Kantz *et al.* 1990) or complete 18SrDNA sequences (Steinkötter *et al.* 1994). Non-monophyletic relationship of the Prasinophyceae is not surprising, which has been suggested from the structural heterogeneity of this class (e.g. Norris 1980; Mattox and Stewart 1984; Melkonian 1982a, 1984; Sym and Pienaar 1993). So, the circumscribed characters of the class Prasinophyceae (e.g. scales, parallel basal bodies, parabasal golgi bodies) are now regarded as symplesiomorphic features (see General discussion). Paraphyletic nature of the Prasinophyceae suggest that the taxonomy of prasinophycean algae should be changed drastically, and I think that each of five prasinophycean lineages mentioned above should be treated as independent taxon, at least at class level (see General discussion).

Steinkötter *et al.* (1994) analyzed 18SrDNA from four prasinophytes (*Pseudoscourfieldia*, *Nephroselmis*, *Scherffelia* and *Tetraselmis*), and indicated that *Pseudoscourfieldia* and *Nephroselmis* diverged earlier, and that *Scherffelia* and *Tetraselmis* formed a clade with the Chlorophyta *sensu stricto* (i.e. Ulvophyceae/Trebouxiophyceae/Chlorophyceae). This analysis clearly indicated paraphyletic nature of the Chlorodendraceae and Chlorodendrales *sensu* Moestrup and Throndsen (1988), and supported the Melkonian's classification system that these prasinophytes are treated as two different orders (Pseudoscourfieldiales and Chlorodendrales *sensu* Melkonian) (Melkonian 1990a). Present study provides further support to Melkonian's classification of the Prasinophyceae, not to that of Moestrup and Throndsen (1988). The algae classified into the order Chlorodendrales, family Halosphaeraceae by Moestrup and Throndsen (1988) are not closely related to any other chlorodendralean prasinophytes in the 18SrDNA tree. Melkonian (1990a) classified these algae into a different order, the Pyramimonadales, and 18SrDNA trees agree to this systematic hypothesis. The definitive character of the Chlorodendrales *sensu* Moestrup and Throndsen (1988) is a layer of square (or diamond) shaped scales covering the flagella and cell body. However, now this character is regarded as a symplesiomorphic one within the Viridiplantae, because this type of scales is present in flagellate cells of some members of the Charophyceae, Ulvophyceae and Chlorophyceae (Pickett-Heaps 1975; Mattox and Stewart 1984; O'Kelly and Floyd 1984a).

Melkonian (1990a) divided prasinophytes into four orders (i.e. Chlorodendrales *sensu* Melkonian, Pseudoscourfieldiales, Mamiellales and Pyramimonadales), and as mentioned above, the results of 18SrDNA analyses almost agree with this grouping, except for the position of *Mesostigma*. This biflagellate prasinophyte is classified into the

Pyramimonadales in the Melkonian's classification (Melkonian 1990a), and ultrastractural characters suggested that the genus *Pyramimonas* is presumably the closest extant relative of *Mesostigma* (Rogers *et al.* 1981; Melkonian 1989). However, 18SrDNA trees indicate distant relationship between *Mesostigma* and all other pyramimonadalean algae including *Pyramimonas*. The phylogenetic position of *Mesostigma* is uncertain (see below), but paraphyletic relationship of the Pyramimonadales is supported in the bootstrap analyses. This unique phylogenetic position of *Mesostigma* is understandable at least in part from unique ultrastractural characters. These are as follows (Melkonian 1989; Sym and Pienaar 1993): 1) *Mesostigma* has two flagella and one Golgi body, while other pyramimonadalean algae have four (or its multiple) flagella and two Golgi bodies. 2) The flagella of *Mesostigma* are covered by only square (or diamond) shaped scales, and lack limuloid and hair scales which are found in other pyramimonadalean prasinophytes. 3) *Mesostigma* has two MLS in its flagellar apparatus in contrast to single MLS of other pyramimonadaleans (exception: *Pyramimonas*). 4) Microbody is associated with 1d root in *Mesostigma*, whereas that of other pyramimonadalean algae shows the characteristically associated with the rhizoplast and chloroplast. Of course, these unique features of *Mesostigma* can be explained as derived (autapomorphic) or primitive (if so, *Mesostigma* should be first divergence in the Pyramimonadales) characters in the Pyramimonadales. However, I cannot find any synapomorphic features shared between *Mesostigma* and other pyramimonadalean members. Only possible unique (apomorphic) character of the Pyramimonadales *sensu* Melkonian is the body scale situated on top of a layer of square-shaped scales. *Mesostigma* has the basket-shaped outer large scales, and very similar scales have been found in a certain species of *Pyramimonas* (*P. longicauda*) (Manton and Ettl 1965; Inouye *et al.* 1984; McFadden *et al.* 1986). However, "basket-shaped scales" of *P. longicauda* do not comprise outermost but intermediate scale layer, and are regarded as specialized scales evolved from the spider-web scale within the Pyramimonadales (see Chapter 3). So, a common origin of "basket-shaped scales" of *Mesostigma* and *Pyramimonas* seems to be doubtful. In conclusion, both morphological and molecular characters suggest that *Mesostigma* is an independent lineage from other prasinophytes.

Recently, Daugbjerg *et al.* (1995) reported the prasinophycean phylogeny inferred from *rbc*L sequences, in which almost simultaneous radiation of the prasinophycean genera was suggested. Only congruence between 18SrDNA and *rbc*L phylogeny is the monophyletic origin of the Mamiellales. Monophyletic natures of the Pyramimonadales and

Pseudoscourfieldiales, which is supported by 18SrDNA and morphological data, were not resolved in *rbc*L analysis. Although phylogeny based on gene sequences which code protein is sometimes considered to be more reliable than that of rDNA sequences (e.g. Hasegawa *et al.* 1992), *rbc*L sequences seem to be less informative than 18SrDNA in the analysis of prasinophycean phylogeny.

Phylogenetic relationships between the Prasinophyceae and other green plants

Though phylogenetic position of *Mesostigma* is not comprehensive in the 18SrDNA analyses (see bootstrap values in Figs. 2-5), it is important to consider the global phylogeny of the Viridiplantae. Present study suggests two possiblities in terms of the position of this unique prasinophyte; 1) the first divergence in whole green plants (in MP analysis) or 2) the sister taxon of the Streptophyta (Charophyceae/land plants) (in NJ analysis). Interestingly, some ultrastructural characters support both possibilities. The former is suitable for the presence of two MLS-roots (1d, 2d) in *Mesostigma*. This is in contrast to the Streptophyta and other prasinophytes where the MLS is single and associated with 1d root. Interestingly, *Cyanophora*, a member of the Graucocystophyta, which is possible sister taxon of the Viridiplantae (see Chapter 1), has two "putative" MLS-roots associated with each of two basal bodies (Mignot *et al.* 1969; Nakayama unpubl. data). It is therefore possible to evaluate that the presence of two MLSs is primitive state in the Viridiplantae and single MLS is synapomorphic character. If this evaluation is correct, the first divergence of *Mesostigma* within the green plants is understandable. However, biochemical comparison of the "putative" MLS of Glaucocystophyta and "true" MLS of Viridiplantae has not been made, which is needed to clarify the homology of these structures.

The close relationship *Mesostigma* and the Streptophyta was suggested by Rogers *et al.* (1981), because both group shared the MLS at d-root. However, now the MLS has been found in other prasinophytes such as the Pyramimonadales (Hori *et al.* 1985; Inouye *et al.* 1990; Inouye pers. comm.) and Mamiellales (see Chapter 3). So the presence of MLS associated with the d-root are regarded as a symplesiomorphic character of the Viridiplantae. The habitat (freshwater) and stellate structure (Melkonian 1984) are also common features between *Mesostigma* and the Streptophyta. However, it is difficult to evaluate these features, because parallel evolution of these probably have often occurred. *Mesostigma* and the scaly motile cells of charophytes have "square" flagellar scales which is different from pentagonal

flagellar scales of other scaly flgellates (e.g. Manton and Ettl 1965; Turner 1968; Pickett-Heaps 1975; Melkonian 1989; Sym and Pienaar 1993), but it is uncertain which type of scales is symplesiomorphic. The considerable characters in common between *Mesostigma* and the Streptophyta are futures of microbody and axoneme. They share elongated microbody associated with ld flagellar root (Rogers *et al.* 1981; Sluiman 1983). Furthermore, the enzyme for oxidation of glycolate in the *Mesostigma* has some similar features to that of the Streptophyta (Iwamoto pers. comm.). Melkonian (1989) reported that *Mesostigme* had no outer dynein arms on all peripheral doublets at the distal region of the axoneme, and the flagella of the Streptophyta are also known to lack all outer dynein arms (Hyams *et al.* 1979). Because the absence of all outer dynein arms is apparently apomorphic, it seems to supports the monophyly of *Mesostigma* and the Streptophyta. So, I feel that the phylogenetic position of *Mesostigma* suggested by the NJ analyses is more acceptable.

All prasinophytes except *Mesostigma* form a clade with the Chlorophyta *sensu stricto* in the 18SrDNA analyses with 73-87% bootstrap supports. However, it is difficult to assign the apomorphic morphological characters of this large clade. Possible characters are the presence of the hair scales and Melkonian's row (paired longitudal rows of square scales situated on two opposite sides of flagellum; Hori and Moestrup 1987) on flagellum which are found in all prasinophytes forming a clade with the Chlorophyta *sensu stricto*. Hair scales and Melkonian's row are absent in *Mesostigma* and, probably, in the member of the Streptophyta (e.g. Manton and Ettl 1965; Melkonian 1984, 1989; Fig. 27 in Turner 1968; Figs. 20,21 in Graham and Taylor 1986)[1]. So these features seems to be synapomorphic characters of this large clade. However, hair scales and Melkonian's row are completely absent in the Ulvophyceae, Trebouxiophyceae and Chlorophyceae. These would probably have lost secondarily. Other considerable feature is the absence of the outer dynein arm on one of the peripheral doublet (no.1 doublet: Hoops and Witman 1983; Melkonian 1984) in these algae. The members of the Pseudoscourfieldiales and Chlorophyta *sensu stricto* lack one of the outer dynein arms (e.g. Melkonian 1984) and this may be synapomorphic character of these algae. Interestingly, *Pyramimonas octopus* is known to possess all dynein arms, but one of these, which is regarded as homologue to that of no.1 doublet, is shorter

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[1] Flagellar "hairs" of motile cells of *Coleochaete* (Pickett-Heaps 1975; Graham and McBride 1979; Graham and Taylor 1986) are probably not homologous to the hair scales because of their structural differences.

than the others and is considered to be retained proximal segment of the bipartite dynein arm (Hori and Moestrup 1987). This situation is comfortable to the earliest divergence of the Pyramimonadales in the green algae except *Mesostigma* and the Streptophyta. However, it should be noted that one flagellum of *Pseudoscourfieldia marina* has no.1 doublet without outer dynein arm, but the other flagellum (I cannot judge whether it is no.1 or 2 flagellum from the micrograph) of this alga seems to have all dynein arms (Fig. 74 in Moestrup and Throndsen 1988). This significant exception suggest that more studies need to evaluate the usefulness and evolution of this structure.

Within the five prasinophycean lineages, only one sister-group relationship, the Pyramimonadales and Mamiellales, is suggested in the NJ analyses (Figs. 3,5; especially 85% bootstrap support in Fig. 3). These two orders, especially primitive members of each order (*Pterosperma* and *Mamiella*), share the considerable morphological features (see Chapter 3). So, this relationship in the NJ trees seems to be acceptable. However, the MP analyses do not support monophyletic relationship between the Pyramimonadales and Mamiellales (but not denied).

Kantz *et al.* (1990) inferred phylogenetic relationships between some prasinophycean algae and other green algae using partial 18S/26S rRNA sequences, and suggested that close affinities of *Tetraselmis* and *Pyramimonas* to the Ulvophyceae/Trebouxiophyceae/Chlorophyceae clade (especially to the Trebouxiophyceae). However, present study clearly showed the distant relationship between the Pyramimonadales (including *Pyramimonas*) and the Chlorophyta *sensu stricto*. I determined 18SrDNA sequences from the same species used in Kantz *et al.* (1990) (*P. parkeae*), but the sequence given by Kantz *et al.* (1990) and that newly determined in this study are significantly different at many sites. This difference is too much to be regarded as intraspecific variation, and this would be the reason why different phylogenetic position were suggested for *Pyramimonas* in Kantz *et al.* (1990). Because 18SrDNA sequences from other pyramimonadalean algae (including three *Pyramimonas* species) agree to that of *P. parkeae* determined in this study, I suppose two probable reasons of this situation; 1) sequence in Kantz *et al.* (1990) is not from *Pyramimonas* but from other organism (e.g. contamination), 2) sequence of gene is modified through/after the transcription in *Pyramimonas* (e.g. RNA editing). The extensive modification of rRNA sequence has never been known, and RNA sequence by Kantz *et al.* (1990) showed considerable similarity to the 18SrDNA sequence of *Chlorella*. So, I think that the first possibility is more convincing.

Steinkötter *et al.* (1994) suggested based on comparison of 18SrDNA sequences that the Chlorodendrales *sensu* Melkonian (=Tetraselmidales) is most close relative of the Ulvophyceae/Chlorophyceae clade. This result is congruent with the hypothesis proposed from morphological characters (e.g. Moestrup 1982; Melkonian 1990a; van den Hoek 1995). Steinkötter *et al.* (1994) also indicated that the Tetraselmidales is independent lineage, and not included in the "Pleurastrophyceae" (see Mattox and Stewart 1984). Their analysis, however, did not include the sequences from another hypothetical "ancestor" of the Ulvophyceae/Chlorophyceae clade, *Pyramimonas*. Present analyses includes sequences of both *Tetraselmis* and *Pyramimonas*, and clearly reveals the distant relationship between the *Pyramimonas* and the Ulvophyceae/Chlorophyceae clade. So, the hypothesis that *Pyramimonas* is closely related to the Ulvophyceae/Chlorophyceae clade (O'Kelly and Floyd 1984a; Floyd and O'Kelly 1990; Segaar 1991; O'Kelly 1992) is denied, and common features between these (e.g. nearly symmetrical flagellar apparatus) are regarded as characters evolved independently (see Chapter 3).

Present study also provided some information about the nature of ancestral green plant. Many phycologist have considered that primitive green plant would be scaly flagellate, and this hypothesis was supported in the present analyses. Two contrastive scenarios about the nature of the ancestral green flagellate (AGF, termed by Mattox and Stewart 1984) have been supposed; 1) AGF is a cell with one or two flagella and possess simple intracellular organization (Norris 1980; Melkonian 1982, 1984; Moestrup 1982, 1990, 1991; Moestrup and Throndsen 1988), 2) AGF is quadriflagellate with complicated cellular organization (Stewart and Mattox 1978; Mattox and Stewart 1984; O'Kelly and Floyd 1984a; O'Kelly 1992). The former is more likely based on the 18SrDNA analyses. Symplesiomorphic state of the biflagellate condition is more parsimonious than that of quadriflagellate condition in the 18SrDNA tree. Quadriflagellation seems to have occurred independently in two groups, the Pyramimonadales and Chlorophyta *sensu stricto*.

O'Kelly (1992) considered that AGF should have phagotrophic ability, because it had obtained chloroplast by phagocytosis. However, now monophyletic origin of the chloroplast is apparent, and colorless origin (i.e. phagotrophic ability) of the green plants must retrace to the common ancestor of the kingdom Plantae *sensu* Cavalier-Smith (Rhodophyta, Glaucocystophyta, Viridiplantae) (see Chapter 1). Because phagocytosis is never found in the Rhodophyta, Glaucocystophyta and most Viridiplantae (see below), phagotrophic ability seems to have been lost in the common ancestor of the Plantae after the acquisition of

chloroplast. So, I think that AGF should have no phagotrophic ability, and feel that reports of phagocytosis in several pyramimonadalean algae (reviewed by O'Kelly 1992) are not clear enough and thus doubtful.

In consideration of the flagellar position in the cell, the condition of laterally (against to the swimming direction) inserted flagella is considered to be symplesiomorphic, because it is found in all lineages of the "Prasinophyceae" (except for the Tetraselmidales). The square-shaped scales covering the flagellar and cell body surface is also a primitive character (see above).

Although 18SrDNA analyses support the biflagellate AGF scenario, flagellar apparatus of the AGF would not be so simple. Moestrup (1990) suggested that green algae with one flagellum (e.g. *Mantoniella*) or devoid of it are more primitive (see also Norris 1980; Melkonian 1984; Moestrup and Throndsen 1988). However, simple organization known in some mamiellalean algae is not regarded as primitive but as reduced form. Morphological and molecular data clearly suggest that biflagellate state is primitive condition in the Mamiellales (see Chapter 3). Furthermore, lack of the 2d/2s root and MLS in the Mamiellales is also considered to be reduced characters (see Chapter 3). In conclusion, molecular and morphological data suggest that the AGF would be an alga cell with laterally inserted two flagella, square-shaped scales, MLS and lacking phagocytic ability.

CHAPTER 3. PHYLOGENY WITHIN THE PRASINOPHYCEAE

INTRODUCTION

Despite detailed studies and discussions continuously undertaken for a long time, strict phylogenetic relationships of the Prasinophyceae have been uncertain. To reveal the phylogeny of the Prasinophyceae is important to consider the evolution of the Viridiplantae. The question of the AGF (ancestral green flagellate) is the one that depends on understanding of the phylogeny of the Prasinophyceae to be answared (see Chapter 2). A great heterogeneity of cell morphology in the Prasinophyceae caused a question about AGF, i.e., which is ancestral condition, simple or complicated flagellate? In many caces, to recognize the primitive extreme in each pair of morphological homologues is difficult.

The molecular data, which evolves independently from morphological characters, is a powerful tool to infer the phylogenetic relationships of organisms. Of course, analyses of molecular data do not provide direct information about evolution of morphological characters and nature of the ancestor. However, molecular data is helpful in understanding and recognition of the primitive extreme in each pair of morphological homologues. I believe that analyses and comparisons of phylogenies inferred from independent data sets (e.g. molecular and morphology) lead us to more reliable phylogenetic relationships (see General introduction).

In Chapter 2, I analyzed global phylogeny of the Viridiplantae, and found five independent lineages in the Prasinophyceae. The recognition of the monophyly of these lineages aid to understand the primitive extreme and evolution of the morphological characters within each lineage because it enable us to perform the outgroup comparisons (e.g. Hennig 1966; Wiley 1981). In Chapter 2, I suggested that some characters of Mamiellales, which is considered to be the most primitive member in the simple-ancestral hypothesis (e.g. Norris 1980), are not primitive but derived states. The Pyramimonadales, which is regarded to retain the primitive condition expected by the complicated-ancestral hypothesis (e.g. O'Kelly 1992), is also not primitive in global phylogeny of the Viridiplantae. However, the strict phylogenetic analysis within the each group of the Prasinophyceae is needed to clarify the evolution of morphological characters.

In this chapter, I analyse the morphological features of the Mamiellales including a new genus, and discuss phylogeny and evolution within the each lineage of prasinophytes,

especially the Mamiellales and Pyramimonadales.

MATERIALS AND METHODS

Morphological study

Unialgal cultures of *Crustomastix didyma* and *Mantoniella antarctica* were provided by Dr. Masanobu Kawachi, Marine Biothechnology Institute, Kamaishi Laboratory. Strains of *Mantoniella squamata* was established by isolation of cells from enrichment culture of a sample collected from the Bay of Minamata, Kumamoto Prefecture, Japan. These cultures were grown in PES medium (Provasoli 1968) at 4°C (*Mantoniella antarctica*) or 15°C (*C. didyma* and *M. squamata*) under the light of 20-30 μEM^{-1} s^{-1} from white fluorescent tubes.

For whole mount preparations, I used the method in Marin and Melkonian (1994). 4 µl of cell suspension was added to an equal volume of fixative (5% glutaraldehyde in 0.2 M cacodylate buffer) placed on formvar-coated grid. After 5 min, the liquid was removed with filter paper, then 4µl distilled water and 4 µl 2% aqueous uranyl acetate were added immediately. After 90 s, the liquid was removed as before, and the grid was immediately washed once with 4µl distilled water.

Materials for thin sections were prepared as follows. Equal volume of fixative (5% glutaraldehyde, 0.5 M sucrose, in 0.2 M cacodylate buffer [pH 7.2]) and culture medium containing growing cells were mixed together at 4°C for 1 h. The fixative containing small amount of 2% osmium tetraoxide was also used. After rinsing twice (10 min each) with 0.1 M cacodylate buffer, cells were postfixed with 2% osmium tetraoxide at 4 °C for 1 h, then rinsed once with the same buffer. Cells were embedded in Spurr's resin (Spurr 1969) after dehydration in a graded ethanol series. Sections were cut with diamond knife and collected on slot grids coated by formvar. Sections on grids were double stained with 2% uranyl acetate and Reynolds' lead citrate (Reynolds 1963). Observations were carried out using a JEOL JEM 100C XII transmission electron microscope.

Molecular study

To infer the phylogenetic relationships within the Mamiellales and Pyramimonadales, I used the data set including 18SrDNA sequences from 12 prasinophytes (five mamiellalean and seven pyramimonadalean algae). This data set included 1764 bp. Based on results in Chapter 2, one order could be treated as outgroup of another order. The MP (by PAUP) and

NJ (by Clustal W) methods were used to infer the phylogenetic relationship of these algae. Bootstrap analyses (100 replications), in both MP and NJ methods, performed to evaluate statistical reliability.

RESULTS

The orientation of the flagellate cell is described here for later references (see Figs. 6,8). The side of the cell that faces forward while swimming is the anterior side (A); the opposite side of the cell is the posterior side (P). The side where the flagella arise is the ventral side (V), so the opposite side is the dorsal side (D). When the cell is arranged so that its ventral side faces the observer and its anterior side at the top, the lateral sides are designated right (R) and left (L). The numbering system of the basal bodies and the terminology of the microtubular roots follow Moestrup and Hori (1989) (see also O'Kelly and Floyd 1984a; Heimann *et al.* 1989; Marin and Melkonian 1994).

1. Morphological study of *Crustomastix*

This green alga had unique features which distinguish this alga from any other green flagellate so far described. The detailed comparisons of light and electron microscopical features have led to the consideration that this alga should be classified as a new taxon of the Prasinophyceae at generic rank.

Diagnosis

***Crustomastix* gen. nov.**

Cellula elongata reniformis, flagellis subaequalibus e medio latere cavo ortis. Cellula et flagella crusta induta. Flagella pilis vestita. Chloroplastus parietalis lateri convexo cellulae appositus.

Species typifica: Crustomastix didyma *sp. nov.*

Elongate bean-shaped cells with two lateral flagella, sub-equal in length and arising from the concave side. Cell and flagella covered with crust. Flagella bearing hairs. Chloroplast parietal, lying against the convex surface of the cell body.

Type species: *Crustomastix didyma* sp. nov.

Crustomastix didyma sp. nov.

Alga crustosa, unicellularis, viva libera, elongata reniformis, 4-7 μm longa et 2-4μm lata; Flagella bina, subaequalia, inserta in latere cavo ortis; Chloroplastus unus, viridis, sine pyrenoide.

Holotypus: Pl. 6-2.

Alga crustaceous, unicellular, free-living, elongate bean-shaped, 4-7 μm in length and 2-4μm in width; Flagella two, subequal, inserted in concave side; A single chloroplast, green, without pyrenoid.

Holotype: Pl. 6-2.

General cell structures

Living cells of *Crustomastix didyma* are elongated bean-shaped in lateral view (Plate 1-1), and this characteristic shape is also seen in the EM images (Plate 1-2; 4). Cells have mean length 4-7 μm and a width of 2-3 μm. The two subequal flagella emerge from concave side of the cell, point in one third to quarter the cell length away from anterior end of the cell. The long flagellum is approximately three to four times the length of the cell, and the short flagellum has nearly the same length to the long one or is shorter than long one in cell length. Both flagella have hair point at the tip. In the swimming cell, one flagellum extends posteriorly along the concave side of the cell. Another flagellum usually extends anteriorly, but often recurves along the cell body, and finally reaches posteriorly. Swimming cells sometimes stop suddenly and put round the flagella to the cell body.

Along the convex side of the cell body is a single chloroplast which comprises two lens-shaped lobes interconnected with central narrow bridge (Plate 4). Large starch grains are often seen in the center of both chloroplast lobes. A faint eyespot, which consists of several osmiophilic globules, is located at the connection site of the chloroplast lobes (Plate 4). Pyrenoid is absent.

A long and narrow penetration of plasmalemma, which opens to the slightly posterior position of the ventral side of the cell, extends to the connection site of the chloroplast lobes (Plate 3-1,2; 4). This structure resembles the "scale duct" found in the pyramimonadalean algae (e.g. Moestrup and Thomsen 1974; Inouye *et al.* 1990), and detailed study about the association to the flagellar apparatus suggests that they are homologous structures (see below). So, I call this structure scale duct.

The nucleus is located at the left-anterior side of the cell. The single Golgi apparatus which

consists of a single dictyosome made up of several cisternae is situated in the right-anterior portion of the cell. Cistarnae of the Golgi apparatus are arranged along dorsiventral axis, but incline to the region of the basal bodies. It seems that maturing face of the Golgi apparatus confronts to the ventral surface (and basal bodies) of the cell. The extension of the nuclear envelop (or ER) is sometimes seen at the dorsal side (probably forming face) of the Golgi apparatus. The characteristic vesicular system is distributed in the region from the maturing face of the Golgi apparatus to the scale duct along the plasmalemma. The membranes of this vesicular system seem to be thicker than other membranes such as of mitochondrion (Plate 3-3). High magnification images suggest that it is caused by the presence of fibrous inner coat in these membrane systems. This fibrous inner coat is also seen in the cisternae of the Golgi apparatus, but it is less developed than that of the vesicular system.

Elongated mitochondria are located along the inner surface of the chloroplast (Plate 4). Examination of the serial sections suggests that sausage-shaped single mitochondrion is situated along the anterior-posterior axis. The small microbody, which is usually elongated in shape, is present at the similar position of the mitochondrion (Plate 3-2; 4). The lipoidal globules are scattered in the cytoplasm. The extensive endoplasmic reticulum system ramifies throughout the cell. The ER is usually rough, and sometimes contains fibrillar structures.

The cell covering

The cell body of *Crustomastix didyma* is covered by no scales, but surrounded by the electron-dense material which usually show fibrous appearance. This fibrous coat continues to the flagella and scale duct, so the cell is entirely covered by this structure. This is one of the most characteristic features of this alga and has resulted in the generic name "*Crustomastix*". The fibrous inner coats of the vesicular system and the Golgi cistarnae (see above) seem to be the same material. This fibrous coat does not attach directly to the membrane, but the gap exists between them.

The only cell covering structure which is common between *Crustomastix didyma* and other prasinophytes is hair scale on the flagellar surface. Three types of hair scales are found in *Crustomastix didyma*, and these can be named as T-hairs (Plate 2-3), P_1-hairs (Plate 2-1) and tip hairs (Plate 2-2) according to Marin and Melkonian (1994). The T-hairs are approximately 1.1 μm long and occur on both the long and short flagella. This type of hairs consists of at least three parts: 1) a shaft which is about 500 nm long, 2) a distal part that consists of ca. 30 globular subunits, and 3) thin distal filament. As the fourth part, the

proximal filament may be present, but I was not able to observe it. The P_1-hair is long (ca. 2 μm) and consists of two parts: 1) a shaft which has characteristic rough appearance, and 2) a distal part that consists of ca. 35 globular subunits. This type of hairs occurs only on the short flagellum. The tip hairs which attain ca. 420 nm occur at the tip of both flagella. This type of hairs consists of ca. 20 irregular sized subunits.

The flagellar apparatus

The basal body of *Crustomastix didyma* is very long (ca. 800 nm) and terminates at the transitional region possessing an undivided stellate structure underlain by the transitional plate (= traverse septum; Melkonian 1984) (Plate 5-6). Two basal bodies are arranged at right and left (Bb1 and Bb2, respectively), and extend to the posterior-ventral direction. However, the Bb2 is more tilted ventrally, so that there is an angle of about 30° between two basal bodies. The basal bodies do not show a strict parallel arrangement in the ventral view, but are close at their proximal portion. Two basal bodies are connected by a distal and a proximal fiber. Both connecting fibers show no conspicuous striation (Plate 5).

Two microtubular roots associated to the Bb1 are found in *Crustomastix didyma* (Plate 5). The right microtubular root (1d root), which originates from the right side of the Bb1, extends posteriorly along the plasmalemma. This root consists of three microtubules, but the number of microtubule decreases posteriorly (3-2-1). Near the starting point of the 1d root, characteristic structure is situated at the dorsal side of the root. In lateral view, this structure shows lamellar appearance which consists of regularly spaced vertical plates, so these lamellae seem to be oriented perpendicularly to the microtubules of the 1d root (Plate 3-3). An electron dense region under the lamellar structure and a plate lying on the 1d root are also recognized (Plate 3-3). This structural complex associated with the 1d root shows considerable resemblance to the MLS (multi-layered structure) found in the Streptophyta and some prasinophytes. At the MLS region, an electron dense material connects between the Bb1 and 1d root. An extension of the distal fiber is closely associated to the 1d root (Plate 5-4). An another dense fiber, which is assumed to be duct fiber found in pyramimonadalean algae (e.g. Moestrup and Hori 1989; Inouye *et al.* 1990), extends along the right side of the ld root (Plate 5). The 1d root has a close association to the scale duct, and at this position this root situated left side of the duct (Plate 4). Under the 1d root, conspicuous distribution of the vesicular system which has fibrous inner coat (see above) is seen (plate 3-3).

Another microtubular root (1s root) originates from the space between the Bb1 and Bb2,

and passes posteriorly along the plasmalemma and the nucleus. This root consists of four microtubules, and shows characteristic 3 over 1 configuration near the basal bodies (Plate 5). The broad extension of the distal fiber is connected to the ventral surface of the 1s root. At the portion that the 1s root situated at the one edge of the nucleus, electron dense material extends dorsally from the 1s root along the right side of the nucleus (Plate 5-5).

An inconspicuous fibrous structure, which is assumed to be the rhizoplast (= system II fiber; Melkonian 1980), arises from the dorsal side of the Bb2, extends toward the chloroplast along the right side of the nucleus (Plate 5).

2. Morphological study of *Mantoniella*

I examined the ultrastructural characters of two species of *Mantoniella*, *M. antarctica* and *M. squamata*. The general features of *Mantoniella* species have been described in the previous reports (Manton and Park 1960; Barlow and Cattolico 1980; Marchant *et al.* 1989; Moestrup 1990; Marin and Melkonian 1994). Since present observations are in good agreement with the published accounts, I will present here only the features undescribed before, such as the arrangement of the major organelles and the flagellar apparatus of *Mantoniella* species.

The arrangement of the major organelles

In both species of *Mantoniella*, the major organelles such as nucleus, chloroplast, Golgi apparatus and basal bodies are arranged in the same manner (Plate 6). The convex (ventral) side of the cell is filled by the saucer-shaped chloroplast with central pyrenoid. The nucleus is situated in the left-posterior region of the cell, and the Golgi apparatus is located right-anterior side of the cell.

The flagellar apparatus

Two basal bodies connected by the distal and proximal fibers are arranged in the same manner of *Crustomastix didyma* (see above). Two microtubular roots (1d and 1s) arise from right basal body (Bb1), as reported previously (Barlow and Cattolico 1980; Marchant *et al.* 1989). The right microtubular root (1d root; "distal rootlet" in Barlow and Cattolico 1980) consists of two microtubules and originates from ventral side of the Bb1 (Plate 7). This root extends through the right side of the Bb1 and to the posterior portion of the cell. An electron

dense fiber is associated to the right side of the 1d root in some length. The 1s root ("proximal rootlet" in Barlow and Cattolico 1980) originates from the dorsal side of the Bb1 and extends along ventral-right surface of the nucleus toward the posterior side of the cell. The 1s root consists of four microtubules arranged in a 3 over 1 configuration (Plate 7-6). As this root extends posteriorly, the microtubules form a linear array and decrease a number of microtubules. The root containing three microtubules reported by Marchant *et al.* (1989) is regarded as this reduced 1s root. From the mid point of the 1s root, a fibrous structure extends along the right side of the nucleus to the ventral surface of the chloroplast where a microbody is situated (Plate 7-8). The rhizoplast (= system II fiber; Melkonian 1980) arises from the proximal part of Bb2 and extends to the pyrenoid with 1s-associated fiber (Plate 7-8).

Mantoniella antarctica has a microtubular band that is never found in the *M. squamata*. This may be only a difference in the flagellar apparatus between two *Mantoniella* species. This band is composed of 5-9 microtubules arising from the right portion of the Bb1, but this microtubular band never associates to the basal bodies (Plate 8-1,2). This structure would not be a flagellar root. An attentive comparison of micrographs suggest that one of the two microtubular "roots" reported by Marchant *et al.* (1989) is identical structure to it, so that the 1d root may be overlooked in Marchant *et al.* (1989).

3. Morphological study of *Micromonas*

Although *Micromonas* is known as an organism which is difficult to fix well for EM, Dr. Masanobu Kawachi provided me a well fixed sample of *Micromonas* collected from the Antarctic Ocean. I observed this sample and found some undescribed features.

Ultrastructural features

The single lens-shaped chloroplast is situated at the dorsal side of the cell. The nucleus is located anteriorly, but some images suggest slightly left portion of the cell. A Golgi apparatus faces to the basal body. In the anterior region of the cell, a few vesicle containing dense core and surrounding fibrous material are present (Plate 8-3,4). This structure resembles the "extrusome" found in mamiellalean algae (Moestrup 1984、1990; Marchant *et al.* 1989) and *Pterosperma* (Inouye *et al.* 1990). There are two microtubular roots (?) at least in the dividing cell. Both root composed of two microtubules (Plate 8).

4. Molecular phylogeny within the Mamiellales and Pyramimonadales

Phylogenetic analyses based on 18SrDNA sequences clearly reveals the branching order, (*Crustomastix*, (*Mamiella*, (*Mantoniella* spp., *Micromonas*))), within the Mamiellales. These relationships were supported by high bootstrap values (Fig. 10). Within the Pyramimonadales, *Halosphaera* represented the first divergence, and *Pterosperma* and *Pyramimonas* spp. assembled in a group. However, these relationships including the monophyly of *Pyramimonas* spp. were not supported by the bootstrap analyses (Fig. 10).

DISCUSSION

Ultrastructure and phylogenetic position of *Crustomastix didyma*

The light microscopical and ultrastructural features of *Crustomastix didyma* (especially the presence of hair scales) indicate that this alga is a member of the Prasinophyceae (Note: this taxon is not monophyletic; see Chapter 2). This alga has unique characters by which it is distinguishable from any other prasinophytes. These features are 1) its elongated bean-like cell shape, 2) a chloroplast divided into two lens-shaped lobes, 3) fibrous coat covered entire cell (including flagella) and 4) the absence of a pyrenoid. The third is the most characteristic feature of this alga.

Electron microscopy suggests that this fibrous coat originates from the Golgi apparatus, and is transported to the "scale duct" through the vesicular system situated beneath the 1d root. The organic scales of the prasinophytes are also produced in the Golgi apparatus (e.g. Becker *et al.* 1994). The scale duct of *Crustomastix didyma* is undoubtedly homologous with that of the Pyramimonadales, because they have the same positional relationship with respect to other organelles, especially to the 1d root. In the pyramimonadalean algae, the scale duct has a role to release scales to the cell surface (e.g. Moestrup and Thomsen 1974). These evidences indicate homologous origin between the fibrous coat of *Crustomastix didyma* and organic scales of the other prasinophytes. This hypothesis needs examination of chemical composition of the fibrous coat of *Crustomastix didyma*, and I suppose the presence of 2-keto sugar acid which is principal component of prasinophycean organic scales (Becker *et al.* 1991, 1994). The continuous cell covering derived from scales is also known in the Tetraselmidales as thecae (e.g. McFadden *et al.* 1986b). However, it covers only cell

body (flagella covered by scales), and scales remain in the Golgi apparatus. Furthermore, it shows different ultrastructural appearance from that of *Crustomastix didyma* (e.g. McFadden *et al.* 1986b). So, I think that they evolved independently from normal scaly covering. Some members of the Pedinomonadales (e.g. *Marsupiomonas pelliculata*), which is another "primitive" green flagellate group (Moestrup 1991), have thecate covering a part of cell body (Jones *et al.* 1994). It also seems to have an independent origin from the former, because of different distribution and appearance.

Although *Crustomastix didyma* is a unique prasinophyte as mentioned above, it has also several features common to other prasinophytes. In the five prasinophycean lineages recognized from the morphological and molecular data (see Chapter 2), the Mamiellales is the most probable group which has close relatedness to the *Crustomastix didyma*. The common features between the mamiellalean algae and *Crustomastix didyma* are 1) two flagella, 2) identical configuration of the major cell organelles, 3) the transitional region possessing an undivided stellate structure underlain by the transitional plate, 4) the absence of the microtubular roots associated to the Bb2 and 5) a rhizoplast connected only to the Bb2 (Balrow and Cattolico 1980; Moestrup 1984; Inouye pers. comm.; see also present study about *Mantroniella*). Furthermore, in two features, 1) the flagellar direction and movement in a swimming cell and 2) types and its distribution of hair scales, *Crustomastix didyma* resembles to *Mamiella* (Parke and Rayns 1964; Marin and Melkonian 1994; Inouye pers. comm.; Nakayama unpubl. observ.). Some (not all) of these features, however, may not be synapomorphic but symplesiomorphic (see below), which suggests the close phylogenetic relationship between *Crustomastix didyma* and other mamiellalean algae. I therefore propose that *Crustomastix didyma* belongs to the Mamiellales. The global phylogeny of the Viridiplantae based on 18SrDNA strongly supports this idea (Figs. 2-5).

In addition to the mamiellalean features, *Crustomastix didyma* also shares two remarkable characters with the pyramimonadalean algae. One is the MLS associated with the 1d root, and the other is the scale duct connected to the 1d root. These characters provide meaningful information to the consideration of the phylogeny and evolution of the Mamiellales (see below). It is a unique feature of *Crustomastix didyma* that only three microtubules comprised the MLS, because 1d root associated to the MLS usually consists of many (at least six; Melkonian 1989) microtubules in the other green plants and so it is called "spline". The MLS in *Crustomastix* seems to be similar to that of other prasinophytes rather than to that of charophytes and land plants, because the lamellar structure of MLS is composed of three

layers (S2 - S4) in the Charophyceae and land plants (e.g. Carothers and Kreitner 1967; Sluiman 1983) but is simpler in prasinophytes including *Crustomastix* (Melkonian 1989; Inouye *et al.* 1990). A plate (which probably comprises "keels" on microtubules) lying on the MLS root (1d root) is detected in *Crustomastix* (present study), *Pterosperma* (Figs. 31, 32 in Inouye *et al.* 1990), *Cymbomonas* (Inouye pers. comm.), *Halosphaera* (Fig. 5 in Hori *et al.* 1985), *Mesostigma* (Melkonian 1989) and charophycean algae (e.g. Sluiman 1983), but are absent in land plants (e.g. Carothers and Kreitner 1967). The homologue of this keel is also found in *Pseudoscourfieldia* (Moestrup and Throndsen 1988) and *Nephroselmis* (Moestrup and Ettl 1979; Inouye pers. comm.), although they lack the MLS. In *Crustomastix*, as in *Mesostigma* and charophycean algae (Sluiman 1983; Melkonian 1989), the lamellae of MLS oriented perpendicular to the "spline". This angle is oblique in the Pyramimonadales and land plants (Carothers and Kreitner 1967; Norstog 1974; Inouye *et al.* 1990; Inouye pers. comm.).

Some comments on the ultrastructural features of the Mamiellales

Present study provides the first information about the configuration of major organelles and the details of the flagellar apparatus in mamiellalean algae. The identical positional relationships of the chloroplast (ventral), nucleus (left), Golgi apparatus (right) and basal bodies (right Bb1 and left Bb2) in *Crustomastix* and *Mantoniella* are also suggested from micrographs of *Mamiella* (Moestrup 1984; Inouye pers. comm.). Furthermore, this situation is found in the other Prasinophyceae (Moestrup and Throndsen 1988; Melkonian 1989; Inouye *et al.* 1990; Inouye pers. comm.) except for the Tetraselmidales (e.g. Melkonian and Preisig 1986) and some species of *Pyramimonas* (e.g. Hori *et al.* 1995). So this configuration of major organelles is considered to be a common feature of the Prasinophyceae including mamiellalean algae (Sym and Pienaar 1993), and may be a symplesiomorphic character of the Viridiplantae.

Inouye *et al.* (1990) reported that a fibrous structure, which was associated with 1s root ("R2-associated fiber"), extends down to the microbody/chloroplast in *Pterosperma cristatum*. *Mantoniella* species have very similar structure which is more weakly developed. They have the same orientation and are probably homologous. I propose the term 1s-associated fiber for this structure. The 1s-associated fiber is found in a micrograph of Barlow and Cattolico (1981; "electron-dense region" in Fig. 7), and is also present in *Mamiella* sp. and *Pyramimonas* sp. (Sym and Pienaar 1993; Inouye pers. comm.). An

electron dense material extends dorsally from 1s root in *Crustomastix*. Although I could not find its terminus at the surface of chloroplast, it seems to be a homologous structure to the 1s-associated fiber. Same situations are also found in *Nephroselmis* spp. and *Cymbomonas tetramitiformis* (Moestrup and Ettl 1979; Inouye pers. comm.). So, it is probably a universal character of prasinophycean algae.

The fiber which extends along the 1d root of the Mamiellales (*Crustomastix*, *Mantoniella*) has never been reported previously. However, it is found in micrographs of *Mamiella* (Moestrup 1984; Inouye pers. comm.). The position and orientation of this fiber are consistent within the Mamiellales (except for *Micromonas*), and show close similarity to the duct fiber of Pyramimonadales (e.g. Moestrup and Hori 1989; Inouye *et al.* 1990). Although there is no duct in the Mamiellales except for *Crustomastix* and the duct fibers of pyramimonadalean algae are more complicated than that of the Mamiellales, they may be homologous structure (see Sym and Pienaar 1993). At the 1d root of *Nephroselmis olivacea*, a similar structure was also reported (Moestrup and Ettl 1979). This fiber is known to include protein that reacts to the antibody of assemblin, a phosphoprotein and a principle component of the system I fiber (Lechtreck and Melkonian 1991). It is interesting to study immunochemically the constituents of the duct fibers in the Mamiellales and Pyramimonadales to better understand homology of fibrous components.

Phylogeny of the Mamiellales

To evaluate the phylogeny and evolution of the Mamiellales, morphological characters of the algae classified into the Mamiellales (*Mamiella, Mantoniella, Micromonas* and *Crustomastix*) and *Pterosperma* (Pyramimonadales) were compared (Table. 4). This comparison apparently suggest the stepwise simplification or complication of morphological characters within these algae. Numbers of organelles such as the Golgi apparatus, lobes of chloroplast, flagella and basal bodies are many in the *Pterosperma*, but these decrease in order *Crustomastix/Mamiella - Mantoniella - Micromonas*. This tendency also exists in the components of the flagellar apparatus. These are the number of microtubules comprising the 1d and 1s roots, and presence or absence of the microtubular roots associated with the Bb2, MLS, duct fiber, 1s-associated fiber and rhizoplast. However, morphological analyses cannot resolve where the evolutionary root point is in this stepwise character-change. Of course, the characters of *Micromonas* is too simple to consider as the ancient organism (Moestrup 1991; but see Norris 1980), but primitive position of other algae cannot be ruled

out.

Molecular analysis provides an answer to this question. The global phylogenetic study of the Viridiplantae based on the 18SrDNA comparison clearly indicated that *Pterosperma* belongs to the independent lineage (Pyramimonadales) from the mamiellalean lineage (see Chapter 2). So, it is apparent that there is a evolutionary root between *Pterosperma* and *Crustomastix*, and that a morphological simplification occurred in the Mamiellales (fig. 11). Furthermore, the branching order within the Mamiellales by the 18SrDNA analysis is completely correspond to that inferred from morphological data (Fig. 10). These results clearly indicate that the simple structures of some mamiellalean algae are not "primitive" as suggested previously (e.g. van den Hoek *et al.* 1988; Moestrup 1990) but derived characters (see also Chapter 2). Recently, basically same consideration was discussed based on the *rbc*L sequence analysis which showed a monophyly of the Mamiellales and a branching order (*Mamiella*, (*Mantoniella*, *Micromonas*)) (Daugbjerg *et al.* 1995, but this order was not supported statistically). These results and distribution of characters in other green algae provide information about the hypothetical ancestor of the Mamiellales. Hypothetical ancestor of the Mamiellales would be a biflagellate covered by hair scales, square-shaped scales and spider-web scales showing morphological variation (see Moestrup 1990). It may have laterally inserted flagella and a characteristic configuration of organelles (see above). Furthermore, the characters of *Crustomastix* clearly suggest that the ancestor would have the MLS and a scale duct, both associated with the 1d root

Three more genera, *Dolichomastix*, *Batycoccus* and *Prasinococcus* have been described in the Mamiellaes. The long flagella of *Dolicomastix* (Manton 1977) seem to be an ancestral position of this alga in the Mamiellales, because most members of the Pyramimonadales have long flagella. Further ultrastructural study (especially about the flagellar apparatus) is needed to clarify its phylogenetic position. *Batycoccus* is coccid alga covered by spider-web scales of identical shapes (Eikrem and Throndsen 1990). Daugbjerg *et al.* (1995) indicate that the first divergence of this alga in the Mamiellales from *rbc*L sequence comparison. The similarity of the chloroplast between *Batycoccus* and *Crustomastix* (e.g. lack of pyrenoid, weakly developed tylakoids, large starch body stored in the central region) is noteworthy (Eikrem and Throndsen 1990). Whichever they are monophyletic or not, the simple characters (e.g. lack of flagella, monotypic scales) of *Batycoccus* would not be primitive but would have caused by an independent simplification from *Mantoniella/Micromonas* clade. Another coccoid prasinophyte, *Prasinococus* is also regarded as a member of the Mamiellales,

because of the presence of uriolide which is a unique carotenoid of this order (Miyashita *et al.* 1993; Miyashita pers. comm.). The presence of the cell wall which is not derivative of scales suggest that *Prasinococcus* is an independent coccoid alga and would be distantly related to *Batycoccus*.

Phylogeny of the Pyramimonadales

The primitive extremes of the morphological characters in the Pyramimonadales can be evaluated by the evidence that this order is monophyletic (except for *Mesostigma*, see Chapter 2, I use the term Pyramimonadales in this sense hereafter). An analysis of morphological characters of the Pyramimonadales suggests the branching order (*Pterosperma*, (*Cymbomonas*, (*Halosphaera*, *Pyramimonas*))) (Table 5, Fig. 12). *Pterosperma* (and maybe *Tasmanites* [= *Pachysphaera*]) has many characters (e.g. pyrenoid with cytoplasmic penetrations, backward swimming, extrusome, spider-web sales) shared with *Mamiella* (Mamiellales) (Inouye *et al.* 1990; Inouye and Hori 1991), and these features are regarded as symplesiomorphic in the Pyramimonadales. *Halosphaera* and *Pyramimonas* may form a clade supported by the presence of deep flagellar pit, a rotational symmetry of the cell, crown scales and eyespot without close association to the pyrenoid (Manton *et al.* 1963; Parke and Den Hartog-Adams 1965; Hori *et al.* 1985; Inouye *et al.* 1985; Nakayama unpubl. observ.). The genus *Cymbomonas* is regarded as an intermediate between *Pterosperma* and *Halosphaera*/*Pyramimonas*. This alga has some plesiomorphic features (e.g. asymmetrical cell shape, lack of crown scales) in addition synapomorphic characters (e.g. forward swimming, pyrenoid traversed by thylakoids, box scales) (Throndsen 1988; Inouye and Hori 1991; Inouye pers. comm.).

A trend of simplification is found in the Pyramimonadales. The genus *Pyramimonas* seems to be a "simplificated" pyramimonadalean alga. Phylogenetic analysis of morphological data suggest that *Pyramimonas* is a representative which has lost and simplified several structures such as the MLS, duct fiber, connecting fibers between basal bodies and components associated to the microtubular roots. The cell size reduction would have occurred in this genus (subgenus *Vestigifera*, *Trichocystis* subgroup I, *Hexactis*). The last subgenus (including single species, *P. virginica*), which is the most simplest alga in the Pyramimonadales, had lost a rhizoplast (system II fiber), scale duct and fibers associated to the microtubular roots (Hori *et al.* 1995). *Pyramimonas* also has another trend, parallelism with the chlorophytan algae (*sensu stricto*). This parallelism is found in a forward swimming

with ciliary beat and a nearly rotationally-symmetrical flagellar apparatus (Inouye and Hori 1991; Sym and Pienaar 1993). Such similarities have made confusion in terms of the phylogenetic position of the Pyramimonadales, and sometimes misled to the opinion that *Pyramimonas* is closely related to the Chlorophyta *sensu stricto* (e.g. O'Kelly 1992). Phylogenetic analysis of morphological and molecular data clearly reveals that these characters evolved in the Pyramimonadales independently from the Chlorophyta *sensu stricto*.

In contrast to the Mamiellales, the branching order within the Pyramimonadales inferred from morphological data is not supported by the 18SrDNA sequence comparison. The 18SrDNA analysis suggest that *Pterosperma* and *Pyramimonas* form a monophyletic clade (Fig. 10). I cannot find any morphological characters which support this relationship. I feel that the 18SrDNA is too conservative to analyze the relationship between genera of this order. Furthermore, short branch length of pyramimonadalean algae in the phylogenetic trees (e.g. Fig. 3) suggest the slower evolutionary rate of 18SrDNA gene in this order than that of other algae. The phylogenetic analysis based on the *rbc*L sequences (including third codon positions) showed branching order, *Pterosperma*, (*Cymbomonas*, *Pyramimonas*), which coincide better with the hypothesis from morphological data (Daugbjerg *et al.* 1995).

Phylogeny of the other prasinophytes and other "primitive" green flagellate

The Pseudoscourfieldiales includes *Nephroselmis*, *Pseudoscourfieldia* and maybe *Pycnococcus* (this genus was originally classified in the Mamiellales, but see Fawley 1992 and Daugbjerg *et al.* 1995). This order is characterized by the presence of small scales that cover a layer comprised of square-shaped (pentagonal) scales. These small scales are rod-shaped on the flagella, and are double arrow-, Maltese cross- or windmill-shaped at the cell body (Moestrup and Ettl 1979; Moestrup 1983; Inouye and Pienaar 1983; Moestrup and Throndsen 1988). The former is also present in the Tetraselmidales, so it is not apomorphic but symplesiomorphic character of the Pseudoscourfieldiales-Chlorophyta *sensu stricto* lineage (see discussion in Steinkötter *et al.* 1994). Furthermore, the evidence that a theca of the Tetraselmidales is generated from two layers of scales (e.g. stellate and disc-shaped scales in *Scherffelia*, McFadden *et al.* 1986b) suggests that the latter is also a symplesiomorphic character. The origin of large stellate or spiny scales comprised of outer layer(s) of body scales in most species of *Nephroselmis* is uncertain. However, it is noteworthy that some of these scales (e.g. third and fourth type body scales of *N. astigmatica*, Inouye and Pienaar 1984) have eight- or four-radial axes which are typical in

spider-web scales of the Mamiellales and Pyramimonadales. Other futures of this order are a flagellar apparatus lacking 2s root and a fused rhizoplast connected to two basal bodies (e.g. Moestrup and Ettl 1979; Inouye and Pienaar 1984; Moestrup and Throndsen 1988). In this order, *Pseudoscourfieldia* is usually regarded as a primitive (e.g. Moestrup and Throndsen 1988). However, I suppose the opposite hypothesis that *Nephroselmis*-like ancestor (more complicated form) evolved to *Pseudoscourfieldia* (simple form), because the latter is too simple to represent ancestral position of this order and *Nephroselmis* has several characters which are evaluated to be plesiomorphic (e.g. lateral inserted flagella, 1s-associated fiber, system I fiber [=? duct fiber]; see above and Chapter 2). So, the "simplification" would have also occurred in this order. The backward-swimming with posterior-inserted flagella of *Pseudoscourfieldia* is considered to be primitive feature (e.g. Inouye and Hori 1991), however it seems unusual in the Viridiplantae and may be an apomorphyic condition. The plesiomorphic condition of the Mamiellales and Pyramimonadales is a backward-swimming with laterally inserted flagella (see above), and it may suit for the Pseudoscourfieldiales (*Nephroselmis*).

The Pedinomonadales (*Pedinomonas, Resulter, Marspinomonas*), *Monomastix* and *Scourfieldia* are another "primitive" green flagellates. Some phycologists include these algae into the Prasinophyceae, and others classified into different taxa (e.g. Loxophyceae, Pedinophyceae) (see Cristensen 1962; Norris 1980; Moestrup 1982, 1991; Melkonian 1990d). Because of their simple organization (e.g. uniflagellation in Pedinomonadales and *Monomastix*, lack of microtubular roots in *Scourfieldia*) (e.g. Manton and Parke 1960; Manton 1967, 1975; Moestrup 1991), phylogenetic positions of these algae are uncertain, and sometimes regarded as "primitive" members of green algae (e.g. Pickett-Heaps and Ott 1974; Moestrup 1991). Kantz *et al.* (1990) reported the first divergence of *Pedinomonas minutissima* in the Viridiplantae based on the comparisons of 18S/26S rRNA sequences. However, it is now apparent that this strain is not a green algae but a member of the Chlorarachniophyta (Daugbjerg *et al.* 1995). I feel that, because of their simple organization, they are not primitive members of the Viridiplantae. As it is difficult to evaluate their morphological characters, molecular analysis is needed to clarify the phylogenetic positions of these algae.

CHAPTER 4. PHYLOGENY WITHIN THE CHLOROPHYTA

INTRODUCTION

The phylum Chlorophyta is usually used for the group including all green algae (e.g. Bold and Wine 1985; Mattox and Stewart 1984). However, this taxon has been criticized by cladistic taxonomists because of its non-monophyletic nature (e.g. Bremer 1985; Bremer *et al.* 1987). The close relationship between the Charophyceae and land plants has strongly suggested by ultrastructural and biochemical data (see General Introduction). Furthermore, molecular data including 18SrDNA sequences clearly support this opinion (see Chapter 2). So, I use the term Chlorophyta in strict sense (e.g. Bremer 1985). This phylum can be characterized by the presence of cruciate flagellar apparatus with 180° rotational symmetry (e.g. Mattox and Stewart 1984).

The Chlorophyta *sensu stricto* includes three major taxa, the Ulvophyceae, Chlorophyceae and Pleurastrophyceae (e.g. Bremer 1985). The Ulvophyceae, which contains most macro green algae inhabiting in the marine, is characterized by counterclockwise (CCW) orientation in the arrangement of basal bodies and cytokinesis mediated by a cleavage furrow. By contrast, the Chlorophyceae, which includes most green microalgae living in freshwater, has directly opposed (DO) or clockwise (CW) basal bodies in their motile cells and uses microtubular bundles parallel to the division plane, a phycoplast, for cytokinesis. The third class, the Pleurastrophyceae, which was described originally by Mattox and Stewart (1984), seems to have intermediate characters between former two classes. The members of this class also have motile cells with basal bodies displaced in the CCW direction as in the Ulvophyceae, but, on the other hand, they use phycoplast for cytokinesis as in the Chlorophyceae. Mattox and Stewart (1984) included two distinctive green algal groups, the Pleurastrales (= Microthamniales *sensu* Melkonian, see Melkonian 1990c) and Tetraselmidales, in the Pleurastrophyceae, because they share characteristic type of spindle in mitosis (metacentric spindle, see Stewart *et al.* 1974; Molnar *et al.* 1975). However, close relationship between the Pleurastrales and Tetraselmidales has been criticized by many phycologists, and the latter is often regarded as a member of the Prasinophyceae (e.g. Moestrup and Throndsen 1988; Melkonian 1990a). Recently, 18SrDNA analyses revealed distant relationship between this two orders and independent nature of these two lineage from the Ulvophyceae and Chlorophyceae (Friedl and Zeltner 1994; Steinkötter *et al.* 1994).

Furthermore, molecular analysis cleared that the type species of *Pleurastrum* (etymology of the Pleurastrophyceae and Pleurastrales), *P. insigne* was not assigned to the Pleurastrophyceae and Pleurastrales (Friedl and Zeltner 1994). Based on these evidences, Friedl (1995) proposed a new class, Trebouxiophyceae, for the Pleurastrales *sensu* Stewart and Mattox. So, now three classes are recognized in the Chlorophyta *sensu stricto*. 18SrDNA analyses suggest that the Tetraselmidales is also independent lineage in the Chlorophyta *sensu stricto* (see Chapter 2). However, the phylogenetic relationships within this phylum is still obscure.

From recent ultrastructural studies especially those based on flagellar apparatus comparison, some distinct groups have been recognized in the Chlorophyceae. These include the Chaetopeltidales, Chaetophorales, Sphaeropleales, Chlorococcales and Chlamydomonadales. The Chaetopeltidales, a taxon recently erected by O'Kelly *et al.* (1994), includes the algae that have zoospores with body scales, four flagella and basal bodies arranged essentially in a cruciate pattern (both upper and lower basal body pairs are arranged in DO pattern). The members of the Chaetophorales have quadriflagellate zoospores like the Chaetopeltidales, but have CW absolute orientation at the lower pair of basal bodies and have no scale (Floyd *et al.* 1980, Watanabe and Floyd 1989a). Zoospores of the algae classified in the Sphaeropleales *sensu* Deason *et al.* (1991) have two directly opposed basal bodies. Recent ultrastructural studies show that many algae traditionally classified in the Chlorococcales *sensu* Bold and Wynne (1978) belong to this order (Wilcox and Floyd 1988, Watanabe *et al.* 1988, Watanabe and Floyd 1989b, Floyd *et al.* 1993). The Chlorococcales *sensu* Deason *et al.* (1991) and Chlamydomonadales have basal bodies with CW absolute orientation (Watanabe and Floyd 1989b,c, Floyd *et al.* 1993). Because ultrastructural characters of these two orders are essentially the same, in this thesis I refer this group as "CW group".

Many phycologists considered that CCW condition is the primitive state in the green algae (e.g. Mattox and Stewart 1984). Based on this point of view, O'Kelly and Floyd (1984) suggested that the progressive clockwise rotation of the basal bodies occurred in the evolutionary line of the Chlorophyceae. The flagellar apparatus of the Chaetopeltidales were regarded as the most primitive form in the Chlorophyceae because of its strictly cruciate basal bodies, and the CW condition was thought to be derived from this form. As the zoospore of most ulvophycean and chaetopeltidalean algae have four flagella, O'Kelly and Floyd (1984) also suggested the quadriflagellate condition as primitive state.

Based on these hypotheses mentioned above, the phylogenetic positions of the quadriflagellate algae classified in the Dunaliellales *sensu* Ettl (1983) (*Hafniomonas*, *Oltmannsiellopsis* , *Polytomella*) and Chlamydomonadales (*Carteria*) are very interesting. These algae have basal bodies with CCW (*Hafniomonas*, *Oltmannsiellopsis*) or CW (*Polytomella*) absolute orientation. Some phycologists suggest that *Hafniomonas* represents the most ancestral position in the chlorophycean lineage (O'Kelly and Floyd 1984, O'Kelly *et al.* 1994, Segaar 1991). The genus *Carteria* is also thought to be one of primitive chlorophycean algae (O'Kelly 1992).

In this chapter, I analyzed 18SrDNA from chlorophycean algae which have quadriflagellate cell (*Planophila* [Chaetopeltidales], *Chaetophora* [Chaetophorales], *Carteria, Hafniomonas*, *Oltmannsiellopsis* and *Polytomella*) to infer the phylogenetic positions of these algae and relationships of various clades in the Chlorophyta *sensu stricto*. Determining phylogenetic positions of these green algae using molecular data would help our understanding of green algal phylogeny and character changes that have occurred during the evolution of green algae.

MATERIALS AND METHODS

I used two data sets for the analyses. The first set (set A) included twenty four taxa representing the members of the Tetraselmidales, Ulvophyceae, Trebouxiophyceae and Chlorophyceae. Two pseudoscourfieldialean prasinophytes (*Nephroselmis olivacea* and *Pseudoscourfieldia marina*) were also included as outgroups (Steinkötter *et al.* 1990, Friedl and Zeltner 1994). The second set (set B) included twenty-one chlorophycean algae and three outgroup taxa (*Trebouxia asymmetrica*, *Chlorella vurgalis*, *Oltmannsiellopsis viridis*). Each data set exclusive of ambiguously aligned regions was 1746 bp (set A) and 1770 bp (set B) in total length.

The maximum likelihood (ML), maximum parsimony (MP) and neighbor-joining (NJ) methods were used to construct the phylogenetic trees. These analyses were performed by fastDNAml, PAUP and clustal W computer programs. The phylogenetic relationships between major clades and phylogenetic position of certain taxa (e.g. *Oltmannsiellopsis*) were tested with user-defined tree analyses using likelihood ratio test (LRT). Bootstrap analyses were used with both the MP and NJ methods to evaluate statistical reliability.

RESULTS

Phylogenetic trees using data set A are shown in Fig. 13 (a; ML tree, b; MP tree, c; NJ tree). All trees demonstrated the first divergence of the tetraselmidalean algae (*Tetraselmis striata, Scherffelia dubia*) with relatively high bootstrap values (71-89%). The branching order of the Ulvophyceae, Trebouxiophyceae and Chlorophyceae contradicted between the MP and NJ trees. All topologies (fifteen) of these three classes (Ulvophyceae, Trebouxiophyceae, Chlorophyceae) (and *Oltmannsiellopsis viridis*, see below) phylogenies were tested using the likelihood ratio test (LRT). Result of this analysis also could not resolve these relationships, because six topologies can not be rejected statistically (Table 6). In the ML and MP trees, *Oltmannsiellopsis viridis* formed a clade with *Gloeotilopsis planctonica* (Ulvophyceae) with high bootstrap support (90% in the MP analysis) (Fig. 13a,b). However, this relationship was not found in the NJ tree (Fig. 13c), and result of the LRT analysis demonstrates that topologies including that of *O. viridis* form a clade either with the Chlorophyceae or with the Trebouxiophyceae, cannot be statistically rejected (Table 6). The monophyly of the Chlorophyceae was well resolved in both MP and NJ methods (bootstrap support of 97% in the MP analysis, 80% in the NJ analysis).

Phylogenetic trees constructed from data set B are shown in Fig 14. Because topology of the NJ tree was identical to that of the MP tree, I showed only the ML (Fig. 14a) and MP trees (Fig. 14b). All trees indicated that two large clades, which can be assigned to the Sphaeropleales and the CW group, existed in the Chlorophyceae. Monophyly of each clade was supported by high bootstrap values in the NJ and MP methods. Phylogenetic positions of *Planophila terrestris* and *Chaetophora incrassata* were not clearly resolved (Fig. 14, Table 7), but early divergence of these algae in the Chlorophyceae was suggested (bootstrap support of 91% in the MP analysis, 65% in the NJ analysis).

DISCUSSION

Phylogenetic relationships within the Chlorophyta

Present analyses demonstrated that prasinophycean genera classified in the Tetraselmidales (= Chlorodendrales *sensu* Melkonian 1990a) (i.e. *Tetraselmis* and *Scherffelia*) form a monophyletic clade and are diverged first in the Chlorophyta *sensu stricto*. This implies that the Tetraselmidales is the sister group of the clade containing the

Ulvophyceae, Trebouxiophyceae and Chlorophyceae. The same relationships have been reported in the recent 18SrDNA studies (Steinkötter *et al.* 1994, Friedl and Zeltner 1994, Friedl 1995). The first radiation of the Tetraselmidales and monophyly of the Ulvophyceae/Trebouxiophyceae/Chlorophyceae clade favors the hypothesis that the loss of the apical groove (flagellar pit) occurred only once in this clade. So, the lack of apical groove and basal bodies inserted into apical papilla can be regarded as a synapomorphic feature of these algae (Melkonian 1982), and they would not have multiple origins as suggested previously (e.g. O'Kelly and Floyd 1984a, Mattox and Stewart 1984).

Phylogenetic relationships between three classes, Ulvophyceae, Trebouxiophyceae and Chlorophyceae were not resolved in the bootstrap analyses and LRT. These results suggest that the divergence of these three classes (and *Oltmannsiellopsis*, see below) may be interpreted as a near-simultaneous radiation and may not be resolved only by 18SrDNA analyses. Mattox and Stewart (1984) suggested sister group relationship between the Chlorophyceae and "Pleurastrophyceae" because they shared phycoplast as cytokinetic apparatus (see also Bremer 1985; Mischler and Churchile 1985). However, non-monophyletic nature of the Pleurastrophyceae *sensu* Mattox and Stewart (1984) is now apparent, and this class divided into Tetraselmidales (Prasinophyceae) and Trebouxiophyceae (Steinkötter *et al.* 1994; Friedl and Zeltner 1994; Friedl 1995). Recent finding of the presence of phycoplast in some ulvophycean algae (Sluiman 1991) and the first divergence of the Tetraselmidales in the Chlorophyta *sensu stricto* indicate that the cytokinesis mediated by a phycoplast is symplesiomorphic feature of the Chlorophyta *sensu stricto* (Steinkötter *et al.* 1994). So, there is no morphological characters which support a sister group relationship between the Chlorophyceae and "Pleurastrophyceae". However, I cannot find any synapomorphic features shared by the Ulvophyceae and Chlorophyceae or Trebouxiophyceae. Therefor, it is not possible to infer the phylogenetic relationships between these three classes from morphological data, as from molecular data.

Phylogenetic position of *Oltmannsiellopsis*

Because of the lack of a cell wall and cell division taking place in the motile form, Chihara *et al.* (1986) placed the genus *Oltmannsiellopsis* in the Dunaliellaceae. However, the present analyses demonstrate that *Dunaliella parva*, a typical dunaliellalean alga, is distantly related to *O. viridis* but included in the CW group (Chlamydomonadales/Chlorococcales) (Fig. 13).

Present analyses were insufficient to confirm the phylogenetic position of

Oltmannsiellopsis, but suggested that *O. viridis* is an early divergence in the Ulvophyceae/Trebouxiophyceae/Chlorophyceae clade, and is distantly related to other members of the Dunaliellales. A recent ultrastructural study of its flagellar apparatus showed that *O. viridis* has basal bodies with a CCW orientation (Lokhorst and Star 1993). As suggested by many phycologists (e.g. O'Kelly and Floyd 1984), the CCW condition of basal bodies is thought to be the symplesiomorphic state of the green algae. Pienaar (1985) reported the presence of diamond-shaped scales on an alga that was otherwise assignable to *Oltmannsiellopsis*. The scaly cell covering is also clearly symplesiomorphic in the green algae, because it is present in most green algal classes including the Prasinophyceae. These ultrastructural characters of *Oltmannsiellopsis* agree with the phylogenetic position inferred from 18SrDNA data, and support the taxonomical view that this alga should be removed from the Dunaliellales (Sym and Pienaar 1991).

Oltmannsiellopsis is known to have some similarities to the prasinophycean algae. Sym and Pienaar (1991) suggested that there are close relationships between *Oltmannsiellopsis* and *Pyramimonas* based on some ultrastructural characters (e.g. muciferous body), but 18SrDNA study do not support this opinion (see Chapter 2, 3). *Oltmannsiellopsis* also has some similarities with tetraselmidalean prasinophytes in the flagellar apparatus architecture, that is, in apical view, the four basal bodies of *Oltmannsiellopsis* and *Tetraselmis* are aligned in a straight line (Salisbury *et al.* 1981; Chihara *et al.* 1986; Segaar 1991; Lokhorst and Star 1993). If this feature is symplesiomorphic, then it suggests that *Oltmannsiellopsis* is the first divergence in the Ulvophyceae/Trebouxiophyceae/Chlorophyceae clade. However, there is some differences between *Oltmannsiellopsis* and *Tetraselmis* in the direction of #1 and #4 flagella. Those of *Oltmannsiellopsis* extend in the same direction, but extend in the opposite direction in *Tetraselmis* (Norris *et al.* 1980; Chihara *et al.* 1986; Inouye and Hori 1991; Inouye pers. comm.). The user-defined trees that *Oltmannsiellopsis* is the first divergence in the Ulvophyceae/Trebouxiophyceae/Chlorophyceae clade (trees 2-4 in Table 6) are significantly "worse" in the LRT. So the arrangement of basal bodies in *Oltmannsiellopsis* may not be symplesiomorphic but autapomorphic.

In conclusion, *Oltmannsiellopsis* has ancestral and unique ultrastructural characters. The present analyses support an ancestral and distinctive phylogenetic position of *Oltmannsiellopsis* in the Chlorophyta, and *Oltmannsiellopsis* cannot be treated as a member of the Dunaliellales or any other green algal taxa. I therefore propose a new order and family for this alga.

Oltmannsiellopsidales ord. nov.

Algae virides, unicellulosae vel coloniales. Cellulae motiles, nudae vel squamatae, quadriflagellatae. Apparatus flagellorum cruciatus. Flagellum primo et flagellum quarto ad directiones identicas extensa. Corporae basalia in absoluta dispositione antihelicte.

Unicellular or colonial green algae. Cell quadriflagellate, naked or covered with scales. Flagellar apparatus is cruciate. Flagellum 1 and 4 extending to same direction. Basal bodies displaced in counterclockwise orientation.

Oltmannsiellopsidaceae fam. nov.

Characteribus ordinis.

Genus typificum: *Oltmannsiellopsis* Chihara et Inouye.

Characters are the same as the order.

Type genus: *Oltmannsiellopsis* Chihara et Inouye.

It is at present difficult to clarify the algal class to which the Oltmannsiellopsidales belongs, because this order shares no synapomorphic character with other green algae. The best choice for its taxonomic treatment is that the order be treated as taxon *incertae sedis* in the Chlorophyta until more information is obtained.

Phylogenetic position of *Hafniomonas*

The phylogenetic position of *Hafniomonas reticulata* inferred from 18SrDNA sequence is most unexpected. The nature of the flagellar apparatus (Ettl and Moestrup 1980; Segaar 1991; Inouye pers. comm.) and cytokinesis (Segaar 1991) suggests that *Hafniomonas* occupies the ancestral position of the Chlorophyceae (O'Kelly and Floyd 1984; O'Kelly 1992; O'Kelly *et al.* 1994; Segaar 1991; Sym and Pienaar 1991). However, 18SrDNA trees demonstrate that *H. reticulata* is a member of the CW group (Figs. 13,14). The LRT also rejected the user-defined trees that *H. reticulata* is at the base of the Chlorophyceae (tree 16 in Table 6, tree 16 in Table 7).

O'Kelly *et al.* (1994) also showed similarities between *Hafniomonas* and the Chaetopeltidales in the flagellar apparatus architecture, such as tetralobose distal fiber and electron-dense components that surround the d rootlets. However, the LRT also denied the

sister group relationship between *H. reticulata* and *Planophila terrestris* (tree 17 in Table 7). Therefore, it is suggested from the present analyses that characters of *Hafniomonas* such as flagellar pit, CCW offset of basal bodies, and daughter nuclei remaining widely separated during cytokinesis, are not primitive but apomorphic (reversion), and *Hafniomonas* is not an "ancestral" chlorophycean alga.

Phylogeny within the Chlorophyceae

Analysis of the 18SrDNA from the Chaetopeltidales (*Planophila terrestris*) and Chaetophorales (*Chaetophora incrassata*) provided some suggestions about the systematics and phylogeny of the Chlorophyceae. In 18SrDNA trees, these algae are included in the Chlorophyceae, which supports the hypothesis that progressive clockwise rotation of the basal bodies has occurred in the chlorophycean line (O'Kelly and Floyd 1984), because all algae that have DO or CW basal bodies form a clade (*Hafniomonas* is an exception, see above). The Chlorophyceae seems to share DO flagellar apparatus as a synapomorphic character, and CW basal bodies in the CW group is thought to be the character derived from DO basal bodies. The Sphaeropleales and the CW group form a clade in all trees and this relationship is supported by bootstrap analyses (especially in the MP method; Fig. 14). Buchheim and Chapman (1992) reported same relationship based on partial 18S/26S rRNA sequences (but not including the Chaetopeltidales). This topology seems to support the idea that the Chaetopeltidales is the ancestral chlorophycean algae and the hypothesis that the quadriflagellate condition (seen in the Chaetopeltidales and Chaetophorales) is primitive in the Chlorophyceae (O'Kelly and Floyd 1984, O'Kelly 1992, O'Kelly *et al.* 1994). However, results of LRT demonstrate that there are no significant differences between the ML tree and most of user-defined trees representing the phylogenetic relationships of the Sphaeropleales, the CW group, *Planophila* and *Chaetophora* (trees 1-15 in Table 7). So, I cannot confirm the phylogenetic relationships of these four clades from the present analyses. From the results it appears that *Planophila* and *Chaetophora* have no close relationship to each other or to two large clades (Sphaeropleales and the CW group) and that they do not belong to either the Sphaeropleales or the CW group (Chlamydomonadales/Chlorococcales) but belong to independent orders (i.e. Chaetopeltidales and Chaetophorales) (Watanabe and Floyd 1989a; O'Kelly *et al.* 1994). So, ultrastructural and molecular characters are congruent. However, I analyzed 18SrDNA sequences from only one representative of the Chaetopeltidales and one from the Chaetophorales. A limited taxon sampling will influence the resolution of

phylogenetic relationships (taxon sampling effect; Buchheim and Chapman 1992, Helmchen *et al.* 1995). The relationships within the Chlorophyceae may be resolved with the addition of more sequences from various species.

The Sphaeropleales

The clade of sphaeroplealean algae (*Neochloris*, *Characiopodium*, *Hydrodictyon*, *Pediastrum*) includes some autosporic coccoid algae (*Scenedesmus* and *Grasiella*) as reported by Wilcox *et al.* (1992), and the monophyly of this clade is supported by high bootstrap values (Fig. 14). Synapomorphic morphological features would also support the monophyly of the clade. These autosporic algae and the sphaeroplealean algae share pyrenoids covered by a continuous starch sheath with no invaginations and no appressed membranes (Kalina and Puncochárová 1987, Pickett-Heaps 1975, Watanabe and Floyd 1989b, Floyd *et al.* 1993). This pyrenoid type can be estimated as a synapomorphic feature of the Sphaeropleales. The trilaminor (TL) layer containing sporopollenin on the outermost cell wall may be also a synapomorphic feature of these algae (Millington and Gawlik 1970, Atkinson *et al.* 1972, Pickett-Heaps 1975). Some species of the genus *Prototheca* also have TL-layer probably containing sporopollenin (Atkinson *et al.* 1972). However, Kalina (1993) demonstrated that cell wall development in *Auxenochlorella protothecoides* (Kremér) Kalina et Puncochárová (= *Chlorella protothecoides*), an alga closely related to *Prototheca* (Huss and Sogin 1990), differs from that of *Scenedesmus* (Pickett-Heaps 1975). This fact suggests that TL-layers of *Prototheca* and *Scenedesmus* are not homologous. Distant relationship of these algae in 18SrDNA tree (Fig. 13) supports this inference.

Although 18SrDNA phylolgeny supports the monophyly of the Sphaeropleales *sensu* Deason *et al.* (1991) at present, I suggest the possibility that the Sphaeropleales is not monophyletic but paraphyletic, because no synapomorphic characters are found between the Sphaeropleaceae (*Sphaeroplea, Atractomorpha*) and other members of this order. The characters shared by the Neochloridaceae and Hydrodictyaceae *sensu* Deason *et al.* (1991), such as distal fiber with ribbed structure, partial caps, striated fiber and (probably) trilaminor layer, are absent in the sphaeropleacean algae (e.g. Hoffman 1984; Buchheim and Hoffman 1986). Motile cells with directly opposed basal bodies, which is definitive character of the Sphaeropleales *sensu* Deason *et al.* (1991), is apparently symplesiomorphic feature. Another common character in this order is multinucleate vegetative cell, but frequent occurrences of multinucleate condition in the Chlorophyta (see Chapter 5, 6) decrease the importance of this

feature.

Phylogeny of quadriflagellate green algae

O'Kelly and Floyd (1984) pointed out the possibility that quadriflagellate condition is ancestral state in the Chlorophyceae. If it is so, how can quadriflagellate state of *Carteria radiosa* and *Polytomella parva* be interpreted?

Carteria radiosa has basal bodies displaced slightly CW configuration (Watanabe pers. comm.). *Pseudocarteria mucosa* (Korshikov) Ettl, which has similar flagellar apparatus to *C. radiosa* and probably closely related to it, also has CW basal bodies (Suda *et al.* 1990). Phylogenetic position of *C. radiosa* inferred from 18SrDNA data agree with this ultrastructural character. 18SrDNA trees favor that quadriflagellate condition of *C. radiosa* derived from biflagellate condition. This would be understandable if sexual reproduction of *Carteria* species is considered. The quadriflagellate state of the genus *Carteria* would not be homologous with that of the Chaetophorales or Ulvophyceae. Gametes of the Chaetophorales are biflagellate and not quadriflagellate (e.g. Singh 1942). As far as known, gametes are also biflagellate in the Ulvophyceae without any exception. In contrast, gametes of *Carteria* and a related genus, *Pseudocarteria* have four flagella as vegetative cell (Wandschneider and Kies 1978, Suda and Watanabe 1995). This fact suggests that quadriflagellate condition of *Carteria* is not primitive but derived character originated by doubling of flagella from biflagellate ancestor like *Chlamydomonas*.

Polytomella parva is also included in the CW group, and is closely related to *Chlamydomonas reinhardtii* and *Volvox carteri*. High bootstrap values support the monophyly of *Polytomella/C. reinhardtii/Volvox* clade (Fig. 14). However, it should be noted that the 18SrDNA of *P. parva* had a low GC content (44.9%). Because base compositional bias has an influence on the reconstruction of phylogeny, and distantly related sequences that have similar GC content sometimes group together (Hasegawa and Hashimoto 1993; Steel *et al.* 1993; Lockhart *et al.* 1994), care is need when analyzing the phylogenetic position of this alga. However, slightly higher GC contents are found in 18SrDNA from *Chlamydomonas reinhardtii* and *Volvox carteri* (50.1%, 49.5% respectively) in comparison with other chlorophycean algae in the analysis (48.6% ±1.1%), and so a low GC content of *P. parva* would have no effect on the phylogenetic trees obtained in this study. This phylogenetic position of *P. parva* strongly suggests that quadriflagellate condition of this alga is not ancestral but derived. Brown *et al.* (1976) demonstrated

ultrastructure of the flagellar apparatus of *Polytomella agilis*. Basal bodies of *P. agilis* are connected by a distal fiber, two proximal fibers and extension of proximal sheaths. Brown *et al.* (1976) showed non-overlapping CCW basal bodies, but this is most likely a mirror-image, because in Fig. 10 of their paper "s" rootlet is situated in the right side of the basal body. So, *P. agilis* possibly has the flagellar apparatus of CW condition. These ultrastructural characters support that *P. agilis* is a member of the CW group. Furthermore, the presence of eight microtubular rootlets strongly suggest that their quadriflagellate condition is derived from biflagellate condition by doubling.

In conclusion, 18SrDNA analyses and organismal characters suggest that quadriflagellate condition of *Carteria* and *Polytomella* are not primitive but derived characters.

Phylogeny and taxonomy of the Dunaliellales

Cell walls of chlamydomonadalean algae have been considered to arisen from the theca of the Tetraselmidales (Mattox and Stewart 1977). However, recent chemical analyses (Becker *et al.* 1991) demonstrated that the sugar composition of the theca of tetraselmidalean algae was the same as that of prasinophycean scales but distinct from that of *Chlamydomonas*. From this result it may be possible to deduce that the "cell wall" of the Tetraselmidales and that of the CW group are not homologous and may have independent origins (Becker *et al.* 1991). If so, ancestral chlorophycean alga would have had naked flagellate cells, and the wall-less nature of flagellate cells of the Ulvophyceae, Trebouxiophyceae and Chlorophyceae (except the CW group) would be regarded as a primitive character. Our study suggests that the absence of a cell wall in *Oltmannsiellopsis* is also a symplesiomorphic state. The halophilic dunaliellalean algae, *Dunaliella* and *Asteromonas,* are sometimes considered to be more primitive than the Chlamydomonadales, and their wall-less state is thought to be primitive (Melkonian 1990b; Segaar 1991). Our present analysis, however, shows that the secondary loss of cell wall in these algae is in agreement with the views of other phycologists (Domozych *et al.* 1980; Chappell *et al.* 1989). 18SrDNA analysis demonstrated two monophyletic groups that are comprised of naked and walled flagellates (*Dunaliella/Asteromonas* and *Chlamydomonas applanata*; *Polytomella* and *C. reinhardtii/Volvox*) in the CW group. Because ultrastructural, chemical and molecular similarities strongly suggest that the cell walls of chlamydomonadalean algae are not homoplasies but homologous characters (Miller 1978; Woessner and Goodenough 1994; see discussion in Nakayama *et al.* 1996), wall-less states in the *Dunaliella/Asteromonas* clade

and *Polytomella* probably originated independently from each other by secondary losses of the cell wall. Chappell *et al.* (1989) implied that the surface coat of *Dunaliella* and strip-like covering of *Asteromonas* was evolutionarily reduced from the cell wall, and this hypothesis is supported by the present study.

In conclusion, 18SrDNA analyses partially support but mostly deny the hypothesis that dunaliellalean algae are intermediate between primitive green flagellates such as prasinophytes and advanced chlorophycean algae (O'Kelly and Floyd 1984, O'Kelly *et al.* 1994, Segaar 1991, Sym and Pienaar 1991). *Oltmannsiellopsis* is an early divergence of the Ulvophyceae/Trebouxiophyceae/Chlorophyceae clade, and its CCW basal bodies and lack of cell wall are interpreted as primitive characters. However, 18SrDNA analyses suggest that the CCW flagellar apparatus and other "ancestral" characters of *Hafniomonas* are not primitive but reversionary. Furthermore, present study provides strong grounds that the wall-less nature of *Dunaliella*, *Asteromonas* and *Polytomella* is not symplesiomorphic but an advanced character (secondarily-lost).

The present study also gives some taxonomic implications about the concept of the Dunaliellales. It is now evident that the wall-less condition in the dunaliellalean algae (*Oltmannsiellopsis*, *Polytomella*, *Dunaliella*) is of multiple origins, and thus the Dunaliellales *sensu* Ettl (1981) is a polyphyletic group (*sensu* Hennig 1966). Melkonian (1990b) restricted the Dunaliellales to two halophilic algal genera, *Dunaliella* and *Asteromonas*. The monophyly of the Dunaliellales *sensu* Melkonian was supported by 18SrDNA analysis, but the phylogenetic trees show that *Chlamydomonas applanata* is more closely related to the Dunaliellales *sensu* Melkonian than to other members of the CW group. This suggests that the Dunaliellales *sensu* Melkonian is only a part of the CW group (Chlamydomonadales/Chlorococcales complex) and I believe it is not necessary to separate them at the order level.

CHAPTER 5. PHYLOGENY WITHIN THE ULVOPHYCEAE

INTRODUCTION

Ultrastructural studies have raised an unexpected view that marine macroscopic green algae belong to different evolutionary lineage from the chlorophycean member such as *Chlamydomonas*, and these algae were classified into the Ulvophyceae (Stewart and Mattox 1978; Mattox and Stewart 1984). The class Ulvophyceae is characterized by basal bodies arranged in counterclockwise (CCW) absolute orientation, a persistent interzonal spindle and cytokinesis mediated by a centripetal furrow associated with neither phycoplast nor phragmoplast (e.g. Mattox and Stewart 1984; O'Kelly and Floyd 1984b). However, this concept of the Ulvophyceae has been criticized by cladistic taxonomists, because definitive characters of this class mentioned above are not apomorphic but symplesiomorphic (e.g. Bremer 1985; Mishler and Churchill 1985). The CCW configuration of basal bodies is widely found in the green algae (except Chlorophyceae) including the "primitive" green flagellates, the Prasinophyceae (e.g. O'Kelly and Floyd 1984; Sym and Pienaar 1993). It is difficult to evaluate mitotic/cytokinetic features of the Ulvophyceae. These characters were estimated to be primitive condition of the green plants as CCW basal bodies (e.g. Bremer 1985). However, recent finding of early divergence of the Tetraselmidales in the Chlorophyta *sensu stricto* (Steinkötter *et al.* 1994; Friedl and Zeltner 1994; see also Chapter 4) suggest that a phycoplast is synapomorphic character of this division and secondarily lost in the Ulvophyceae. If so, loss of phycoplast should be an apomorphic feature of the Ulvophyceae. Using freeze fixation, Sluiman (1991), however, showed that ulovophycean alga, *Gloeotilopsis planctonica*, had a cytokinesis mediated by microtubular system (phycoplast) which originates from centrioles (see also Sluiman 1993). The persistent interzonal spindle at telophase, which is another definitive feature of the Ulvophyceae, also seems to be problematic (see Sluiman 1993). Furthermore, vegetative cell division (desmoschisis or "Zellteilung"; see Sluiman *et al.* 1989) of the multinuclear ulvophytes has not been studied ultrastructurally, or in these algae vegetative cell division itself is absent. These evidences suggest that presently well accepted mitotic/cytokinetic features of the Ulvophyceae should be re-investigated. Therefore, it is uncertain whether or not, the definitive characters of the Ulvophyceae is plesiomorphic. So, monophyletic nature of the Ulvophyceae is ambiguous.

The Ulvophyceae includes many macroscopic and microscopic green algae inhabiting marine water (rarely in freshwater or soil), and these algae show great diversity in vegetative morphology, reproduction, life history and ultrastructure. O'Kelly and Floyd (1984b) reviewed various characters (flgellar apparatus, reproduction, development, life history) of the Ulvophyceae, and discussed the classification and evolution within this class. They recognized five orders in the Ulvophyceae. Those are the Ulotrichales, Ulvales, Siphonocladales, Dasycladales and Caulerpales (Table 8). Different systems were proposed by Sluiman (1989) and van den Hoek *et al.* (1995). Sluiman (1989) included the Pleurastrales (and two enigmatic groups, Trentepohliales and Ctenocladales) in the Ulvophyceae, and treated the Ulotrichales and Ulvales as single order (Ulotrichales *sensu* Sluiman). In his system, the Acrosiphoniaceae, which is a member of the Ulotrichales in O'Kelly and Floyd's system, is regarded as an independent order (Table 8). The classification system of van den Hoek *et al.* (1995) is different from both systems, that is, they raised most orders to independent classes (Table 8). The Codiolales (= Ulotrichales *sensu* O'Kelly and Floyd) and Ulvales *sensu* O'Kelly and Floyd are merged into a single group as in Sluiman's system but at the class level, the Ulvophyceae *sensu stricto*. The two orders are retained as separate orders in this class. van den Hoek *et al.* (1995) included acrosiphoniacean algae into the Codiolales as in O'Kelly and Floyd's system (Table 8).

In this chapter, I analyzed phylogeny of the Ulvophyceae using 18SrDNA sequences to answer the following questions.

1) Is the Ulvophyceae monophyly or not?
2) How can groups be recognized in the Ulvophyceae?
3) what are the phylogenetic relationships and evolution of morphological characters within the Ulvophyceae?

MATERIALS AND METHODS

I used two data sets of 18SrDNA sequences of various ulvophycean algae. First data set (data set A; 1676 bp) consisted of thirty three sequences including those from the Ulotrichales, Ulvales, Siphonocladales, Dasycladales, Chlorophyceae, Trebouxiophyceae and Prasinophyceae. Two pseudoscourfieldialean sequences were used as outgroups. Second data set (data set B; 1772 bp) include ten sequences from the Ulotrichales and Ulvales, and four sequences of outgroups. The MP (by PAUP with heuristic search from

unweighted sequences) and NJ (by Clustal W) methods were used to infer the phylogenetic relationship of these algae. Bootstrap analyses (100 replications), in both MP and NJ methods, were performed to evaluate statistical reliability (see General Materials and Methods).

I investigated ultrastructure of the motile cells of *Monostroma latissimum* and *Halochlorococcum* sp. Biflagellate motile cells of the former were collected from natural samples (see Table 1). Strains of *Halochlorococcum* sp. was established by isolation of cells from enrichment culture of a sample collected from Funaura, Iriomote Is., Japan. This cultures was grown in ESM medium (see Watanabe and Nozaki 1994) at 20 °C under the light of 20-30 μEM^{-1} s^{-1} from white fluorescent tubes. Quadriflagellate zoospores were produced when the old cells (after one month) were transferred to new medium.

Materials for thin sections were prepared as follows. Equal volume of fixative (5% glutaraldehyde, 0.5 M sucrose, in 0.2 M cacodylate buffer [pH 7.2]) and culture medium containing growing cells were mixed together at 4 °C for 1 h. The fixative containing small amount of 2% osmium tetraoxide was also used. After rinsing twice (10 min each) with 0.1 M cacodylate buffer, cells were postfixed with 2% osmium tetraoxide at 4 °C for 1 h, then rinsed once with the same buffer. Cells were embedded in Spurr's resin (Spurr 1969) after dehydration in a graded ethanol series. Sections were cut with diamond knife and collected on slot grids coated by formvar. Grids were double stained with 2% uranyl acetate and Reynolds' lead citrate (Reynolds 1963). Observations were carried out using a JEOL JEM 100CXII transmission electron microscope.

RESULTS

Three MP trees were found in the MP analysis using data set A (1922 steps, CI = 0.540). Strict consensus tree of theses MP trees is shown in Fig. 15. NJ tree constructed from data set A is shown in Fig. 16. Both trees indicated that the ulvophycean algae used in this study divided into two distinct lineages. The one lineage consisted of the Ulotrichales and Ulvales *sensu* O'Kelly and Floyd (1984b) and this lineage was supported by 100% bootstrap values in both the MP and NJ methods. The other lineage included the Siphonocladales (= Cladophorales) and Dasycladales and it was also supported by 100% bootstrap values. In this lineage, monophyly of both orders were also clearly supported by bootstrap analyses. The monophyly of the Ulvophyceae *sensu* Mattox and Stewart (1984) was not resolved in

these analyses. *Prasiola japonica*, a member of the Prasiolaceae, formed a clade with *Chlorella ellipsoidea* (89 - 92% bootstrap supports) and was situated in the Trebouxiophyceae (monophyly of this class was not resolved in the MP analysis, but see Friedl 1995).

The MP (663 steps, CI = 0.698) and NJ trees constructed from data set B are shown in Fig. 17a and b, respectively. These trees revealed that four distinct clades exist in the Ulotrichales/Ulvales clade. These were the *Monostroma/Gloeotilopsis* clade, Acrosiphoniaceae (*Urospora, Acrosiphonia*), *Halochlorococcum* spp. and Ulvales *sensu* O'Kelly and Floyd (*Entocladia, Enteromorpha, Ulva*). Monophyly of each clade was well resolved in the bootstrap analyses (≥95%). Both MP and NJ tree suggested that *Halochlorococcum* was sister group of the Ulvales (76 - 95% bootstrap supports). The Acrosiphoniaceae formed a clade with the *Halochlorococcum*/Ulvales lineage in the MP tree (with 56% bootstrap value), but this family showed sister group relationship to the *Monostroma/Gloeotilopsis* clade in the NJ analyses (100% bootstrap support). In the *Monostroma/Gloeotilopsis* clade, *Monostroma* was not monophyletic in both analyses (with 53 - 83% bootstrap values). The Ulvaceae (*Ulva* and *Enteromorpha*) was apparently monophyletic in both analyses (100% bootstrap supports).

I determined the complete 18SrDNA sequence from *Bryopsis maxima* Okamura. However, this sequence was unusual in comparison with sequences of other green algae. I found that this sequence had different nucleotides at many sites which had consistent nucleotides in all green plants, and it was difficult to align this sequence with others. Phylogenetic position of this sequence was unstable, because some trees showed sister-group relationship with the Siphonocladales/Dasycladales, and other trees failed to include this sequence in the Viridiplantae.

The biflagellate motile cells of *Monostroma latissimum* have no scales but are covered by amorphous material (Plate 9-5,6). Other features including the flagellar apparatus of this swamer are consistent with those of *Monostroma oxyspermum* (= *Gayralia oxysperma*) (Hoops *et al.* 1982; O'Kelly *et al.* 1984) as will be mentioned below.

1) Each basal body has Ulotrichales-type terminal cap (Plate 9-2), proximal sheath (Plate 9-2)consists of two unequal subunits and circular structure (Plate 9-1,2,4) at the proximal end (the last structure is absent in *M. oxyspermum*; Hoops *et al.* 1982).

2) CCW overlapping basal bodies (Plate 9-1) are connected each other by distal fiber, proximal fiber (not cited in Hoops *et al.* [1982], but see Fig. 14) and connection between the

proximal sheathes (Plate 9-3,4).
3) The flagellar apparatus has cruciate flagellar roots (Plate 9-1) and contains d (two microtubules) and s (three over one microtubules) microtubular roots, system I fibers (= SMAC in Hoops *et al.* 1982) underlying d roots and two unequal rhizoplasts extending under the d root and system I fiber (Plate 9-3,4,5,6).
4) Cells posses membrane-associated plaque (O'Kelly *et al.* 1984) which has association with one of two d roots and rhizoplasts (Plate 9-6).

The strain of *Halochlorococcum* sp. produced only quadriflagellate zoospores which are covered by body scales which lack distinct substructure (Plate 9-8). The flagellar apparatus of this zoospore is similar to those of the quadriflagellate cell of ulotrichalean algae (Sluiman *et al.* 1980; Floyd and O'Kelly 1984; Lockhorst and Star 1986; Watanabe and Floyd 1992). Characterization of the flagellar apparatus of *Halochlorococcum* sp. is as follows.
1) Upper basal body has proximal sheath consisting of two unequal subunits (Plate 9-12) and terminal cap which completely cover the proximal end of the basal body (Plate 9-7,12). Terminal cap has no distinct bilobed structure found in the Ulvales (e.g. O'Kelly *et al.* 1984). All basal bodies have electron dense core (Plate 9-7,10).
2) Upper basal bodies overlap and are arranged in CCW absolute orientation. They are connected by distal fiber, proximal fiber and the connection between the proximal sheathes.
3) The flagellar apparatus is cruciate and has d (two microtubules; Plate 9-10) and s (three over one microtubules; Plate 9-9) microtubular roots. Rhizoplast which originates from each lower basal body extends under the s root (Plate 9-11,12). System I fibers (= SMAC) underlie d roots.

The flagellar apparatuses of motile cells from *Monostroma latissimum* and *Halochlorococcum* sp. are diagrammed in Fig. 18.

DISCUSSION

Enigmatic sequence of *Bryopsis maxima*

Unusual 18SrDNA sequence from *Bryopsis maxima* (Caulerpales *sensu* O'Kelly and Floyd) is enigmatic. This sequence is inconsistent not only with that of other ulvophycean algae but also with partial 18SrRNA sequences from various caulerpalean algae reported by Zechman *et al.* (1990). Five representatives of the Caulerpales in Zechman *et al.* (1990) have consistent sequences, and phylogenetic tree generated from these sequences is suited for the

traditional hypothesis of the caulerpalean phylogeny. So, my sequence may not be of *Bryopsis*. However, partial 18SrDNA sequence from *Codium divaricatum,* which was collected from different site from that of *Bryopsis*, corresponds to sequence of *Bryopsis*. I suppose three probable reasons of this situation; 1) sequence of gene (DNA) is modified through or after the transcription in caulerpalean algae (e.g. RNA editing), 2) present sequence is of pseudogene (nonfunctional copy of the 18SrDNA), or 3) sequences in this study are not from *Bryopsis* and *Codium* but from DNAs of contaminants. Caulerpalean algae have internal endophytes or endozoa which may cause contamination of DNA, and present sequence of *Bryopsis* can be reconstructed into normally accepted secondary structure. So, I feel that the last reason is more possible. However, it should be noted that I found no similar sequence from the large database of eukaryotic 18SrDNA sequences.

Is the Ulvophyceae monophyletic?

18SrDNA analyses clearly suggested that the Ulvophyceae *sensu lato* is divided into two distinctive groups, Ulotrichales/Ulvales and Siphonocladales/Dasycladales. The monophyly of each clade is supported by long branch and high bootstrap values in both MP and NJ analyses, and therefore seems to be acceptable (but see later). Zechman *et al.* (1990) reported the same phylogeny based on partial 18S/26S rRNA sequences (but their analysis included the Caulerpales and Trentepohliales, and statistical test was not performed).

18SrDNA analyses cannot resolve whether or not the Ulotrichales/Ulvales and Siphonocladales/Dasycladales, namely, the Ulvophyceae, form a monophyletic clade. Phylogenetic trees suggest that the divergence of these two lineages is almost simultaneous to that of the Trebouxiophyceae and Chlorophyceae. To resolve the branching order between these clades, more species or sequences of 18SrDNA or other genes may be required. However, it is apparent that there is no synapomorphic character shared by the Ulvophyceae *sensu* Mattox and Stewart (see Introduction), so classification system of van den Hoek *et al.* (1995), in which most ulvophycean orders are treated as independent classes, seems to be acceptable. The presence of fossil record of dasycladalean algae in the early Cambrian period (ca. 600 million years ago) (Tappan 1980; Berger and Kaever 1992) suggests that radiation of major ulvophycean clades (and other chlorophytan clades) took place in the Precambrian period.

O'Kelly and Floyd (1984b) proposed the phylogenetic hypothesis of the Ulvophyceae that the Ulotrichales is most primitive member and the Siphonocladales, Dasycladales and

Caulerpales evolved through the multinucleate member of the Ulvales (see also Floyd and O'Kelly 1990). Present study, however, apparently contradicts to this hypothesis, because the Ulotrichales and Ulvales form a robust monophyletic clade. So the direct evolution of three multinucleate orders from the Ulvales is unlikely (see also Chappell *et al.* 1990). Furthermore, present result suggests that elimination of "primitive" features, such as scale covering, is not a single event but have occurs at least twice in the Ulotrichales/Ulvales and the Siphonocladales/Dasycladales (see also later).

Close relationship between the Siphonocladales and Dasycladales was suggested especially based on similarity of the flagellar apparatuses (O'Kelly and Floyd 1984b; Floyd *et al.* 1985; see also Roberts *et al.* 1984). They share flattened aspect of the flagellar apparatus and "wing" which is the connection between upper and lower microtubules of s root. However, it should be noted that flattened flagellar apparatus is not only characteristic of this two orders, but also of the Trentepohliales and most members of the Trebouxiophyceae (e.g. Chapman and Henk 1985; Melkonian and Peveling 1988). The "wing" is also found in other green algae such as *Oltmannsiellopsis*, *Nephroselmis* and *Mesostigma* (Moestrup and Ettl 1979; Melkonian 1989; Lokhorst and Star 1993). So, it should be careful to evaluate morphological synapomorphic features shared by the Siphonocladales and Dasycladales. The monophyly of these two orders is supported by 18SrDNA analyses, but their long branch lengths warn its artificiality caused by "long branch effect" (Felsenstein 1978).

Phylogeny within the Ulotrichales and Ulvales

As discussed above, present study clearly suggest that the monophyly of the Ulotrichales and Ulvales. This evidence supports the taxonomic treatment that this two orders are merged to a single taxon (e.g. class Ulvophyceae *sensu* van den Hoek *et al.* 1995). This clade shares the flagellar apparatus with developed proximal sheathes, system I fibers underlying d roots, rhizoplasts associated (but not connected) with microtubular roots (d roots in biflagellate cells, s [and d] roots in quadriflagellate cells) and terminal caps (e.g. Floyd and O'Kelly 1984). Some of these may be symplesiomorphic, but the last seems to be synapomorphic feature of the Ulvophyceae *sensu* van den Hoek *et al.* Terminal caps are also reported from other green algae (e.g. Caulerpales, Trebouxiophyceae; e.g. Roberts *et al.* 1982; Melkonian and Berns 1983), but they are not directly attached to the proximal end of the basal bodies, so that they may not be homologous to those of the Ulvophyceae *sensu* van den Hoek *et al.*

Terminal caps of the Ulotrichales and Ulvales show distinctive appearance from each other, but share the bilobed structure directly attached to the proximal end of the basal bodies (e.g. O'Kelly *et al.* 1984; Watanabe and Floyd 1992), so they can be regarded as synapomorphic character of the Ulvophyceae *sensu* van den Hoek *et al.*

The Acrosiphoniaceae has some specialized characters such as multinucleate cell and synchronous mitosis (but some members lack these features), and is sometimes treated as member of the Cladophorales (e.g. Jónsson 1959) or an independent taxon (e.g. Sluiman 1989). Present study suggest that this family is ingroup of the Ulotrichales/Ulvales. The inclusion of the Acrosiphoniaceae into the Cladophorales (= Siphonocladales *sensu* O'Kelly and Floyd 1984b) is not acceptable as suggested from features of life cycle, cell wall and ultrastructure (e.g. Kornmann 1973; Floyd and O'Kelly 1984). So, the multinuclear condition would have evolved several times in the Chlorophyta *sensu stricto* (e.g. Acrosiphoniaceae, Cladophorales and some members of Chlorophyceae [see Chapter 6]). The MP analysis showed that the Acrosiphoniaceae (*Acrosiphonia* and *Urospora*) is more closely related to the Ulvales than to the Ulotrichales *sensu* O'Kelly and Floyd (1984b) (but bootstrap value is very low). However, this family apparently formed a clade with ulotrichalean algae in the NJ analysis (100% bootstrap support). The latter result is more agreeable to the evidence that the Acrosiphoniaceae usually has *Codiolum*-type sporophyte (or zygote, see van den Hoek *et al.* 1995) as the other ulotrichalean algae (e.g. Kornmann 1963, 1973). Presence of intermediate species (*Chlorothrix*) between *Ulothrix* and *Urospora* supports this consideration (e.g. Berger-Perrot 1980). The flagellar apparatus also seems to support the merger of the Acrosiphoniaceae to the Ulotrichales (e.g. Floyd and O'Kelly 1984), but it should be noted that common ultrastructural characters of these algae may be symplesiomorphic. So, it should be careful to assess the phylogenetic position of the Acrosiphoniaceae, but the classification systems that the Ulotrichales/Ulvales clade is divided into the Acrosiphoniales and other algae (i.e. Ulotrichales *sensu* Sluiman [1989]) is not acceptable based on the molecular analyses.

The 18SrDNA analyses showed an interesting phylogenetic position of the Chlorocystidaceae (i.e. *Halochlorococcum* spp.). *Halochlorococcum* is usually classified into the Ulotrichales *sensu* O'Kelly and Floyd (= Codiolales) because closely related genus, *Chlorocystis*, has *Codiolum*-type sporophyte (zygote) (Kornmann and Sahling 1983). However, molecular trees indicate close relationship of *Halochlorococcum* to the Ulvales. This suggestion contradicts the evaluation of *Codiolum*-type sporophyte as synapomorphic

feature (see Floyd and O'Kelly 1984). I feel, however, it is difficult to estimate pattern of life cycle and development in the Chlorocystidaceae because of its unicellular nature. Interestingly, the flagellar apparatus of *Halochlorococcum* seems to support the affinity of this alga to the Ulvales. *Halochlorococcum* sp. has the terminal cap, which has simple structure and almost completely covers the proximal end of the upper basal body. This condition seems to be intermediate form between the simple ulotrichalean terminal cap covering only anterior side of the basal body and the ulvalean distinct bilobed terminal cap covering completely proximal end of basal body (see Floyd and O'Kelly 1984; O'Kelly and Floyd 1984b).

It is difficult to evaluate synapomorphic feature of the *Monostroma/Gloeotilopsis* clade, because their most ultrastructural features seem to be symplesiomorphic. However, I suggest that the loss of electron dense core in the basal body is possible synapomorphic feature of the Ulotrichales *sensu* O'Kelly and Floyd excluding the Acrosiphoniaceae and Chlorocystidaceae. This structure is present in the Acrosiphoniaceae (Floyd and O'Kelly 1984; Miyaji and Hori 1984; but absent in *Urospora*, e.g. Sluiman *et al.* 1982), Chlorocystidaceae (this study) and Ulvales (e.g. O'Kelly and Floyd 1983; O'Kelly *et al.* 1984), but it is absent in other ulotrichalean algae (e.g. Sluiman *et al.* 1980; Hoops *et al.* 1982; Floyd and O'Kelly 1984; Lokhorst and Star 1986; Watanabe and Floyd 1992). O'Kelly and Floyd (1984b) classified within the Ulotrichales using the presence or absence of scales and shape of thallus in early development of gametophyte. Because available 18SrDNA sequences from the Ulotrichales are limited, usefulness of the latter is uncertain, but that of the former is questionable. The presence of body scales in *Halochlorococcum* and absence in *Monostroma latissimum* clearly suggest that lack of body scales occurred independently several times in the Ulvophyceae *sensu* van den Hoek *et al.* (at least three times). So it should be careful to evaluate the evolutionary change of this character.

The 18SrDNA analyses clearly support that leaf-like frond evolved many times in the green algae. This organization had been principal character of the Ulvales in traditional sense, but would have been acquired independently in the Ulotrichales, Ulvales and Prasiolales (and may be in the Chaetophorales). Close relationship between *Entocladia* and the Ulvaceae also indicates the convergence of vegetative organization. *Entocladia* has been often classified into the Chaetophorales in traditional sense, but this alga is apparently a member of the Ulvales as suggested from its flagellar apparatus (O'Kelly and Floyd 1983). These results further supports the consideration that convergent evolution of the vegetative

morphology frequently occurred in the green algae.

Phylogenetic position of *Prasiola*

The genus *Prasiola*, which is monostromatic macroscopic green algae, has unique characters such as stellate chloroplast, oogamy and haploid gametophyte remaining part of the original diploid thallus (but there are contradictory reports; see van den Hoek *et al.* 1995). *Prasiola* is classified into the Prasiolaceae with some other algae possessing the same features (e.g. *Rosenvingiella*), but the taxonomic position of this family has been uncertain. Traditionally, this family was included in the Ulvales (in traditional sense) based on its leaf-like thallus (e.g. Bold and Wyne 1985), but recently it has been treated as independent order because of its unique features mentioned above (e.g. van den Hoek *et al.* 1995).

18SrDNA analyses clearly suggest the close affinity of *Prasiola japonica* to the Trebouxiophyceae. This relationship is not unexpected but has been suggested by the ultrastructural architecture of the flagellar apparatus. The male gametes (only motile stage in the Prasiolaceae) have "terminal cap" which resembles more to the plate-like platform (sometimes called "terminal cap") of some trebouxiophycean algae than to the terminal caps of ulvophycean algae (O'Kelly *et al.* 1989). Other unique features of the prasiolacean flagellar apparatus such as non-overlapping CCW basal bodies, lack of d root, striated root (O'Kelly *et al.* 1989) are regarded as specialized (autapomorphic) characters of this family. However, limited information about the mitosis and cytokinesis of *Prasiola* (Lokhorst and Star 1988) contradicts the assignment of this alga to the Trebouxiophyceae. Presence of a central stellate chloroplast is another character which suggests trebouxiophycean affinities of *Prasiola*. 18SrDNA analysis, however, did not indicate close relationship between *Prasiola* and *Trebouxia*, a trebouxiophycean representative which has a stellate chloroplast. In conclusion, present study suggested that the Prasiolaceae is a member of the Trebouxiophyceae, but further studies (molecular and ultrastructural) is needed to clarify the robust phylogenetic position of this enigmatic green alga.

CHAPTER 6. PHYLOGENY OF THE CHLAMYDOMONADALES AND CHLOROCOCCALES

INTRODUCTION

Lewis *et al.* (1992) studied nuclear-encoded small subunit ribosomal RNA gene (18SrDNA) of 'chlorococcalean' algae possessing three different flagellar apparatus types (CCW, DO and CW). The phylogenetic tree based on 18SrDNA sequences did not support taxonomic system based on the vegetative morphology, but supported that based on the flagellar apparatus (Lewis *et al.* 1992). The flagellar apparatus is therefore thought to reflect the evolutionary relationships of these algae. From this point of view, the relationship between the Chlamydomonadales and Chlorococcales *sensu stricto* should be reevaluated. These two orders share the CW flagellar apparatus, and differ from each other only in vegetative form (flagellate vs. coccoid) (Watanabe and Floyd 1989a). A question to be answered is whether the vegetative morphology reflects the phylogenetic relationships within and between these two orders or not.

Ettl (1981) established the class Chlamydophyceae to include the green algae that produce walled zoospores. In the Chlamydophyceae, he recognized four orders: the Chlamydomonadales, Volvocales, Tetrasporales and Chlorococcales. Coccoid green algae producing wall-less zoospores were assigned to the Chlorophyceae *sensu* Ettl (Ettl 1981, Ettl and Komárek 1982). However, from the ultrastructural view mentioned above, the Chlamydophyceae and Chlorophyceae *sensu* Ettl are difficult to distinguish. For example, *Protosiphon* is assigned to the Chlorophyceae *sensu* Ettl because it has wall-less zoospores, but these zoospores have the CW flagellar apparatuses like those of *Chlorococcum* which produces walled zoospores (Watanabe and Floyd 1989a). Deason (1984) and Mattox and Stewart (1984) raised the question about the concept of the Chlamydophyceae, and it should be resolved whether the Chlamydophyceae and Chlorophyceae *sensu* Ettl are natural assemblages.

In the present study, I determined 18SrDNA sequences from *Chlamydomonas moewusii* and five chlorococcalean algae. I analyzed these sequences to infer the phylogenetic relationship between the Chlamydomonadales and Chlorococcales. The concept of the Chlamydophyceae and taxonomic value of wall condition was also evaluated by sequence comparison.

MATERIALS AND METHODS

I used data set including sequences from twenty five chlorophycean algae and outgroups. The sequences of *Chlorella vulgaris* and *Trebouxia impressa* were used as outgroups, because these algae were situated in the sister group of the Chlorophyceae in previous 18SrDNA studies (see Chapter 4). The data set excluded the ambiguously aligned regions and was 1706 bp in total length. The NJ (by the Clustal W) and MP (by the PAUP) methods were used to construct the phylogenetic trees. Bootstrap analyses (1000 replications) were used with both the NJ and MP methods to evaluate statistical reliability.

RESULTS

The phylogenetic tree obtained from NJ analysis is shown in Fig. 19a. MP analysis of 18SrDNA sequences resulted in twelve most parsimonious trees (length = 1011 steps, consistency index = 0.575). A strict consensus tree of these twelve tree is presented in Fig. 19b.

In 18SrDNA trees, four distinct lineages can be recognized in the Chlorophyceae (Fig. 19). These are Chaetopeltidales (*Planophila terrestris*), Chaetophorales (*Chaetophora incrassata*), Sphaeropleales (*Neochloris aquatica*, *Hydrodictyon reticulatum*, *Scenedesmus obliquus*) and the algae with CW flagellar apparatus (all other chlorophycean algae analyzed in this study). For the last lineage, I use hereafter the term 'CW group'.

I can identify three principal clades in the CW group (Fig. 19). The first clade contains *Volvox carteri*, *Chlamydomonas reinhardtii* and *Polytomella parva*. The monophyly of this clade is supported by high bootstrap values (99% in both NJ and MP analyses), and I refer it as the '*Volvox* clade'. The second clade contains only algae whose sequences were determined in the present study: *Chlamydomonas moewusii*, *Chlorococcum hypnosporum*, *Chlorococcum* sp. and *Tetracystis aeria*. This clade is also supported by high bootstrap values in both methods (≥99%), and referred as the '*Tetracystis* clade'. The third clade comprises *Dunaliella* spp., *Asteromonas gracilis*, *Chlamydomonas applanata*, *Chlorococcum oleofaciens*, *Pleurastrum insigne*, *Chlamydopodium* spp., *Ettlia minuta* and *Protosiphon botryoides*. Although the bootstrap value supporting the monophyly of this clade is slightly low in MP analysis (67%), NJ analysis provides 92% bootstrap value. I refer this clade as

the '*Dunaliella* clade'. Phylogenetic relationships among these three principal clades are not well resolved. In NJ analysis, the *Volvox* clade is the sister group to the *Tetracystis* clade, and this relationship is supported by relatively high bootstrap value (80%) (Fig. 19a). However, MP tree demonstrates different branching pattern, that is, the *Tetracystis* clade and *Carteria radiosa* are monophyletic and the *Volvox clade* is a sister group to these clades (Fig. 19b). Two members of the CW group, *Carteria radiosa* and *Spermatozopsis similis*, can not be included in any of three clades. Phylogenetic positions of these two algae are not clearly resolved in bootstrap analyses.

The branching orders within the *Volvox* clade and *Tetracystis* clade in the NJ and MP trees are identical, and well resolved from bootstrap analyses (≥92%). The coccoid algae in the *Tetracystis* clade (*Tetracystis aeria*, *Chlorococcum hypnosporum*, *C.* sp.) apparently have non-monophyletic relationships. In the *Dunaliella* clade, I can detect two lineages, flagellate and coccoid. The flagellate lineage of the *Dunaliella* clade includes *Dunaliella* spp., *Asteromonas gracilis* and *Chlamydomonas applanata*. The monophyly of halophilic dunaliellacean algae (*Dunaliella*, *Asteromonas*) is clearly demonstrated. In coccoid lineage of the *Dunaliella* clade, *Chlorococcum oleofaciens*, *Pleurastrum insigne* and two species of *Chlamydopodium* form a monophyletic clade. This clade is supported by high bootstrap values (≥98%). These four coccoid algae and *Protosiphon botryoides* form the clade that is a sister group to the *Ettlia minuta* in both NJ and MP trees.

DISCUSSION

Phylogenetic relationships within the CW group

In the 18SrDNA trees, the algae possessing the CW flagellar apparatus form a monophyletic clade (CW group). Some of these algae in the CW group have coccoid vegetative form, however, they are distantly related to the coccoid members of the Sphaeropleales and outgroup algae (*Chlorella*, *Trebouxia*) (Fig. 19). This result supports the taxonomic treatment based on the flagellar apparatus, and does not support the traditional concept of the Chlorococcales which is based on the vegetative morphology (e.g. Bold and Wynne 1985).

The recognition of three principal clades in the CW group is rather unexpected, because no morphological character has been recognized to support the monophyly of any of these clades. However, consistency between NJ and MP methods and high bootstrap values

strongly suggest the presence of these monophyletic clades in the CW group, and I believe that some characters will be found in the future that support these relationships.

The members of the Chlamydomonadales are distributed in all three clades, and those of the Chlorococcales are in the *Tetracystis* clade and *Dunaliella* clade, which indicates neither the Chlamydomonadales nor Chlorococcales is monophyletic taxon. Buchheim and Chapman (1992) suggested, based on the analyses of partial 18S and 26S rRNA sequences, that the Chlamydomonadales (chlorophycean flagellates) is monophyletic and the Chlorococcales is the sister group to the Chlamydomonadales. However, their analyses did not include any taxon from the Chlorococcales *sensu* Deason *et al.* (1991). *Scenedesmus obliquus* and *Oocystis minuta* Guillard *et al.* used by Buchheim and Chapman (1992) as representatives of the Chlorococcales actually belong to the Sphaeropleales *sensu* Deason *et al.* (1991).

Each of the three principal clades includes *Chlamydomonas* species, which apparently indicates the non-monophyly of the genus *Chlamydomonas*. The heterogeneity of *Chlamydomonas* has also been suggested by ultrastructure of the cell wall (Roberts 1974) and the partial 18S and 26S rRNA sequences analyses (Buchheim *et al.* 1990, 1994, Buchheim and Chapman 1991, 1992). I suggest that non-monophyly of the *Chlamydomonas* is due to the usage of symplesiomorphic features (unicellular flagellate, cell wall, etc.) (see also later) in definition of the genus.

Turmel *et al.* (1993) studied the chloroplast-encoded large subunit ribosomal RNA gene (cp23SrDNA) from seventeen *Chlamydomonas* species. Phylogenetic tree based on cp23SrDNA demonstrated three distinct lineages in the *Chlamydomonas* (Turmel *et al.* 1993). In this tree, *Chlamydomonas reinhardtii*, *C. moewusii* and *C. humicola* (= *C. applanata*) appear in three different lineages. This situation is the same as the 18SrDNA tree (Fig. 19). I suggest that the three lineages in the cp23SrDNA tree correspond to the *Volvox* clade, *Tetracystis* clade and *Dunaliella* clade, respectively (Table 9).

Buchheim *et al.* (1990, 1994) and Buchheim and Chapman (1991, 1992) determined partial 18S and 26S rRNA sequences from many chlorophycean flagellates and analyzed their phylogenetic relationships. Three *Chlamydomonas* lineages represented by *C. reinhardtii*, *C. moewusii* and *C. humicola* (= *C. applanata*) can also be identified in their tree (Table 9). Buchheim and his coworkers also demonstrated phylogenetic positions of chlorophycean flagellates related to *Chlamydomonas* (Buchheim *et al.* 1990, 1994, Buchheim and Chapman 1991, 1992). In the phylogenetic trees based on partial 18S and

26S rRNA sequences, the members of the Haematococcaceae *sensu* Ettl (1983) (*Chlorogonium, Haematococcus, Stephanosphaera*) are closely related to *Chlamydomonas applanata* (*Dunaliella* clade), while colonial flagellates (Goniaceae and Volvocaceae *sensu* Nozaki and Itoh 1994) are included in the *C. reinhardtii* lineage (*Volvox* clade) (Table 9). The present analysis and these various sequence analyses strongly suggest three lineages, *Volvox* clade, *Tetracystis* clade, *Dunaliella* clade, are major components of the CW group (Table 9).

All the chlorococcalean algae (*sensu* Deason *et al.* 1991) whose 18SrDNA has been previously reported are closely related to each other and are included in the *Dunaliella* clade (Fig. 19) (see also Lewis *et al.* 1992, Wilcox *et al.* 1992a, Friedl and Zeltner 1994). *Chlorococcum oleofaciens* and *Protosiphon botryoides* also belong to the *Dunaliella* clade. However, coccoid algae whose sequences were determined in this study, *Tetracystis aeria* and two species of *Chlorococcum*, form a monophyletic clade with *Chlamydomonas moewusii* (*Tetracystis* clade) and distantly related to other coccoid members of the CW group (Fig. 19). The ultrastructure of the cell wall of the flagellate cells supports the difference between these two chlorococcalean lineages. Zoospores of *Chlorococcum hypnosporum* and *Tetracystis aeria* have a thick cell wall as do most *Chlamydomonas* species (Watanabe and Floyd 1989a), whereas all chlorococcalean members situated in the *Dunaliella* clade possess thin-walled zoospores (Watanabe and Floyd 1989b, Floyd *et al.* 1993, Friedl pers. comm.). Although *Chlorococcum oleofaciens* has the character of the genus *Chlorococcum* (e.g. zoospores not becoming spherical immediately upon quiescence), zoospores of this alga have a thin cell walls like other coccoid members of the *Dunaliella* clade (see Figs 1-3 in Miller 1978). Heterogeneity of the genus *Chlorococcum* is also suggested by the serological studies of Brown and Bold (1964) who demonstrated that *Chlorococcum hypnosporum* has more antigens in common with *Tetracystis aeria* than with *C. oleofaciens*, a result which also would support present 18SrDNA analyses (Fig. 19).

The present analyses clearly demonstrate the non-monophyly of both the Chlamydomonadales and Chlorococcales and indicate that vegetative morphology does not reflect phylogenetic relationships in the CW group and convergence of the same vegetative morphology in the evolution of the CW group. Convergence of vegetative morphology is common in the green algae (e.g. *Ulothrix-Uronema-Klebsormidium*). A question arises. Which is the ancestral (plesiomorphic) form in the CW group, the flagellate or coccoid condition? The topology of the 18SrDNA tree favors the ancestral state of the flagellate

vegetative form, because it is more parsimonious. The walled cell seems to be more advantageous for protecting the cell against bad conditions or parasites than the wall-less cell. If it is true, it is more logical that the flagellate vegetative form acquired the cell wall, rather than that coccoid form acquired it on a flagellate cell that occupies for a very short period of the life cycle. So, I suggest the ancestral state of the flagellate vegetative form and multiple origins of coccoid life-style in the CW group. The 18SrDNA analyses suggest that the acquisition of the coccoid form in the CW group occurred at least three times (*Chlorococcum* sp., *Chlorococcum hypnosporum* / *Tetracystis aeria*, coccoid lineage of the *Dunaliella* clade) (Fig. 20). Some *Chlamydomonas* species have non-motile or palmelloid stages (e.g. Ettl 1983), suggesting that the change of vegetative form from motile to coccoid can occur easily (see also Deason 1984). In conclusion, molecular and organismal data suggest that the Chlamydomonadales and the genus *Chlamydomonas* are paraphyletic taxa, and the Chlorococcales *sensu stricto* and the genus *Chlorococcum* are polyphyletic taxa.

The present study clearly demonstrates the non-monophyly of the Chlamydomonadales and Chlorococcales. The para- or polyphyly of the genus *Chlamydomonas* and *Chlorococcum* are also revealed. However, taxonomical changes of these taxa will cause confusion to biologists in general (especially in the case of the genus *Chlamydomonas*), because because these are widely used in various fields of biology. Even though, I believe that the classification should be changed to agree with the phylogenetic relationships as much as possible. I suggest that the Chlamydomonadales and Chlorococcales *sensu* Deason *et al.* (1991) should be treated as a single order in the near future. This order will be equivalent to the CW group.

Is the Chlamydophycease a natural taxon?

Ettl (1981) proposed the class Chlamydophyceae for the green algae which have walled motile stage. He reclassified the chlamydomonadalean and chlorococcalean algae into the Chlamydophyceae and Chlorophyceae based on the presence or absence of the cell wall on the flagellate stage. The 18SrDNA analyses clearly demonstrate the non-monophyly of the Chlamydophyceae and Chlorophyceae *sensu* Ettl. Among the algae that appear in the 18SrDNA tree (Fig. 19), *Polytomella parva*, *Dunaliella* spp., *Asteromonas gracilis*, *Spermatozopsis similis*, *Protosiphon botryoides* and all the members outside the CW group belong to the Chlorophyceae *sensu* Ettl (Ettl 1981, 1983, Ettl and Komárek 1982, Komárek 1989, Kouwets 1994). These algae are apparently polyphyletic. Although chlamydophycean

algae are restricted in the CW group, they do not form a monophyletic clade.

Some differences have been detected in the cell wall structure of various *Chlamydomonas* species (Roberts 1974), however, basic structure and components of their wall is the same (Woessner and Goodenough 1994). The wall of zoospores of some chlorococcalean algae (*Chlorococcum*, *Ettlia, Pleurastrum*) is similar to that of *Chlamydomonas*, especially in the presence of the crystalline W6 layer (Miller 1978, Watanabe and Floyd 1989a, Woessner and Goodenough 1994, Friedl pers. comm.). Miller (1978) demonstrated that components of cell wall of *Chlorococcum oleofaciens* are similar to that of *Chlamydomonas*. These similarities suggest that the cell wall of flagellate cell in the CW group are homologous. Thus, the cell wall may be an apomorphy gained only once in the CW group, and 18SrDNA trees suggest multiple losses of the cell wall in the CW group. The 18SrDNA trees indicate at least three independent origins of the wall-less state (Fig. 19). Therefore, the principal characters that define the Chlorophyceae *sensu* Ettl are probably symplesiomorphic (e.g. *Neochloris*) or homoplastic (e.g. *Protosiphon*). Although the character defining the Chlamydophyceae may be synapomorphic in the CW group, it was probably lost secondarily in several lineages. I feel it is not necessary to separate the Chlamydophyceae from the Chlorophyceae *sensu* Mattox and Stewart.

Some comments on *Protosiphon*

The genus *Protosiphon* is sometimes regarded as a member of the Siphonales with the multinucleate ulvophycean algae (Fritsch 1935). However, ultrastructural studies indicate a close relationship between *Protosiphon* and some chlorococcalean algae (Deason and O'Kelley 1979, Watanabe and Floyd 1989a). The present study also supports this view. Interestingly, in the 18SrDNA tree, *Protosiphon* is closely related to the chlorococcalean algae that produce thin walled zoospores (coccoid lineage of the *Dunaliella* clade) . This relationship suggests that *Protosiphon* is the end of the progressive degeneration of the cell wall on the flagellate cell in this coccoid lineage. The thin cell wall of zoospore would derive from a thick multilayered cell wall like that of *Chlamydomonas*. Probably, the common ancestor of the coccoid lineage in the *Dunaliella* clade lost the innermost layer (W1) of the cell wall (see Miller 1978, Woessner and Goodenough 1994), and then, the cell wall was completely lost in the ancestor of *Protosiphon*.

Ettl and Komárek (1982) classified *Protosiphon* in the order Protosiphonales, which is defined by naked zoospores and multinucleate vegetative cells (see also Komárek 1989).

However, the present study reveals a distant relationship between *Protosiphon* and other members of the Protosiphonales such as *Hydrodictyon* (Fig. 19). Thus, the 18SrDNA tree suggests multiple origins of the multinucleate state in the Chlorophyceae *sensu* Mattox and Stewart. Although the 18SrDNA data from the multinucleate chlorophycean algae are still limited, the tree suggests that multinucleate state was acquired at least twice in the Chlorophyceae, which resulted in *Protosiphon* and the Sphaeropleales *sensu* Deason *et al.*(1991) (most members of this order are multinucleate, see Watanabe and Floyd 1989b). These multinucleate conditions are apparently independent from that of the Ulvophyceae (e.g. Acrosiphoniaceae, Siphonocladales). Even in the Ulvophyceae, there are multiple acquisitions of the multinucleate state (O'Kelly and Floyd 1984, Nakayama unpubl. data). The trend from uninucleate to multinucleate cells is distributed over the Chlorophyta, and therefore has a limited taxonomic value at higher taxonomic rank.

GENERAL DISCUSSION

EVOLUTION OF MORPHOLOGICAL CHARACTERS IN GREEN ALGAE

In this study, I dealt with representatives selected from most groups of green algae, and analysed phylogeny based on 18SrDNA sequences and ultrastructural data. Based on these, I discussed in the previous chapters the evolution of several morphological characters in particular lineages. Here, I will deal whole green plants and discuss comprehensively the global view of the evolution of morphological characters, especially ultrastructural ones. This discussion is important to evaluate characters to be used in constructing classification systems of green algae and to recognize the synapomorphic features of each algal group.

Vegetative phase organization

As mentioned in the General Introduction, green algae were classified based on their organization of vegetative phase (e.g. flagellate, coccoid, filamentous). However, ultrastructural studies concerning the flagellar apparatus and the process of mitosis/cytokinesis have led to the view that the same organization of vegetative phase independently evolved more than once. For example, unbranched filamentous green algae apparently belong to three separate evolutionary lines (Charophyceae, Ulvophyceae, Chlorophyceae) as revealed based on their ultrastructural features (e.g. Floyd *et al.* 1980). The 18SrDNA analyses clearly supported this view. The phylogenetic tree based on sequences suggest that coccoid organization evolved many times, even within the CW group (see Chapter 6). Leaf-like frond would be also acquired many times in the green algae (see Chapter 5). So, it is difficult to apply this character to the consideration of classification of green algae. In many algae, since organization of vegetative phase is a character easily influenced by environmental conditions, in evolutionary sense it would change rapidly to adapt external environmental changes. Frequent occurrences of convergence of vegetative organization seem to provide interesting subject to the evolutional and developmental biology. What kind of molecular mechanism does perform these changes? Is this mechanism common to all green algae? In the Metazoa, organization and development are partially controlled by homeodomeins encoded in homeobox genes. Recently, homologous genes were found in fungi and land plants (e.g. Bürglin 1994). It is likely that these should also be present in the green algae. It is interesting to know their roles in the green algae, which would be important

to understand the evolution of organization and development in the Viridiplantae.

General features of flagellate cell

As suggested by ultrastructural studies, features of the flagellate cells reflect their evolutionary relationships. 18SrDNA trees suggested that the cell with a characteristic configuration of major organelles (nucleus, chloroplast, Golgi apparatus, basal bodies) and laterally inserted flagella are symplesiomorphic conditions of the Viridiplantae (see Chapter 2, 3). The loss of this organellar configuration would have occurred twice. The one in the common ancestor of the Streptophyta and the other in that of the Chlorophyta *sensu stricto*. The former lineage retains asymmetric configuration of organelles (but further study is needed in this point) and laterally inserted flagella, but the latter gained rotational symmetry of the cell and apically inserted flagella. As the direction of swimming seems to be consistent in *Mesostigma*, Mamiellales and the hypothetical ancestors of the Pyramimonadales and Pseudoscourfieldiales ("forward direction"; see Chapter 3), it is probably plesiomorphic in the green algae. Drastic change of swimming direction would have occurred within the Pyramimonadales, Pseudoscourfieldiales and in a common ancestor of the Chlorophyta *sensu stricto*.

A flagellar pit (groove or depression) was regarded as a distinctive feature of the Prasinophyceae and as a primitive character of green flagellate (Cristensen 1962; Chadefaud 1977). However, ultrastructural surveys of "primitive" green flagellates revealed that some prasinophycean algae had no deep flagellar pit (e.g. Moestrup 1984). As the phylogenetic positions of the flagellates with deep flagellar pit (*Mesostigma*, some pyramimonadaleans, *Tetraselmis*) are dispersed in the 18SrDNA tree, it is difficult to evaluate the nature of this character. However, that of *Pyramimonas/Halosphaera* was apparently acquired within the Pyramimonadales as a synapomorphic feature (Chapter 3). So I suppose that the presence of deep pit is not a primitive condition but appeared in several different lineages as consequences of convergence.

The number of flagella is variable in the Viridiplantae. The ancestral state of this would be biflagellate as suggested from the 18SrDNA analyses (Chapter 2), and uni- or quadriflagellate ancestor suggested previously (e.g. Norris 1980, O'Kelly 1992) would be misinterpretation of morphological characters. Uniflagellate cell might occur independently in the Pedinomonadales, *Monomastix* and *Micromonas* (Chapter 3, see also Heimann *et al.* 1989; Daugbjerg *et al.* 1995). Acquisition of quadriflagellate condition in the

Pyramimonadales would be independent from that of the Chlorophyta *sensu stricto*, and would be caused by duplication of flagella. The similar configuration of basal bodies in *Pterosperma* and preprophase cell of *Mantoniella* supports this idea (Barlow and Cattolico 1981; Inouye *et al.* 1990). In the Chlorophyta *sensu stricto*, quadriflagellate cell appear only in algae which produce biflagellate gametes (except for *Carteria*), so that their quadriflagellate condition is related to the life cycle. I suppose that the common ancestor of the Chlorophyta *sensu stricto* was haploid biflagellate cell (like Pseudoscourfieldiales, Suda *et al.* 1989), and quadriflagellate condition was acquired by simple duplication (without chromosome duplication) or retention of planozygote phase. Because the Tetraselmidales is first divergence in the Chlorophyta *sensu stricto* (Chapter 4), the latter hypothesis is more likely if the suggestion by Huber and Lewin (1986) is considered, that is, based on the isozyme analysis, they suggested that *Tetraselmis* is diploid. However, the fact that reduction division occur at the sporogenesis in ulvophycean algae that also produce quadriflagellate zoospore (e.g. van den Hoek *et al.* 1995) favors the former hypothesis. O'Kelly and Floyd (1984a,b) suggested that there is a trend to lose quadriflagellate cell in the Chlorophyta *sensu stricto*. I agree to this hypothesis. The loss of quadriflagellate condition would happen independently in the Trebouxiophyceae, some lineages of the Ulvophyceae, and probably, in a common origin of the Sphaeropleales and CW group. In the CW group, however, the multiple acquisitions of quadriflagellate condition may have occurred (e.g. *Polytomella*, *Carteria*; Chapter 4).

Cell covering

Non-motile cells of green algae are usually covered by cell wall that comprise diverse chemical compsistion. The nature of the cell wall may be usable as taxonomic or phylogenetic marker in certain case (e.g. Sphaeropleales, Chapter 4; see also Huizing *et al.* 1979). In other cases, however, it is variable even in a single genus (Deason 1983). Therefore, in general, the vegetative cell wall seem to be of limited use to consider phylogeny of the green algae.

Ancestral green plant has been considered to be a flagellate covered by organic scales as seen in most members of the Prasinophyceae (e.g. Pickett-Heaps 1975; Melkonian 1982a; Mattox and Stewart 1984), and this hypothesis was clearly supported by 18SrDNA comparisons (Chapter 2). The organic scales of the green algae can be roughly classified into three types, small, large and hair scales. The small scale is found in some members of

Charophyceae, Ulvophyceae, Chlorophyceae and almost all prasinophytes excluding the Mamiellales, that is, this is the scale most universally present in the green algae. This type of scales would have been originate in a common ancestor of the Viridiplantae and secondarily lost in the Mamiellales (Chapter 2, 3). The double-layered small scale, a unique character shared by the Pseudoscourfieldiales and Tetraselmidales, is not a synapomorphic but a symplesiomorphic feature (Chapter 2). Large scale has been found only in the Prasinophyceae. Their homologies are obscure, and this time, it is not easy to discuss its phylogenetic significance. However, the following hypotheses would be useful for future studies of the Prasinophyceae. Spider-web scale, characteristic feature of the Pyramimonadales and Mamiellales, would diverge to box and crown scales in the former lineage (Chapter 3). Peculiar large scales of both *Mesostigma* and *Nephroselmis* have some similarities to spider-web scale of the Mamiellales/ Pyramimonadales (e.g. eight-radial symmetry, Chapter 3) and would be homologous. Body scales of the Tetraselmidales and all scales (except for hair scales) of *Crustomastix* would transform to theca or fibrous coat (Chapter 3). 18SrDNA analyses indicate that loss or reduction of scary covering very easily occurred, especially in the Chlorophyta *sensu stricto* (i.e. *Oltmannsiellopsis*, Chlorophyceae, Trebouxiophyceae and several times in Ulvophyceae; see Chapter 4, 5).

Flagellate members of the CW group often have crystalline cell wall containing fibrous glycoprotein which is completely different from components of organic scales. So, the evolutionary relationship between cell coverings of Tetraselmidales and CW group (e.g. Domozych 1984; Mattox and Stewrt 1984) is impossible (Becker *et al.* 1991). Homologous nature of cell wall in the CW group suggests that this structure is synapomorphic feature of this group (see Chapter 6). However, 18SrDNA analysis indicates that this type cell covering is independently lost in several lineages of the CW group. Multiple loss of the cell wall would result in the polyphyletic nature of the Dunaliellales and Chlorophyceae *sensu* Ettl (Chapter 4, 6).

As discussed above, evaluation of the cell covering in phylogenetical sense is not easy, as morphology is relatively simple and very different between taxa. To determine homology or to evaluate gains and losses of certain characters are also difficult. However, as shown in previous chapters, phylogenetic analyses of molecules are powerful tool to help our understanding of evolution of very simple but phylogenetically important characters like cell covering.

Flagellar apparatus

basal bodies

Previous studies on phylogeny of the green algae suggest that the condition of parallel or nearly parallel basal bodies is primitive state in the Viridiplantae. This condition remains in the Streptophyta and most prasinophytes (parallel basal bodies in some members of the CW group [e.g. *Heterochlamydomonas*, Floyd *et al.* 1990] seem to be evolved by convergence), but in the Chlorophyta *sensu stricto*, configulation of basal bodies drastically changed to anti parallel, namely, opposite to each other. The apically inserted flagella and a "papilla" would be gained along with this process. Mattox and Stewart (1984) suggested that multiple origins of the papilla (= anteriorly inserted flagella with basal bodies oriented opposite to each other) in the Chlorophyceae, Ulvophyceae and "Pleurastrophyceae" (see also O'Kelly and Floyd 1984a). This hypothesis was based on the consideration that both the Chlorophyceae and "Pleurastrophyceae" includes "primitive" members which have nearly parallel basal bodie and flagellar pit (*Hafniomonas* and Tetraselmidales, respectively). However, the 18SrDNA analyses indicated that *Hafniomonas* is not a primitive member of the Chlorophyceae and the Tetraselmidales did not form a clade with other pleurastrophycean algae (Chapter 4 and 6; see Steinkötter *et al.* 1994; Friedl and Zeltner 1994). The first divergence of the Tetraselmidales in the Chlorophyta *sensu stricto* suggests a single origin of papilla in a common ancestor of the clade including the Chlorophyceae, Ulvophyceae and Trebouxiophyceae (Chapter 4).

O'Kelly and Floyd (1984) discussed the evolution of the flagellar apparatus in the green algae, and suggested that clockwise rotation of basal bodies had occured in the Chlorophyta *sensu stricto*. This hypothesis agrees with the phylogenetic trees deduced from 18SrDNA data which shows the monophyletic nature of the Chlorophyceae and CW group. So, the Chlorophyceae is considered to have gained directly opposed configuration of basal bodies as synapomorphic feature, and clockwise basal bodies of the CW group would have evolved in a common ancestor of the CW group (Chapter 4).

microtubular roots

The microtubular roots connected to the basal bodies is regarded as one of the most important features to consider the phylogeny of the green algae (e.g. Mattox and Stewart 1984; Melkonian 1984). The Streptophyta and Chlorophyta *sensu stricto* can be easily and well defined using conditions of this feature. The Streptophyta has only two (one in land

plant, but see Sluiman 1983) microtubular roots connected to one basal body, whereas the Chlorophyta *sensu stricto* has for that comprise cruciate root system that is characterized by 180° rotational symmetry. Present findings based on 18SrDNA analyses provide some consideration about the evolution of microtubular root system in the green algae. The Mamiellales has been often regarded as primitive green algae and its root system with only two microtubular roots is considered to be plesiomorphic condition (e.g. Mattox and Stewart 1984). Present study indicates an apomorphic nature of the simple cell organization in the Mamiellales (see Chapter 3). So, the lack of the microtubular roots associated with the second basal body seems to be derived condition, and would evolve independently from that of the Streptophyta (see Melkonian 1984). However, ancestral state of two microtubular roots cannot be rulued out in the 18SrDNA analyses. If so, four microtubular roots would have been acquired independently in the Chlorophyta *sensu stricto*, Pyramimonadales and *Mesostigma*.

An early and independent divergence of *Mesostigma* in the 18SrDNA tree makes difficult to evaluate the nature of the cruciate root system. The microtubular roots with 180° rotational symmetry are found in *Mesostigma* and some taxa of *Pyramimonas* (subgenus *Pyramimonas* and *Hexactis*) in addition to the Chlorophyta *sensu stricto* (Melkonian 1989; Moestrup and Hori 1989; Hori *et al.* 1995). Is this type of root system symplesiomorphic, or was this gained independently several times? As for *Pyramimonas*, the phylogenetic analysis based on morphological data clearly suggests that this type of root system originated within the Pyramimonadales independently from the other algae (see Chapter 3). So the convergent hypothesis concerning the origin of the microtubular roots with 180° rotational symmetry is favored here.

MLS (multilayered structure)

The multilayered structure (MLS) has been regarded as a distinctive feature of the Streptophyta. However, the presence of the MLS in the some prasinophycean algae and distribution of this structure within the phylogenetic tree of green plants clearly suggest symplesiomorphic nature of this structure (see Chapter 2, 3). This consideration is supported by the presence of "putative" MLS in other eukaryotes such as Graucocystophyceae, Jacobids and Euglenophyta (e.g. Moestrup 1982; O'Kelly 1993), but chemical study is needed to clarify their homology. The MLS would reduced several times in different lineages, Pseudoscourfieldiales/Chlorophyta *sensu stricto*, Mamiellales (after divergence of

Crustomastix), Pyramimonadales (*Pyramimonas*) and Charales. Some variations have been found in the components of the MLS of the Viridiplantae. I suggest that the primitive MLS had keels on spline (S1) and simple lamellate structure (2 layers?) underlain by electron dense region (S5) (see Chapter 3). The angle between the spline (S1, microtubules of the d root) and lamellae (S2-4) is different between the green plant groups. It is approximately 90° in *Mesostigma*, *Crustomastix* and charophycean algae, and this condition may be a symplesiomorphic state in the Viridiplantae. The stepwise tilting would occur in the land plants, because it is 45° in bryophytes and ferns, and 20° in gymnosperm (e.g. Carothers and Kreitner 1967; Duckett 1973; Norstog 1974). The angle in the pyramimonadalean algae (except for *Pyramimonas*) has been thought to be 45° as in bryophytes (e.g. O'Kelly 1992), however, it is really an opposite angle, nearly 135° (Inouye pers. comm.), which supports independent origins of tilted lamellae of land plants and Pyramimonadales.

Fibrous structure

In the green plants, basal bodies are connected by two basic fibrous structures, distal fiber (= "capping plate" *sensu* Melkonian 1979 and "synistosome" *sensu* Norris and Pearson 1975) and proximal fiber (the term "proximal fiber" is used for many different structures, but I use this *sensu* Moestrup and Hori 1989, not *sensu* Ringo 1967 or *sensu* Hoops *et al.* 1982). There is no report for the proximal fiber of the Streptophyta and Mamiellales, but *Coleochaete* (Fig. 13, 14 in Sluiman 1983) and most mamiellalean algae (Chapter 3) really have this structure. So, both connecting fibers are regarded as a symplesiomorphic character of the Viridiplantae. Possible homologous structure to the proximal fiber seems to retain in the Ulvophyceae (e.g. "proximal connective" in Roberts *et al.* 1982; "amorphous material" in Stuessy *et al.* 1983) and Trebouxiophyceae (e.g. one of the two "small striated connecting fibers" in Melkonian and Berns 1983; "weakly striated fiber" in Watanabe and Floyd 1994), but it would be lost in the Chlorophyceae. Additional connecting fibers between basal bodies found especially in the quadriflagellate cells. However it is difficult to recognize what structures are homologous, and further studies are necessary on this subject.

Melkonian (1982) classified fibrous flagellar roots of green algae into two major types, system I fiber (= SMAC *sensu* Floyd *et al.* 1980) and system II fiber (= rhizoplast). These two structures are now known to consist of different phosphoprotein, assemblin and centrin respectively (e.g. Lechtreck and Melkonian 1991). In the mamiellalean algae, I found a duct fiber which is associated with the 1d root, and this structure is equivalent to that of the

Pyramimonadales and probably to system I fiber (Chapter 3). So, the system I fiber may be a synapomorphic structure of the green algal group excluding the Streptophyta and *Mesostigma*. However, presence of possible homologous protein in other eukaryotes (e.g. β-giardin in *Giardia*) suggests that the origin of system I fiber should retrace to more ancient ancestor and that a loss of this fibrous root is synapomorphic feature of the Streptophyta and *Mesostigma* (Lechtreck and Melkonian 1991). As fibrous roots consisting of centrin are found in many other eukaryotes (e.g. Melkonian *et al.* 1992), system II fiber of the green algae is also considered to be plesiomorphic structure. This fibrous root would be secondarily lost in the land plants.

Conclusive remarks

Present study indicate that the phylogenetic relationships deduced from ultrastructural characters are generally congruent with those from molecular data. Hypotheses of evolution of ultrastructural features are also comprehensible based on the 18SrDNA phylogeny is taken into consideration. So, both ultrastructural (especially of the flagellar apparatus) and 18SrDNA characters are considered to be good markers to trace phylogenetic relationships of the green algae. It is often difficult to recognize a primitive state of morphological features. Another problem is frequent occurrence of "simplification" which sometimes lead misunderstanding of phylogeny and evolution. However, molecular data provide useful information to answer to these problems. Contrary, phylogenetic deduced from molecular data cannot be interpreted comprehensively without ultrastructural data, because monophyletic clades often comprise organisms which are very different at light microscopical level. It would be the best way for understanding phylogeny and constructing natural taxonomic system to employ both ultrastructural and molecular data as mutually testing and supporting tools.

A CLASSIFICATION OF THE GREEN ALGAE

The green algae and land plants apparently form a monophyletic clade in the eukaryotes (see Chapter 1), so they should be treated as a single taxon (e.g. Viridiplantae *sensu* Cavalier-Smith 1981). Other "green" algae such as the Euglenophyta and Chlorarachniophyta have chloroplasts with chlorophylls a and b as in the green plants, but they are undoubtedly originated through secondary symbioses (see Chapter 1). I belive that

classification of eukaryotes should be based on the phylogeny of "host", because symbiotic organelles (i.e. chloroplast and mitochondria) are completely ruled by their host. So, these "pseudo" green algae should be treated as separate taxa, probably at a kingdom-level. Only two algal groups, Glaucocystophyta and Rhodophyta, can be included in the kingdom Plantae with the Viridiplantae (e.g. Cavalier-Smith 1981; see also Chapter 1).

Recent phylogenetic view that there are two major evolutionary lineages in the Viridiplantae is supported by molecular data. This consideration leads to the non-monophyletic nature of the division Chlorophyta in traditional sense (= green algae) (Chapter 2). So, I agree with the proposal that the Viridiplantae should not be divided into land plants and green algae but into two major divisions, the Streptophyta and Chlorophyta *sensu stricto* (e.g. Bremer 1995; Bremer *et al.* 1987; see also Sluiman 1985) (Tables 10, 11). The division Streptophyta includes land plants and related green algae which are usually called the Charophyceae. Morphological (e.g. Pickett-Heaps 1975) and molecular data (e.g. Manhart and Palmer 1990) indicates that the class Charophyceae *sensu* Mattox and Stewart (1984) is paraphyletic, but further study is needed to clarify their relationships. At present, the Bremer's classification seems to be possible to apply to the classification system within the Streptophyta (Bremer 1985) (Tables 10, 11).

The most problematic taxon in terms of constructing taxonomic system of the green plants is the Prasinophyceae. The Prasinophyceae has no synapomorphic character, and 18SrDNA analysis also supports its paraphyletic nature (Chapter 2). These evidences are not surprising, because this class is recognized as a "primitive" green algae, and no phycologist consider that this group is monophyletic in cladistic sense (see Chapter 2). The reasons why prasinophycean algae have been classified into single class may be as follows, 1) the phylogenetic relationships within the Prasinophyceae and to the other classes were obscure (see Mattox and Stewart 1984), or 2) as more positive reason, they share primitive condition of the green plants. However, now combinations of morphological and molecular data provide reliable phylogenetic hypothesis of these algae. It is possible to accept the taxon (i.e. Prasinophyceae) which is defined by symplesiomolphic characters (i.e. primitive condition of the green algae) if the "evolutionary taxonomy" is approved. However, I agree the assertion that only phylogenetic classification can be a general reference system for the knowledge about the evolution of organisms (e.g. Hennig 1966; Wiley 1981). On the basis of the phylogenetic taxonomy, I feel that the Prasinophyceae should be divided into five classes (Chapter 2). I propose five new classes, Mesostigmatophyceae,

Pyramimonadophyceae, Mamiellophyceae, Nephroselmidophyceae and Tetraselmidophyceae (Tables 10, 11). In these classes, Tetraselmidophyceae is able to be classified into the Chlorophyta *sensu stricto*, because its flagellar apparatus of 180° rotational symmetry is synapomorphic feature shared with other chlorophytes (Tables 10, 11). Except for the Mesostigmatophyceae, other three classes form a clade with the Chlorophyta *sensu stricto* in the 18SrDNA tree, however, I cannot find any synapomorphic characters support this clade (Chapter 2, 3; see also above). So, at present, I suppose to put these classes in a "*incertae cedis*" (= uncertain position) of the Viridiplantae (Tables 10, 11). As strict phylogenetic position of *Mesostigma* is unstable (Chapter 2), I also locate the Mesostigmatophyceae in *incertae cedis* of the Viridiplantae (Tables 10, 11).

The Chlorophyta *sensu stricto* originally included three classes, Pleurastrophyceae, Ulvophyceae and Chlorophyceae (Bremer 1985; Bremer *et al.* 1987). The Pleurastrophyceae is apparently a heterogeneous taxon (see Steinkötter *et al.* 1994; Friedl and Zeltner 1994), and is considered to consist of two distinctive groups. One of these is the Pleurastrales *sensu* Mattox and Stewart(1984) (= Microthamniales *sensu* Melkonian 1982b, 1990c), and recently Friedl (1995) proposed a new class, Trebouxiophyceae, for this lineage. Another "pleurastrophycean" group is the Tetraselmidales which is now regarded as an independent class, Tetraselmidophyceae (see above).

The Ulvophyceae Mattox and Stewart (1984) has been considered to be a paraphyletic taxon, because its definitive characters (CCW basal bodies, cytokinesis without phycoplast) are regarded as symplesiomorphic (e.g. Bremer 1985; Mishler and Churchill 1985). However, this problem is confused by the recent finding of the presence of phycoplast in some ulvophycean algae (Sluiman 1991) and that the Tetraselmidophyceae, which also produces a phycoplast, is the first divergence in the Chlorophyta (Steinkötter *et al.* 1994; Friedl and Zeltner 1994; see also Chapter 4). These evidences suggest that more detailed studies (e.g. freeze fixation) are needed to evaluate the cytokinesis process in the ulvophycean algae. On the one hand, the 18SrDNA analysis suggest that at least two independent lineages exist in the Ulvophyceae *sensu* Mattox and Stewart (see Chapter 5). One consists of the Ulotrichales and Ulvales, and the other includes the Siphonocladales and Dasycladales (these orders *sensu* O'Kelly and Floyd 1984b). Unfortunately, complete 18SrDNA sequence data from the Caulerpales, which is one of five orders of O'Kelly and Floyd (1984b), is not available. However, phylogenetic analysis based on partial sequences of 18S/26S rRNA suggested close relationship between the Caulerpales, Siphonocladales

and Dasycladales (Zechmann *et al.* 1990). So, I feel that the Ulvophyceae *sensu* Mattox and Stewart can be divided into two classes (Ulotrichales/Ulvales and Siphonocladales/Dasycladales/Caulerpales), but I cannot designete any synapomorphic characters of the latter for definition. At present, classification of van den Hoek *et al.* (1995) seems to be suitable for the "ulvophycean" algae. In this classification, the clade consisting of the Ulotrichales and Ulvales is treated as a single class, the Ulvophyceae *sensu stricto* (Tables 10, 11). van den Hoek *et al.* (1995) raised the other three orders to the class level (Cladophorophyceae, Dasycladophyceae and Bryopsidophyceae) (Tables 10, 11). Each class in this classification is defined clearly by morphological features such as ultrastructure of the flagellar apparatus (O'Kelly and Floyd 1984b, van den Hoek *et al.* 1995).

The Chlorophyceae is well defined and seems to be monophyletic. However, classification within the Chlorophyceae is problematic, because it includes some traditional groups which are expected to be heterogeneous (e.g. Tetrasporales, Chlorosarcinales) (e.g. Melkonian 1990b). I suppose that the classification by Floyd and his coworkers (e.g. Deason *et al.* 1991; O'Kelly *et al.* 1994) is reliable to apply within the Chlorophyta. They recognized five major orders, the Chaetopeltidales, Chaetophorales, Sphaeropleales, Chlorococcales and Chlamydomonadales. However, I found that the last two orders are non-monophyletic and should be treated as a single order (CW group, Chapter 6). I chose the Chlamydomonadales for the name of this group (Tables 10, 11), because the term Chlorococcales is used traditionally for all coccoid algae which are very heterogeneous. Even in the recent studies, the Chlorococcales is employed in traditional sense. So I feel that this term should not be apply the natural classification system. Another traditional order, the Volvocales, is also often used in narrower sense than before (e.g. Mattox and Stewart 1984), i.e., this group contains only specialized chlamydomonadalean algae. This order should also be included in the Chlamydomonadales.

This new classification (Tables 10, 11) is considered to be more suitable to the hypothesis of green algal phylogeny. However, "natural" systems such as that proposed here have significant disadvantage in practical use, because their definitive characters are usually ultrastructural. A great many green algae have been observed only in light microscopical level, so it is difficult to assign them to correct place (even in divisional level!) in many cases. However, the accumulation of phylogenetic knowledge from light microscopical, ultrastructural and molecular studies will lead us to the recognition of definitive (synapomorphic) characters which are practicable.

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Table 1. List of species examined in this study

Species	Source [1]	18SrDNA	EM
VIRIDIPLANTAE			
Prasinophyceae			
Mamiellales			
Crustomastix didyma	North Pacific	O	O
Mamiella sp.	Shizugawa, Miyagi, Japan	O	
Mantoniella antarctica	Ongul Is., Antarctica	O	O
Mantoniella squamata	Minamata, Kumamoto, Japan		O
Pyramimonadales			
Pterosperma cristatum	Yokohama, Kanagawa, Japan	O	
Cymbomonas tetramitiformis	Shizugawa, Miyagi, Japan	O	
Halosphaera sp.	Shizugawa, Miyagi, Japan	O	O
Pyramimonas propulsa	NIES 251	O	
Pyramimonas parkeae	Hachijo Is., Tokyo, Japan	O	
Pyramimonas olivacea	Shizugawa, Miyagi, Japan	O	
Pyramimonas disomata	Changi, Singapore	O	
Ulvophyceae			
Ulotrichales			
Monostroma angicava	Oshoro, Hokkaido, Japan	O	
Monostroma latissimum	Shimoda, Shizuoka, Japan	O	O
Urospora mirabilis	Choshi, Chiba, Japan	O	
Halochlorococcum marinum	UTEX 1490	O	
Halochlorococcum sp.	Ishigaki Is., Okinawa, Japan	O	O
Ulvales			
Ulva pertusa	Shimoda, Shizuoka, Japan	O	
Enteromorpha linza	Iwaki, Fukushima, Japan	O	
Entocladia viride	UTEX 1430	O	
Siphonocladales			
Chaetomorpha moniligera	Iwaki, Fukushima, Japan	O	
Caurelpales			
Bryopsis maxima	Oarai, Ibaraki, Japan	O	
Codium divaricatum	Tateyama, Chiba, Japan	O	
Chlorophyceae			
Chaetopeltidales			
Planophila terrestris	UTEX 1709	O	
Chaetophorales			
Chaetophora incrassata	UTEX LB1289	O	
Chlorococcales			
Chlorococcum oleofaciens	UTEX 105	O	
Chlorococcum hypnosporum	UTEX 119	O	
Chlorococcum sp.	Sesoko, Okinawa, Japan	O	O
Tetracystis aeria	UTEX 1453	O	
Protosiphon botryoides	UTEX 99	O	
Chlamydomonadales			
Chlamydomonas moewusii	CGC-1419	O	
Polytomella parva	UTEX L193	O	
Carteria radiosa	NIES 432	O	
Carteria crucifera	NIES 421	O	

Species	Source [1]	18SrDNA	EM
incertae cedis			
Oltmannsiellopsis viridis	NIES 360	O	
Hafniomonas reticulata	Iwata, Shizuoka, Japan	O	O
Prasiola japonica	Kiryu, Gunma, Japan	O	
Ignatius tetrasporus	UTEX 2012	O	
Microspora sp.	UTEX LB472	O	
STRAMENOPILES			
Chattonella sp.	DNA from Dr. Ueshima	O	
Opalina sp.	Tsukuba, Ibaraki, Japan	O	
HAPTOPHYTA			
Pleurochrysis carterae	DNA from Dr. Kawachi	O	
Chrysochromulina hirta	DNA from Dr. Kawachi	O	
INCERTAE CEDIS			
Cercomonas sp.	Kiryu, Gunma, Japan	O	

[1] UTEX = Culture Collection of Algae at The University of Texas at Austin; NIES = National Institute for Environmental Studies Collection at Tsukuba; CGC = Chlamydomonas Genetic Center at Duke University.

Table 2. Oligonucleotide primers used for amplification and sequencing of 18SrDNA.

Code	SD[a]	Sequence	Anneals to[b]
SR-1	F	5′-TACCTGGTTGATCCTGCCAG-3′	1-20
SR-2	F	CATTCAAATTTCTGCCCTATC	293-313
SR-3	R	AGGCTCCCTGTCCGGAATC	394-376
SR-4	F	AGCCGCGGTAATTCCAGCT	568-586
SR-5	R	ACTACGAGCTTTTTAACTGC	630-611
SR-6	F	GTCAGAGGTGAAATTCTTGG	891-910
SR-7	R	TCCTTGGGCAAATGCTTTCGC	952-932
SR-8	F	GGATTGACAGATTGAGAGCT	1224-1243
SR-9	R	AACTAAGAACGGCATGCAC	1286-1267
SR-10	F	AGGTCTGTGATGCCCTTAGA	1420-1439
SR-11	R	CGCTTACTAGGAATTCCTCG	1582-1563
SR-12	R	CCTTCCGCAGGTTCACCTAC	1781-1762

[a] SD = synthesis direction, F= forward, R = reverse.

[b] Annealing site in the 18SrDNA of *Volvox carteri* (Rausch et al. 1989).

Table 3. List of sequences used in this study.

Species	Major group	Ac. no. [a]
Tritrichomoans foetus	Parabasalia	M81842
Naegleria gruberi	Heterolobosea	M18732
Bodo caudata	Euglenozoa, Kinetoplastida	X53910
Euglena gracilis	Euglenozoa, Euglenophyta	M12677
Dictyosterilum discoideum	lobose amoeba?, Dictyostelids	K02641
Hartmanella vermiformis	lobose amoeba	M95168
Acanthamoeba castellanii	lobose amoeba	M13435
Euglypha rotunda	filose amoeba	X53235
Chlorarachnion reptans	Chlorarachniophyta	X70809
Pavlova salina	Haptophyta	L34669
Emiliania huxleyi	Haptophyta	M87327
Alexandrium fundyense	Alveolata, Dinophyta	U09048
Prorocentrum micans	Alveolata, Dinophyta	M25952
Perkinsus sp.	Alveolata, Apicomplexa	L07375
Toxoplasma gondii	Alveolata, Apicomplexa	M97703
Blepharisma americanum	Alveolata, Ciliophora	M97909
Paramecium tetraurelia	Alveolata, Ciliophora	M25786
Cafeteria roengergensis	Stramenopiles, Bicosoecids	L27633
Labylinthuloides minuta	Stramenopiles, Labylinthulids	L27634
Lagenidium giganteum	Stramenopiles, Oomycetes	M54939
Costaria coctata	Stramenopiles, Phaeophyceae	X53229
Ochromonas danica	Stramenopiles, Chrysophyceae	M32704
Homo sapiens	Metazoa, Chordata	X03205
Styela plicata	Metazoa, Chordata	M97577
Spisula solida	Metazoa, Mollusca	L11266
Anemonia sulcata	Metazoa, Cnidaria	X53498
Scypha ciliata	Metazoa, Polifera	L10827
Diaphanoeca grandis	Choanoflagellata	L10824
Blastocladiella emersonii	Fungi, Chytridiomycetes	M54937
Mucor racemosus	Fungi, Zygomycetes	M54863
Saccharomyces cervisiae	Fungi, Ascomycetes	V01335
Neurospora crassa	Fungi, Ascomycetes	X04971
Ustilago maydis	Fungi, Basidiomycetes	X62396
Auricularia auricula	Fungi, Basidiomycetes	L22254
Goniomonas truncata	Cryptophyta	U03072
Teleaulax sp.	Cryptophyta	X57162
Porphyridium aergineum	Rhodophyta	L27635
Dixoniella grisea	Rhodophyta	L26187
Rhodella maculata	Rhodophyta	U21217
Erithrotrichia carnea	Rhodophyta	L26189
Porphyra miniata	Rhodophyta	L26200
Gracilaria verrucosa	Rhodophyta	L26179
Cyanophora paradoxa	Glaucocystophyta	X68483
Glaucocystis nostochinearum	Glaucocystophyta	X70803
Oryza sativa	Viridiplantae, Spermatopsida	X00755
Abies lasiocarpa	Viridiplantae, Spermatopsida	X79407
Zamia pumila	Viridiplantae, Spermatopsida	M20017

Pteridium aquilium	Viridiplantae, Polypodiopsida	U18628
Equisetum hyemale	Viridiplantae, Equisetopsida	U18500
Tmesipteris tannensis	Viridiplantae, Psilotopsida	U18103
Isoetes engelmannii	Viridiplantae, Lycopodiopsida	U18506
Atricum angustatum	Viridiplantae, Bryopsida	U18492
Anthoceros laevis	Viridiplantae, Anthocerotopsida	U18491
Sphaerocarpos donnelli	Viridiplantae, Marchantiopsida	X85094
Chara foetida	Viridiplantae, Charophyceae, Charales	X70704
Nitella flexilis	Viridiplantae, Charophyceae, Charales	U05261
Coleochaete scutata	Viridiplantae, Charophyceae, Coleochaetales	X68825
Coleochaete orbicularis	Viridiplantae, Charophyceae, Coleochaetales	M95611
Klebsormidium flaccidum	Viridiplantae, Charophyceae, Klebsormidiales	X75550
Chlorokybus atomophticus	Viridiplantae, Charophyceae, Chlorokybales	M95612
Mesothaenium caldarium	Viridiplantae, Charophyceae, Zygnematales	X75763
Mougeotia scalaris	Viridiplantae, Charophyceae, Zygnematales	X70705
Staurastrum sp.	Viridiplantae, Charophyceae, Zygnematales	X74752
Mesostigma viride	Viridiplantae, Prasinophyceae, Pyramimonadales	*1
Micromonas pusilla	Viridiplantae, Prasinophyceae, Mamiellales	*1
Mantoniella sqamata	Viridiplantae, Prasinophyceae, Mamiellales	X73999
Nephroselmis olivacea	Viridiplantae, Prasinophyceae, Pseudoscourfieldiales	X74754
Pseudoscourfieldia marina	Viridiplantae, Prasinophyceae, Pseudoscourfieldiales	X75565
Tetraselmis striata	Viridiplantae, Prasinophyceae, Chlorodendrales	X70802
Tetraselmis sp.	Viridiplantae, Prasinophyceae, Chlorodendrales	U05039
Scherffelia dubia	Viridiplantae, Prasinophyceae, Chlorodendrales	X68484
Gloeotilopsis planctonica	Viridiplantae, Ulvophyceae, Ulotrichales	Z28970
Acrosiphonia sp.	Viridiplantae, Ulvophyceae, Ulotrichales	U03757
Cladophora albida	Viridiplantae, Ulvophyceae, Siphonocladales	Z35421
Valonia utricularis	Viridiplantae, Ulvophyceae, Siphonocladales	Z35323
Microdictyon boergesenii	Viridiplantae, Ulvophyceae, Siphonocladales	Z35324
Acetabularia acetabulum	Viridiplantae, Ulvophyceae, Dasycladales	Z33461
Cympolia van bosseae	Viridiplantae, Ulvophyceae, Dasycladales	Z33467
Batophora occidentalis	Viridiplantae, Ulvophyceae, Dasycladales	Z33465
Bordnetella nitida	Viridiplantae, Ulvophyceae, Dasycladales	Z33464
Chlorella vulgaris	Viridiplantae, Trebouxiophyceae	X13688
Chlorella kessleri	Viridiplantae, Trebouxiophyceae	X56105
Chlorella saccharophila	Viridiplantae, Trebouxiophyceae	X63505
Chlorella ellipsoidea	Viridiplantae, Trebouxiophyceae	X63520
Nannochloris eukaryotum	Viridiplantae, Trebouxiophyceae	X06425
Auxenochlorella protothecoides	Viridiplantae, Trebouxiophyceae	X56101
Prototheca wickerhamii	Viridiplantae, Trebouxiophyceae	X56099
Choricystis minor	Viridiplantae, Trebouxiophyceae	Z55697
Parietochloris pseudoalveolaris	Viridiplantae, Trebouxiophyceae	M63002
Dictyochloropsis reticulata	Viridiplantae, Trebouxiophyceae	Z47207
Pleurastrum terrestrre	Viridiplantae, Trebouxiophyceae	Z28973
Trebouxia asymmetrica	Viridiplantae, Trebouxiophyceae	Z21553
Trebouxia imprexa	Viridiplantae, Trebouxiophyceae	Z21551
Myrmecia biatorellae	Viridiplantae, Trebouxiophyceae	Z28971
Microthamnion kuetzingianum	Viridiplantae, Trebouxiophyceae	Z28974
Mychonastis zofingiensis	Viridiplantae, Chlorophyceae, Sphaeropleales?	X74004
Ankistrodesmus stipitatus	Viridiplantae, Chlorophyceae, Sphaeropleales?	X56100

Neochloris aquatica	Viridiplantae, Chlorophyceae, Sphaeropleales	M62861
Characiopodium hindakii	Viridiplantae, Chlorophyceae, Sphaeropleales	M63000
Pediastrum duplex	Viridiplantae, Chlorophyceae, Sphaeropleales	M62997
Hydrodictyon reticulatum	Viridiplantae, Chlorophyceae, Sphaeropleales	M74497
Grasiella vacuolata	Viridiplantae, Chlorophyceae, Sphaeropleales	X56104
Scenedesmus obliquus	Viridiplantae, Chlorophyceae, Sphaeropleales	X56103
Ettlia minuta	Viridiplantae, Chlorophyceae, Chlorococcales	M62996
Pleurastrum insigne	Viridiplantae, Chlorophyceae, Chlorococcales	Z28972
Chlamydopodium starrii	Viridiplantae, Chlorophyceae, Chlorococcales	M84319
Chlamydopodium vacuolatum	Viridiplantae, Chlorophyceae, Chlorococcales	M63001
Botryococcus braunii	Viridiplantae, Chlorophyceae, Chlorococcales?	X78276
Dunaliella parva	Viridiplantae, Chlorophyceae, Dunaliellales	M62998
Dunaliella salina	Viridiplantae, Chlorophyceae, Dunaliellales	M84320
Asteromonas gracilis	Viridiplantae, Chlorophyceae, Dunaliellales	M95614
Spermatozopsis similis	Viridiplantae, Chlorophyceae, Chlamydomonadales?	X65557
Chlamydomonas applanata	Viridiplantae, Chlorophyceae, Chlamydomonadales	U13984
Chlamydomonas reinhardtii	Viridiplantae, Chlorophyceae, Chlamydomonadales	M32703
Polytoma uvella	Viridiplantae, Chlorophyceae, Chlamydomonadales	U22940
Volvox carteri	Viridiplantae, Chlorophyceae, Volvocales	X53904

[a] Accession number in GenBank, EMBL and DDBJ nucleotide libraries.

*1 Sequences are provided by Dr. M. Melkonian, Universität zu Köln.

Table 4. Comparison of the selected characters in the Mamiellales. MT s= microtubules.

Characters	*Pterosperma* [1]	*Crustomastix* [2]	*Mamiella* [3]	*Mantoniella* [4]	*Micromonas* [5]
1.Cell length	6-11	4-7	4-6.5	2.5-6.5	1-3
2. Golgi apparatus	2	1	1	1	1
3. lobes of Chloroplast	4	2	2	2	1
4. Pyrenoid	present	absent	present	present	present
5. Penetration in pyrenoid	cytoplasmic	-	cytoplasmic	absent	absent
6. Eyespot	absent	absent	present	present	absent
7. Extrusome	absent	absent	absent	absent	absent
8. Scale duct	present	present	absent	absent	absent
9. Pentagonal scales	present	absent	absent	absent	absent
10. Spider-web scales	present	absent	present	present	absent
11. Hair scales	present	present	present	present	absent
12. Two rows subunits in T-hairs	absent	absent	present	present	-
13. Number of flagella	4	2	2	1.5	0.5
14. Number of basal bodies	4	2	2	2	1
15. 2d, 2s roots	present	absent	absent	absent	absent
16. 1d root	10-12 MTs	3 MTs	3 MTs	2 MTs	2 MTs
17. 1s root	4 MTs	4 MTs	4 MTs	4 MTs	2 MTs
18. MLS	present	present	absent	absent	absent
19. duct fiber	present	present	present	present	absent
20. 1s-associated fiber	present	present	present	present	absent
21. Rhizoplast	present	present	present	present	absent

[1] Inouye et al. (1990), Inouye (unpubl. data)

[2] present study

[3] Moestrup (1984), Inouye (unpubl. data)

[4] present study, Barlow and Cattolico (1980), Marchant et al. (1989)

[5] present study, Manton (1959)

Table 5. Comparison of the selected characters in the Pyramimonadales. MT s= microtubules.

Characters	*Mamiella* [1]	*Crustomastix* [2]	*Pterosperma* [3]	*Cymbomonas* [4]	*Halosphaera* [5]	*Pyramimonas* [6]
1. Swimming direction [a]	forward	forward	forward	backward	backward	backward
2. Cilialy beat	absent	absent	absent	absent	absent?	present
3. Deep flagellar pit	absent	absent	absent	absent	present	present
4. Golgi apparatus	1	1	2	2	2	2
5. Extrusome	present	absent	present	absent	absent?	absent
6. Penetration in pyrenoid	cytoplasmic	-	cytoplasmic	thylacoid	thylacoid	thylacoid
7. Eyespot	present	present	absent	present	present	present
8. Eyespot - pyrenoid association	present	-	-	present	absent	absent
9. Eyespot layers	1	1	-	1	1	1-many
10. Scale duct	absent	present	present	present	present	present
11. Pentagonal scales	absent	absent	present	present	present	present
12. Spider-web scales	present	absent	present	absent	absent	absent
13. Box scales	absent	absent	absent	present	present	present
14. Crown scales	absent	absent	absent	absent	present	present
15. Second projection of Limuloid scales	absent	absent	present	present	present	present
16. Number of flagella	2	2	4	4	4	4-16
17. Coiled fiber	absent	absent	present	absent	present	present
18. Distal cartwheel	absent	absent	absent	absent?	present	present
19. 3 over 1 basal bodies	-	-	absent	present	present	present
20. Block-like distal fiber	absent	absent	absent	absent	present	present
21. Bb1-4 connecting fiber	-	-	absent	absent	present	present
22. 1d root	3 MTs	3 MTs	many MTs	many MTs	many MTs	2-3 MTs
23. 1s root	4 MTs	4 MTs	4 MTs	4 MTs	4 MTs	2-4 MTs
24. 2d, 2s roots	absent	absent	present	present	present	present
25. MLS	absent	present	present	present	present	absent
26. 1s-associated fiber	present	present	present	present	absent?	present
27. Plate overlaying 1s root	absent	absent	present	present	present	absent

[1] Moestrup (1984), Inouye (unpubl. data); [2] present study; [3] Inouye et al. (1990), Inouye (unpubl. data); [4] Inouye (unpubl. data);
[5] Hori et al. (1985), Hori (unpubl. data), Nakayama (unpubl. data); [6] Moestrup and Hori (1989), Sym and Pienaar (1995).
[a] Acoording to description in Chapter 3.

Table 6. Results of the loglikelihood ratio test for the phylogenetic relationships of the Chlorophyta in the maximum-likelihood analyses

Tree[1]	Difference in log-likelihood*	Standard deviation	Significantly worse?
1. ML tree (Tre, (Chl, (Olt, Ulv)))	-	-	-
2. Olt, (Tre, (Ulv, Chl))	-35.00	15.43	Yes
3. Olt, (Ulv, (Tre, Chl))	-33.66	16.52	Yes
4. Olt, (Chl, (Ulv, Tre))	-39.11	16.10	Yes
5. (Olt, Tre), (Ulv, Chl)	-37.45	16.02	Yes
6. (Olt, Chl), (Ulv, Tre)	-36.97	14.37	Yes
7. (Olt, Ulv) (Tre, Chl)	-10.99	10.78	No
8. Tre, (Olt, (Ulv, Chl))	-24.25	10.37	Yes
9. Tre, (Ulv, (Olt, Chl))	-21.52	11.06	No
10. Ulv, (Olt, (Tre, Chl))	-20.31	18.77	No
11. Ulv, (Chl, (Olt, Tre))	-21.05	19.48	No
12. Ulv, (Tre, (Olt, Chl))	-17.41	17.39	No
13. Chl, (Olt, (Ulv, Tre))	-46.35	14.85	Yes
14. Chl, (Ulv, (Olt, Tre))	-38.85	16.79	Yes
15. Chl, (Tre, (Olt, Ulv))	-15.41	9.60	No
16. *Hafniomonas* is base of Chlorophyceae	-77.27	21.93	Yes

[1] Olt =*Oltmansiellopsis* , Ulv = Ulvophyceae (*Gloeotilopsis*), Tre = Trebouxiophyceae, Chl = Chlorophyceae.

* Log-likelihood of the ML tree is -9494.64.

Table 7. Results of the loglikelihood ratio test for the phylogenetic relationships of the Chlorophyceae in the maximum-likelihood analyses

Tree[1]	Difference in log-likelihood*	Standard deviation	Significantly worse?
1. ML tree (CP, (CH, (SP, CW)))	-	-	-
2. CP, (SP, (CH, CW))	-13.66	8.80	No
3. CP, (CW, (CH, SP))	-15.55	8.28	No
4. (CP, CH), (SP, CW)	-4.62	10.45	No
5. (CP, SP), (CH, CW)	-21.21	18.55	No
6. (CP, CW), (CH, SP)	-38.43	14.57	Yes
7. CH, (CP, (SP, CW))	-11.65	8.68	No
8. CH, (CW, (CP, SP))	-18.32	17.97	No
9. CH, (SP, (CP, CW))	-32.93	14.51	Yes
10. SP, (CP, (CH, CW))	-39.04	14.58	Yes
11. SP, (CW, (CP, CH))	-23.71	14.65	No
12. SP, (CH, (CP, CW))	-39.02	14.66	Yes
13. CW, (CP, (CH, SP))	-30.30	16.20	No
14. CW, (SP, (CP, CH))	-14.67	16.18	No
15. CW, (CH, (CP, SP))	-20.38	18.52	No
16. *Hafniomonas* is base of Chlorophyceae	-76.88	23.16	Yes
17. *Hafniomonas* with *Planophila*	-73.77	24.08	Yes

[1] CP = Chaetopeltidales (*Planophila*), CH = Chaetophorales (*Chaetophora*), SP = Sphaeropleales, CW = CW group (Chlamydomonadales/Chlorococcales).
* Log-likelihood of the ML tree is -8274.56.

Table. 8 . Classification of the Ulvophyceae sensu Mattox and Stewart (1984).

O'Kelly and Floyd (1984b)	Sluiman (1989)[1]	Hoek *et al.* (1995)
ULVOPHYCEAE	ULVOPHYCEAE	ULVOPHYCEAE
Ulotrichales		Codiolales
Acrosiphoniaceae	Acrosiphoniales	
other families		
	Ulotrichales	
Ulvales		Ulvales
Siphonocladales	Cladophorales	CLADOPHOROPHYCEAE
Dasycladales	Dasycladales	DASYCLADOPHYCEAE
Caurelpales	Bryopsidales	BRYOPSIDOPHYCEAE

[1] He also included the Trentepohliales and Ctenocladales in the Ulvophyceae.

Table 9. Possible grouping of members of the CW group based on molecular data.

	complete 18SrDNA[a]	partial 18S/26SrRNA[b]	chloroplast 23SrDNA[c]
Volvox clade	*Chlamydomonas reinhardtii* Dangeard *Volvox carterii* Stein *Polytomella parva* Pringsheim	*Chlamydomonas reinhardtii* Dangeard *C. komma* Skuja *C. callosa* Gerloff *C. zebra* Korschikoff *C. mexicana* Lewin *C. peterfii* Gerloff *Asterophomene gubernaculifera* Pocok *Eudorina elegans* Ehrenberg *Gonium pectorale* Müller *Pandorina morum* (Müller) Bory *Platydorina caudata* Kofoid *Pleodorina californica* Shaw *Volvulina boldii* O'Neil et Starr *Volvox aureus* Ehrenberg	*Chlamydomonas reinhardtii* Dangeard *C. komma* Skuja *C. iyengarii* Mitra *C. starrii* Ettl *C. zebra* Korschikoff *C. gelatinosa* Korschikoff *C. mexicana* Lewin *C. peterfii* Gerloff *C. frankii* Pascher *C. pallidostigmatica* King
Tetracystis clade	*Chlamydomonas moewusii* Gerloff *Chlorococcum hypnosporum* Starr *C.*sp. *Tetracystis aeria* Brown et Bold	*Chlamydomonas moewusii* Gerloff[d] *C. pitschmannii* Ettl *C. noctigama* Korschikoff[e]	*Chlamydomonas moewusii* Gerloff[d] *C. pitschmannii* Ettl *C. noctigama* Korschikoff[e] *C.* sp. (SAG 66.72)
Dunaliella clade	*Chlamydomonas applanata* Pringsheim *Dunaliella salina* Teodorescu *D. parva* Lerche *Asteromonas gracilis* Artari *Chlamydopodium starii* (Fott) Ettl et Gärtner *C. vacuolata* (Lee et Bold) Ettl et KomÜJek *Chlorococcum oleofaciens* Trainor et Bold *Ettlia minuta* (Arce et Bold) KomÜJek *Pleurastrum insigne* Chodat *Protosiphon botryoides* (Kützing) Klebs	*Chlamydomonas applanata* Pringsheim[f] *Chlorogonium elongatum* Dangeard *Haematococcus lacustris* (Girod.) Rostaf. *H. zimbabwiensis* Pocock *Stephanosphaera pluvialis* Cohn	*Chlamydomonas applanata* Pringsheim[f]

[a] Present study.
[b] Buchheim et al. (1990) and Buchheim and Chapman (1991, 1992).
[c] Turmel et al. (1993).
[d] Listed as *C. eugamatos* Moewus and *C. indica* Mitrain in Bucheim et al. (1990), Buchheim and Chapman (1991, 1992) and Turmel et al. (1993).
[e] Listed as *C. geitleri* Ettl in Bucheim et al. (1990), Buchheim and Chapman (1991) and Turmel et al. (1993).
[f] Listed as *C. humicola* Lucksch in Buchheim et al. (1990), Buchheim and Chapman (1991), Turmel et al. (1993).

Table 10. New classification system for the green plants. Only orders discussed in present study are listed.

Subkingdom Viridiplantae

Division Streptophyta
- Class Chlorokybophyceae
- Class Klebsormidiophyceae
- Class Zygophyceae
- Class Coleochaetophyceae
- Class Charophyceae
- Class Antoceratopsida
- Class Marchantiopsida
- Class Bryopsida
- Class Lycopodiopsida
- Class Psilotopsida
- Class Equisetopsida
- Class Polypodiopsida
- Class Spermatopsida

Division Chlorophyta
- Class Tetraselmidophyceae
- Class Trebouxiophyceae
- Class Ulvophyceae
- Class Cladophorophyceae
- Class Dasycladophyceae
- Class Bryopsidophyceae
- Class Trentepohliophyceae
- Class Chlorophyceae
  - Order Chaetopeltidales
  - Order Chaetophorales
  - Order Sphaeropleales
  - Order Chlamydomonadales
- *incertae cedis*
  - Order Oltmannsiellopsidales

incertae cedis
- Class Mesostigmatophyceae
- Class Pyramimonadophyceae
- Class Mamiellophyceae
- Class Nephroselmidophyceae
- Class Pedinophyceae

Table 11. Classification of green plants. Divisions, classes, orders and families of green algae and possible genera of each taxa are listed. Families of the Zygnematophyceae and land plant's taxa below class level are not sited. Descriptions of new taxa are included.

Kingdom PLANTAE
Subkingdom VIRIDIPLANTAE Cavalier-Smith 1981

Division: STREPTOPHYTA Jeffrey 1982
Syn. Anthoceratophyta Sluiman 1983
Unicellular, sarcinoid, filamentous and parenchmatous organisms with chloroplasts containing chlorophylls *a* and *b*. Flagellate cell (zoospore or sperm), if produced, which is bi- or multiflagellate (Tracheodatae), has an asymmetric flagellar apparatus without microtubular root associated to no.2 basal body. Square scales often cover cell body and flagella (without Melkonian's row?). Outer dynin arms are completely absent. Basal bodies arranged in parallel to each other and to cell membrane. 1d microtubular root is usually well developed and has a multilayered structure (except Charophyceae). System I fiber, rhizoplast and eyespot are absent. Mitosis is open (except Zygnematophycee). Interzonal mitotic spindle is persistent at telophase. Cell walls consist of crystaline cellulose synthesized by rosette-like cellulose synthese complex. Pyrenoid, if present, has traversings of single thylacoid. Glycolate oxydase located in microbody and Cu/Zn superoxide dismutase are present. Habitat is predominantly freshwater.

Class: Chlorokybophyceae Bremer 1985 (*nom. nud.*)
Sarcinoid green algae; plasmodesmata and phragmoplat absent; scaly zoospore (squre scales) released by disintegration of sarcinoid packet.
Order: Chlorokybales Mattox et Stewart 1984 (*nom. nud.*)
Family: Chlorokybaceae (*nom. nud.*)
Genus: *Chlorokybus* Geitler 1942.

Class: Klebsormidiophyceae Jeffrey 1982 (*nom. nud.*)
Unbranched filaments without holdfast; plasmodesmata and phragmoplat absent; zoospore, if produced, naked and released through pore of wall.
Order: Klebsormidiales Stewart et Mattox 1975 (*nom. nud.*)
Family: Klebsormidiaceae Stewart et Mattox 1975 (*nom. nud.*)
Genera: *Klebsormidium* Silva *et al.* 1972; ?*Stichococcus* Nägeli 1849 (in part?); ?*Raphidonema* Lagerheim 1892 (in part?).

Class: Zygnematophyceae Round 1971 (*num. nud.*)

Syn. Akontae Blackman et Tansley 1903 (*nom. descript.*); Conjugatophyceae Engler 1892; Zygophyceae Widder 1960 (*nom. descript.*).

Coccoid or unbranched filaments; semiclosed or open mitosis; plasmodesmata absent; flagellate cell absent; sexual reproduction by conjugation.

Order: Zygnematales Borge et Pascher 1913

Cell walls lack pores or pore-like modifications. (this order may be paraphyletic)

Genera: *Zygnema* Agardh 1817; *Zygnemopsis* (Skuja) Transeau 1934; *Zygogonium* Kützing 1843; *Pleurodiscus* Lagerheim 1895; *Hallasia* Rosenvinge 1924; *Mougeotia* Agardh 1824; *Mougeotiopsis* Palla 1894; *Temnogametum* W. et G. S. West 1897; *Debarya* Wittrock 1872; *Spirogyra* Link 1820; Temnogyra; *Sirogonium* Kützing 1843; *Mesothaenium* Nägeli 1849; *Roya* W. et G. S. West 1896; *Cylindrocystis* Meneghini 1838; *Spirotaenia* Brébisson 1844; *Geniculus* Prescott 1966; *Netrium* (Nägeli) Itzsigshn et Rothe 1856; *Sirocladium* Randhawa 1941; *Gonatozygon* de Bary 1856; *Genicularia* de Bary 1858.

Order: Desmidiales

Cell consists of two symmetrical halves (semicells); cell walls with pores or pore-like modifications.

Genera: *Closterium* Nitzsch 1817; *Spinoclosterium* Bern. 1909; *Penium* de Brébisson 1844; Haplotaenium; *Pleurotaenium* Nägeli 1849; *Docidum* de Brébisson 1844; *Triploceras* Bailey 1850; *Actinotaenium* (Nägel) Teiling 1954; *Cosmarium* Corda 1834; *Cosmocladium* de Brébisson 1856; *Xantidium* Ehrenberg 1834; *Hyalotheca* Ehrenberg 1840; *Spondylosium* de Brébisson 1844; *Sphaerozoma* Corda 1825; *Desmidium* Agardh 1824; *Tetmemorus* Ralfs 1844; *Euastrum* Ehrenberg 1832; *Staurastrum* Meyen 1829; *Micrasterias* Agardh 1827.

Class: Coleochaetophyceae Bessey ex Woods 1894

Packed cells, branched filaments or discoid thalli; sheathed setae present; plasmodesmata and phragmoplat present; scaly flagellate cells; asexual reproduction by zoospores; oogamy.

Order: Coleochaetales Chadefaud et Emberger 1960

Family: Coleochaetaceae Nägeli 1847

Branched filaments or discoid thalli; oogonia surrounded by sterile cells; eggs retained

in oogonium.

Genus: *Coleochaete* de Brebisson 1844.

?Family: Chaetosphaeridiaceae Blackman et Tansley 1902

Packed cells or filaments with subapical filament growth (?).

Genera: *Chaetosphaeridium* Klebahn 1892; ?*Conochaete* Klebahn 1893; ?*Dicoleon* Klebahn 1893; ? *Chaetotheke* Düringer 1958; ?*Oligochatophora* W. et G. S. West 1903; ?*Polychaetophora* W. et G. S. West 1903.

Class: Charophyceae Rabenhorst 1863

Complex plant body with apical growth and differentiation into nodes and internodes; plasmodesmata and phragmoplat present; zoospore absent; oogamous haplont; sterile cells surrounded antherida and oogonia; eggs retained in oogonium; herical sperms with posteriorly directed two flgella with subapical insertions, scaly covering, reduced MLS and chloroplast, single mitochondrion.

Order: Chalales Borge et Pascher 1913

Family: Characeae Gray 1821

Genera: *Chara* L. ex Vaillant 1719; *Lamprothamnium* Groves 1916; *Lynchnothamnus* Leonhardi 1862; *Nitellopsis* Hy 1889; *Nitella* Agardh 1824; *Tolypella* Leonhardi 1863.

Class: Marchantiopsida, liverworts

Class: Anthoceratopsida, hornworts

Class: Bryopsida, mosses

Class: Lycopodiopsida, lycopods etc.

Class: Psilotopsida, *Psilotum* etc.

Class: Equisetopsida, equisetums

Class: Polypodiopsida, ferns

Class: Spermatopsida, spermatophtes (= seed plants)

Division: CHLOROPHYTA Pasher 1914

Unicellular, sarcinoid, colonial, filamentous thalloid, coenocytic, siphonous and parenchmatous organisms with chloroplasts containing chlorophylls *a* and *b*. Flagellate cell (zoospore or gamete), if produced, which is bi-, quadri- or multiflagellate (Bryopsidales and Oedogoniales), has an cruciate flagellar apparatus with 180° rotational symmetry. Single doublet (#1) lacks outer dynin arm. Basal bodies are usually short (>500nm), arranged end to end and inserted apically (except Tetraselmidophyceae).

System I fiber, rhizoplast and eyespot are usually present. MLS is absent. Mitosis is closed or semiclosed. Glycolate dehydrogenase is located in mitochondria. Cu/Zn superoxide dismutase is absent.

Class: Tetraselmidophyceae classis nov.

Syn. Prasinophyceae Moestrup et Throndsen, Can. J. Bot. 66, p. 1432 (1988).

Algae unicellulares vel coloniales chlorophyllis a *et* b *coloratae. Cellula monadoides quaternis flagellis, theca squamis distromaticis parvis oriunda induda. Flagella squamis distromaticis parvis tecta, partes basales ejus in foveam flagellarirem profundam immersae. Corpuscula basalia flagellorum parallela, modo valde flexuoso dispositis. Duo evoluti rhizoplasti extensi membranae cellulosae. Fusus mitoticus semiclausus, metacentricus et origine rhizoplasto. Fusus mitoticus interzonalis in telophase collabens, trochoplasto tum evoluto. Hab. in mari vel aqua durci.*

Genus typificum: Tetraselmis *Stein 1878.*

Unicellular or colonial alage with chlorophylls *a* and *b*. Monadoid cell with four flagella, invested with a theca derived from two-layered small scales. Flagella covered by two-layered small scales, with base inserted in a deep flagellar pit. Parallel basal bodies arranged in a "zig-zag" pattern. Two well-developed rhizoplasts extending to cell membrane. Mitotic spindle semiclosed, "metacentric" and originating from rhizoplast. Interzonal mitotic spindle collapses at telophase and a trochoplast develops. Habitat is marine or freshwater.

Type genus: *Tetraselmis* Stein 1878.

Order: Tetraselmidales Mattox et Stewart 1984

Syn. Tetraselmidales Ettl 1982 (*nom. nud.*), Chlorodendrales Fritsch 1918.

Familly: Chlorodendraceae Oltmanns 1904

Syn. Tetraselmidaceae Christensen 1962 (nom. nud.); Prasinocladaceae Ettl 1966 (nom. nud.); Platymonadaceae Christensen 1967.

Genera: *Tetraselmis* Stein 1878; *Scherffelia* Pascher 1911.

Class: Ulvophyceae Mattox et Stewart 1984 *sensu* Hoek *et al.* 1995

Syn. Codiolophyceae Kornmann 1973 (*nom. nud.*); Ulotrichophyceae Pascher ex Hollerbach et Polyansky 1951.

Coccoid, filamentous, thalloid and siphonocladous organisms; gamete biflagellate and zoospore usually quadriflagellate; some members have squre scales covering cell bodies of flagellates; flagellar apparatus cruciate with counterclockwise overlapped basal bodies; electron dense core often situated in basal body; terminal cap and developed proximal

sheath present; system I fibers underlie 1d root; rhizoplast, if present, from (#5 triplet of?) basal body, and associated with d root (and mating structure) in biflagellate cell; mating structure usually associated with d root; mitosis semiclosed and centrioles positioned lateral to spindle pole; cytokinesis performed by clevage furrow, vesicles derived from Golgi apparatus and trochoplat (?); plasmodesmata absent; bilenticular pyrenoid with crossing(s) of single tylakoid typical; alternation of generations common; isogamy or anisogamy; predominantly marine, but some members in freshwater and soil habitats.

Order: Codiolales Kornmann 1973

Syn. Ulotrichales Borzi 1895 *sensu* O'Kelly et Floyd 1984b; Acrosiphoniales Kornmann 1965.

Filamentous, thalloid and siphonocladous; terminal caps of (upper) basal bodies small; proximal sheath wedge-shaped; flagellate cells often surrounded by vesicle, produced through nonpapillate aperture; heteromorphic and diplohaplontic life cycle (except *Eugomontia*?);*Codiolum* type sporophyte (zygote?) produced in species with sexual reproduction.

Family: Acrosiphoniaceae Jónsson 1959

Branched or unbranched filaments; uninucleate or multinucleate; flagellate cells lack scales; electron dense materials overlie d roots; biflagellate cell lacks rhizoplast; small vesicles dispersed above the flagellar apparatus; polypyramidal pyrenoid with tubular penetrations of thylakoid.

Genera: *Acrosiphonia* Agardh 1846; *Chlorothlix* Berger-Perrot et Thomas 1982; *Spongomorpha* Kützing 1843; *Urospora* Areschoug 1866; ?*Psendendoclonium* Wille (in part); ?*Ulothrix* Kützing (in part).

Family: Ulotrichaceae Kützing 1843

(incl. Monostromataceae Kunieda 1934 sensu O'Kelly et Floyd 1984b)

Branched or unbranched filaments, thalloid or palmelloid; uninucleate; most members produce scaly flagellates; electron dense core of basal body absent; quadriflagellate zoospore has rhizoplasts associated with s roots and lower basal bodies.

Genera: *Capsosiphon* Gobi 1879; *Chamaetrichon* Tupa 1974; *Collinsiella* Setchell et Gardner 1903; *Eugomonta* Kornmann 1960; *Gayralia* Vinogradova 1969; *Gloeotilopsis* Iyengar et Philipose 1956; *Gomontia* Bornet et Flahault 1888, *Monostroma* Thuret 1854, *Protoderma* Kützing 1843; *Protomonostroma* Vinogradova 1969; *Trichosarcina* Nichols et Bold 1965; *Ulothrix* Kützing 1836,?*Psendendoclonium* Wille (in part) ; ?*Pseudopringsheimia* Wille (in part).

Order: Ulvales Blackman et Tansley 1902

Branched filamentous or thalloid; terminal cap of (upper) basal body distinct bilobed structure; proximal sheath consists of two equal subunit; gametangia and zoosporangia have identical structure and development, and form papillate exit aperture and "capsule"; isomorphic and diplohaplontic life cycle.

Family: Ulvaceae Lamouroux ex Dumortier 1822

Macroscopic thalloid algae; quadriflagellate zoospore has four rhizoplasts associated with all microtubular roots and basal bodies.

Genera: *Chloropelta* Tanner 1986; *Enteromorpha* Link in Nees 1820; *Letterstedtia* Kützing 1843; *Percursaria* Bory de Saint-Vincent 1823; *Ulva* L. 1753; *Ulvaria* Ruprecht 1850.

Family: Ulvellaceae Schmidle 1899 emend. O'Kelly et Floyd 1983

Microscopic branched filaments; rhizoplast is absent; apparent anisogamy.

Genera: *Acrochaete* Pringsheim 1862; *Endophyton* Gardner 1909; *Entocladia* Reinke 1879; *Ochlochaete* Thwaites in Harvey 1849; *Pringsheimiella* Höhnel 1920; *Ulvella* Crouan frat. 1859; ?*Pilinia* Kützing 1843; ?*Pseudendoclonium* Wille 1901; ?*Pseudopringsheimia* Wille in Engler et Prantl 1909; ?*Stromatella* Kornmann et Sahling 1983; ?*Syncoryne* Nielsen et Pedersen 1977; ?*Tellamia* Batters 1895.

Ulvophyceae ***incertae cedis***

Family: Chlorocystidaceae Kornmann et Sahling 1983

Unicellular coccoids; flagellate cells are scaly; terminal caps cover the entire proximal end of the upper basal bodies; other characters almost identical to taht of the Ulotrichaceae.

Genera: *Chlorocystis* Reinhard 1885; *Halochlorococcum* Dangeard 1965.

Family: Phaeophilaceae Chapell *et al.* 1990

Microscopic branchced filaments; quadriflagellate zoospore with six microtubular roots and large eyespot; rhizoplast and dense core of basal bodies absent; proximal end of upper basal body covered by single convex terminal cap; formation of zoospores occures in multinucleate zoosporangia by simultaneous clevage; zoospores liberated through neck apex with "plug" originated from residual cytoplasm.

Genus: *Phaeophila* Hauck 1876

Family: Kornmanniaceae Golden et Cole 1986

Macroscopic thaloid organizations; vegetative cells small (ca. 5μm); alternation of discoid gametophyte and tubular or monostromatic thallus typical; eyespopt absent.

Genera: *Blidingia* Kylin 1947; *Kornmannia* Bliding 1969.

"*Bolbocoleon* group" *sensu* O'Kelly et Floyd 1984b

Microscopic branchced filaments; hairs bear from bulb-shaped cells with chloroplast; zoospores released with plug.

Genera: *Bolbocoleon* Pringsheim 1862; ?*Acroblaste* Reinsch 1879.

Class: Cladophorophyceae

Syn. Confervophyceae Engler 1903; Siphonocladophyceae Wille in Engler 1909. Filamentous to vesicular thalli composed of multinucleate cells (siphonocladous); gamete biflagellate and zoospore usually quadriflagellate; flagellar apparatus cruciate with counterclockwise overlapped basal bodies; flagellar apparatus "flattened"; quadriflagellate cell has tetralobed distal fiber and striated fibers connecting upper and lower basal bodies; terminal cap and proximal sheath weakly developed; 1d root accompanied by electron dense fiber(s); rhizoplast, if present, associated with s root in quadriflagellate cell (?); "wing" present in s root; mating structure, if present, associated with d root; mitosis closed and centric; telophase nucleus dunbbell shape with persistent spindle; plasmodesmata absent; bilenticular pyrenoid with crossing(s) of single tylakoid typical; major component of cell walls crystaline cellulose produced by linear arrays of cellulose synthase particle; typical life cycle isomorphic and diplohaplontic; isogamy; gametangia and zoosporangia have identical structure and development with symultaneous cleavage; flagellate cells are liberated through papillate apertures with "plug"; protoplasmic streaming absent; usually macroscopic; predominantly marine, but some members have freshwater habitats.

Order: Cladophorales G. S. West 1904

Family: Chaetosiphonaceae Blackman et Tansley 1902

Microscopic flamentous algae.

Genera: *Chaetosiphon* Huber 1892; *Blastophysa* Reinke 1888

Family: Arnordiellaceae Fritsch 1935

Genera: *Arnordiella* Miller 1928; *Basicladia* Hoffmann et Tilden 1930; *Dermatophyton* .

Family: Cladophoraceae Wille in Warming 1884

Branched or unbranched filamentous thallus. (paraphyly?)

Genera: *Bryobesia* Weber-van Bosse 1911; *Chaetocladiella* Meyer et Skabistschevsky 1968; *Chaetomorpha* Kützing 1845; Chaetonella Schmidle 1901; *Cladophora* Kützing 1843; *Cladophorella* Fritsch 1944; *Cladostroma* Skuja in Handel-Mazzetti 1937; *Gemmiphora* Skabichevsky 1931; *Pithophora* Wittrock 1877; *Rhizoclonium* Kützing 1843; *Wittrockiella* Wille 1909.

Family: Anadyomenaceae Kützing 1843

Filaments unite latetally to form a net lying in one plane.

Genera: *Anadyomene* Lamouroux 1812; *Cystodictyon* Gray 1866; *Microdictyon* Decaisne 1841; *Valoniopsis* Børgesen 1934;*Willeella* Børgesen 1930.

Family: Siphonocladaceae Schmitz 1879

Segregative division present; branched filamentous thalli.

Genera: *Apjonnia* Harvey 1855; *Boergesenia* Feldmann 1938; *Boodlea* Murray et de Toni 1889; *Chamaedris* Montagne 1842; *Cladophoropsis* Børgesen 1905; *Pseudostruvea*; *Siphonocladus* Schmitz 1879; *Struvea* Sonder 1845.

Family: Valoniaceae Kützing 1849

Segregative division present; aggregation of large vesicular cells and lacks a central axis; small leticular cells usually present.

Genera: *Dictyosphaeria* Decaisne ex Endlicher 1843; *Ernodesmis* Børgesen 1912; *Valonia* C. Agardh 1822; *Ventricaria* Olsen et West 1988.

Class: Dasycladophyceae

Siphonous thalli comsist of erect axis bearing whorls of lateral branches; only motile stage biflagellate gamete; flagellar apparatus cruciate with counterclockwise overlapped basal bodies; flagellar apparatus "flattened"; terminal cap and proximal sheath weakly developed; 1d root underlain by system I fiber; rhizoplast, if present, has no association with microtubular roots; "wing" present in s root; mitosis closed and acentric; telophase nucleus dunbbell shape with persistent spindle; pyrenoid absent; strage products fructan and starch reserved in chloroplast and cytoplasm; major component of cell walls ß-1,4-mannan, but cellulose dominant in gametangial cysts; clcium carbonate often encrusts; vegetative thallus has a single giant diploid nucleus at first (giant cell), and becomes haploid multinucleate thallus by meiosis and following mitoses; gametogenesis occurs in operculate gametangial cysts within lateral branches; simultaneous cleavage produces gametes; isogamy; protoplasmic streaming present; marine.

Order: Dasycladales Pascher 1931

Family: Dasycladaceae Kützing 1843

Genera: *Acetabularia* Lamouroux 1821; *Acicularia* d'Archiac 1843; *Batophora* Agardh 1854; *Bornetella* Munier-Chalmas 1877; *Chalmasia* Solms-Laubach 1895; *Cympohlia* Lamouroux 1816; *Dasycladus* Agardh 1828; *Halycoryne* Harvey 1859; *Neomeris* Lamouroux 1816; *Polyphysa* Lamarck 1816.

Class: Bryopsidophyceae Bessey 1907

Filamentous to vesicular thallus essencially a single multinucleate cell (siphonous); gamete biflagellate and zoospore quadri- (*Osterobium*) or multiflagellate (Bryopsidaceae); flagellar apparatus cruciate with counterclockwise overlapped basal bodies; distal fiber weakly or not at all striated; terminal cap (?) covers half of proximal end of basal body; rhizoplast (?) associated with opposite s root in male gamete, and with d root in female gamete; mating structure, if present, associated with d root; mitosis closed, and centric or acentric; telophase nucleus dunbbell shape with persistent spindle; major component of cell walls xylan or mannan; cellulose, if present, not crystaline; multinucleate haploid gametophyte alternate with zygote containg a giant diploid nucleus (?); after meiosis (?), zygote develop to gametophyta directly or to sporophyte producing zoospores which grow into gametophyta (Bryopsidaceae); anisogamy typical; flagellate cells produced by symultaneous cleavage liberated through exit papillae; siphonéin and siphonaxiantin present; protoplasmic streaming is present; usually macroscopic; marine (except *Dichotomosiphon*).

Order: Codiales Setchell 1929

Syn. Derbesiales Feldmann 1954; Bryopsidales Fott 1959.

Leucoplast absent (homoplastidic); thylakoid organizing body absent; thallus not holokarpic; pyrenoid, if present, has penetration(s) of a single tylakoid.

Family: Bryopsidaceae Bory 1829

Stephanokont zoospore present; alternation of generations between gametophyte and sporophyte (?); major component of cell walls of gametophyte xylan (and cellulose), while that of sporophyte mannan.

Genera: *Bryopsis* Lamouroux 1809; Bryopsidella; *Derbesia* Solier 1847; *Pedobesia* McRaild et Womersley 1974; *Trichosolen* Montagne 1860.

Family: Codiaceae Kützing 1843

Stephanokont zoospore absent; alternation of generation absent (?); cell walls of thalli consists of mannan.

Genus: *Codium* Stackhouse 1797; ?*Pseudocodium* Weber van Bosse 1896.

Order: Caulerpales Setchell 1929

Leucoplast present (heteroplastidic); thylakoid organizing body present (except Dichotomosiphonaceae); thallus holokarpic; cell wall consists predominantly of ß-1,3-xylan; pyrenoid, if present, has no penetration.

Family: Caulerpaceae Kützing 1843

Thallus differrentiates into horizontal stolon attached by rhizoids and bearing erect fronds; thallus with internal trabeculae traversing the lumen.

Genera: *Caulerpa* Lamouroux 1809; *Caulerpella* Pru'dhomme van Reine et

Lokhorst 1992.

Family: Halimedaceae Link 1832

Thallus is composed of tafted subdichotomous filaments or of filaments interwoven to form macroscopic flattened branched thallus.

Genera: *Avrainvillea* Decaisne 1842; *Boodleopsis* Agardh et Gepp 1911; *Callipsygma* Agardh 1887; *Chlorodesmis* Harvey et Bailey 1851; *Cladocephalus* Montagne 1860; *Flabellaria* Lamouroux 1813; *Halimeda* Lamouroux 1812; *Johnson-sea-linkia* Eiseman et Earle 1983; Penicillus Lamarck 1813; *Pseudochlorodesmis* Børgesen 1925; *Rhipilia* Kützing 1858; *Rhipiliopsis* Gepp et Gepp 1911; *Rhipocephalus*; *Tydemania* Weber van Bosse 1901; *Udotea* Lamouroux 1812;.

Family: Dichotomosiphonaceae Chadefaud ex G. M. Śmith 1950

Simple siphonous filaments; siphonaxanthin absent; oogamy; freshwater.

Genus: *Dichotomosiphon* Ernst 1902.

Bryopsidophyceae ***incertae cedis***

Family: Osterobiaceae Silva 1982

Microscopic siphonous filaments; cell wall consists of mannan; quadriflagellate zoospore present.

Genus: *Osterobium* Bornet et Flahault 1889.

Class: Trentepohliophyceae Hoeck (nom. nud.)

Filamentous uninucleate green algae; quadriflagellate zoospores and biflagellate gamates produced; flagella have two oppsing "keels" subtended by microtubules; flagellar apparatus usually flattened and cruciate with counterclockwise overlapped basal bodies; distal fiber is absent; conical terminal cap (?) completely covers proximal end of basal body and s root; columnar structure associated with d root; mitosis closed (?); persistent interzonal spindle situated between doughter nucleus at telophase; vesicles align amid spindle microtubules in plane of division, and produce cell plate like phragmoplast; plasmodesmata present; cells contain several discoid chloroplasts without pyrenoids; hematochrome (mainly ß-carotene) and polyhydroxyalchols reserved; life cycle heteromorphic (?), and alternation of diploid sporophyte (characteristic shape with head cell, recurved suffultory cells and spherical zoosporangia) producing quadriflagellate zoospores and haploid gametophyte producing biflagellate isogametes (?); subaerial habitats.

Order: Trentepohliales Chadefaud et Emberger 1960

Family: Trentepoliaceae Hansgirg 1886

Genera:*Trentepohia* Martius 1817; *Cephaleuros* Kunze 1827; *Stomatochroon* Palm 1934; *Phycopeltis* Millardet 1870, ?*Ctenocladus* Borzi 1883.

Class: Trebouxiophyceae Friedl 1995

Coccoid, sarcinoid, filamentous and membranous green algae; flagellate cells always biflagellate and often compressed; flagellar apparatus cruciate with counterclockwise overlapped basal bodies; plate-like platform covers partly proximal end of basal body; system I fibers absent; rhizoplast, if present, usually originates from each basal body (#5 triplet ?) and merged into single strand; mitosis semiclosed and centrioles positioned close to division plane (metacentric spindle); cytokinesis performed by clevage furrow, vesicles derived from Golgi apparatus and trochoplat; plasmodesmata absent; pyrenoid, if present, with crossing(s) of tylakoid(s) typical; sexual reproduction known only in the Prasiolaceae (oogamy); freshwater, soil, subaerial, marine and phycobiont habitats. Classifications at ordinal and family level are uncertain.

Genera:*Trebouxia* Puymaly 1924; *Pseudotrebouxia* Archibald 1975; *Myrmecia* Printz 1921; *Pleurastrosarcina* Sluiman et Blommers1990; "*Pleurastrum* Chodat" (in part); *Microthamnion* Nägeli 1849; *Fusochloris* Floyd et Watanabe 1993; *Kentrosphaera* Borzi 1883; *Protοtheca* Krüger 1894; *Chlorella* Beijerinck 1890 (*sensu* Kalina et Punkacharova 198X); *Nannochloris* Naumann emend. Sarokin et Carpenter 1982; ?*Stichococcus* Nägeli 1849 (in part?); ?*Raphidonema* Lagerheim 1892 (in part?); ?*Apatococcus* Brand 1925; ?*Lobococcus* Reisigl 1964; ?*Ectogeron* Dangeard 1947; *Prasiola* (Agardh) Meneghini 1838; *Rosenvingiella* Silva 1957; *Prasiolopsis* Visher 1953; ?*Prasiococcus* Vischer 1953.

Class: Chlorophyceae Christensen 1994 *sensu* Mattox and Stewart 1984

Syn. Chlorophyceae Wille in Warming 1884 (*nom. descript.*); Chaetophorophyceae Wille 1909; Chlorococcophyceae Hollerbach et Polyansky 1951; Oedogoniophyceae Round 1963 (*nom. nud.*); Stephanokontae Blackman et Tansley 1902 (*nom. descript.*) Tetrasporophyceae Pascher ex. Hollerbach et Polyansky 1951; Volvocophyceae Schoenichen 1925.

Flagellate, coccoid, colonial, sarcinoid, filamentous or thalloid green algae; flagellate cells biflagellate or quadriflagellate; flagellar apparatus cruciate with directly opposed or clockwise basal bodies; proximal fiber absent; system I fibers associated with d root; mitosis semiclosed and centric; cytokinesis performs by phycoplast (trochoplast,

radichoplast or mesoplast); sexual reproduction isogamy, anisogamy or oogamy; algae predominantly in freshwater but also in soil, subaerial and marine habitats.

Order: Chaetopeltidales O'Kelly *et al.* 1994

Unicellular, colonial, filamentous or thalloid green algae; pseudocilium sometimes present; quadriflagellate zoospores covered with square scales (only cell body); flagellar apparatus cruciate with directly opposed upper and lower basal bodies; distal fiber tetralobate; proximal sheath developed; d root surrounded by four electron dense components (system I fiber?); rhizoplast originates from (#5 triplet of?) lower basal body; plasmodesmata absent; pyrenoid with cytoplasmic invagination typical; sexual reproduction unknown; freshwater.

Family: Chaetopeltidaceae G. S. West 1904

Genera: *Chaetopeltis* Berthold 1878; *Dicranochaete* Hieronymus 1887; *Hormotilopsis* Trainor et Bold 1953; *Planophila* Gerneck *sensu* Groover et Hostetter; *Phylogloea* Silva 1959; *Schizochlamys* Braun 1849; ?*Placosphaera* Dangeard 1889; ?*Porochloris* Pascher 1929.

Order: Chaetophorales Wile in Engler et Prantl 1909

Filamentous or thalloid green algae; quadriflagellate zoospores and biflagellate gametes produced (?); flagellar apparatus cruciate; directly opposed upper basal bodies and clockwise lower basal bodies; basal bodies connected by distal, peripheral and terminal fibers; prominent connecting fiber between basal body and s root; d root sandwiched by electron dense components (system I fiber?); rhizoplast originates from (#7 triplet of?) lower basal body; centrioles situated at poles and without migration in mitosis; cytokinesis performed by mesoplast and cell plate of golgi vesicles; plasmodesmata present; freshwater or soil.

Family: Chaetophoraceae Greville 1824

Filamentous or thalloid; pyrenoid with thylakoids appressed between matrix and starch sheath; diplohaplontic or diplontic life cycle (?); isogamy (?).

Genera: *Chaetophora* Schrank 1789; *Draparnaldia* Bory 1808; *Draparnaldiopsis* Smith et Klyver 1929; *Fritschiella* Iyengar 1932; Pseudoschizomeris Deason et Bold 1960;*Stigeoclonium* Kützing 1843; *Uronema* Lagerheim 1887.

Family: Schzomeridaceae G. M. Smith 1933

Unbranched cylindrical thallus displays of bricklike arrangement of cells; pyrenoid with wavy traversings of thylakoid.

Genus: *Schizomeris* Kützing 1843.

?Family: Aphanochaetaceae Oltmanns 1904

Branched filaments with hairs; pyrenoid with tubules of thylakoid; oogamy (?).

Genera: *Aphanochaete* Braun 1849; ?*Chaetonema* Nowakovsky 1876; ?*Gonatoblaste* Huber 1892; ?*Thamniochaete* Gay 1893.

Order: Sphaeropleales Luerssen 1877

Coccoid, colonial or filamentous green algae; cells usually multinucleate; biflagellate zoospores or gametes produced; cruciate flagellar apparatus with directly opposed basal bodies; in mitosis, centrioles situated at poles at first, but migrate to division plane side of doughter nuclei later; cytokinesis performed by trochoplast and cleavage furrow or centrifugal cleavage with Golgi vesicles; plasmodesmata absent; outer cell wall often consists of sporopollenin-like substance; algae grow predominantly in freshwater but also occur in marine or soil. (paraphyly??)

Family: Sphaeropleaceae Kützing 1849

Unicellular or unbranched filamentous algae with many nuclei; s root consists of many (>7) microtubules; pyrenoid with invagination of cytoplasm; anisogamy or oogamy; freshwater.

Genera: *Atractomorpha* Hoffman 1983; *Sphaeroplea* Agardh 1824; ?*Ankyra* Fott 1957; ?*Korshikoviella* Silva 1959; ?*Schroedeiella* Lemmermann 1898.

Family: Neochloridaceae Ettl et Komárek 1982

Unicellular coccoid algae with many nuclei; basal bodies connected by distal fiber with ribbed structure; partial caps covers the s root side of basal body; system I fiber overlies d root; rhizoplast, if present, originates from (#7 triplet of?) basal body; striated fiber connects basal body with s root; pyrenoid with continuous starch sheath and no invasion is typical; sexual reproduction unkown; freshwater or soil.

Genera: *Neochloris* Starr 1955; *Characiopodium* Floyd et Watanabe 1993.

Family: Hydrodictyaceae Dumortier 1829

Colonial coccoid algae (coenobia); juxtaposed settled zoospores develop to coenibia; basal bodies connected by distal fiber with ribbed structure; partial caps covers the s root side of basal body; system I fiber overlies d root; striated fiber connects basal body with opposite s root; s root consists of many (ca. 6-8) microtubules; pyrenoid with continuous starch sheath and no invasion; isogamey; zygote polyeder; freshwater.

Genera: *Hydrodictyon* Roth 1800; *Pediastrum* Meyen 1829; *Sorastrum* Kützing 1845; *Euastropsis* Lagerheim 1894; ?*Chlorotetraedron* McEntee *et al.* 1978; ?*Tetraedron* Kützing 1845.

?Family: Microsporaceae Bohlin 1901

Unbranched filamentous alga; cell walls composed of overlapping H-shaped segments;

pyrenoid absent; isogamy (?); freshwater.

Genus: *Microspora* Thuret 1850.

Sphaeropleales ***incertae cedis***

Genera: *Scenedesmus* Meyen 1829; *Mychonastes* Simpson et van Valkenburg 1978; *Grassiella* Kalina et Pun; *Halochlorella* Dangeard 1965; *Scotiellopsis* Vinatzer 1975; *Coelastrella* Chodat 1922; *Coelastrum* Nägeli 1849; *Planktosphaeria* G. M. Smith 1918; ?*Actinastrum* Lagerheim 1882; ?*Neodesmus* Hindák 1976; ?*Dicloster* Jao *et al.* 1976; ?*Didymocystis* Korschikoff 1953; ?*Tetradesmus* G. M. Smith 1913; ?*Enallax* Pascher 1943; ?Pseudotetradesmus Hirose et Akiyama 1963; ?*Rayssiella* Edelstein et Prescott 1964; ?*Schmidleia* Woloszynska 1914; ?*Schroederiella* Woloszynska 1914; ?*Tetrallantos* Teling 1916; ?*Gilbertsmithia* Iyengar 1975; ?*Crucigenia* Morren 1830; ?*Suxenella* Srivastava et Nizam 1969; ?*Crucugeniella* Lemmermann 1900; *Didymogenes* Schmidle 1905; ?*Willea* Schmidle 1900; ?*Tetrastrum* Chodat 1895; ?*Westella* De-Wildeman 1897; ?*Tetrachlorella* Korschikoff 1939; ?*Polyedriopsis* Schmidle 1899.

Order: Chlamydomonadales Fritsch in G. S. West et Fritsch 1927

Syn. Volvocales Oltmanns 1904; Chlamydomonadales Pascher 1931; Tetrasporales Pascher 1914; Chlorococcales Marchand 1895; Chlorosarcinales Groover et Bold 1960.

Flagellate, coccoid, colonial, sarcinoid, filamentous or siphonous green algae; flagellate cells typically biflagellate and often covered by crystaline cell wall consists of fibrous glycoproteins; cruciate flagellar apparatus with clockwise basal bodies; basal bodies connected by distal and proximal fibers (*sensu* Ringo 1967); system I fiber overlies d root; rhizoplast originates from (#7 triplet of?) basal body; centrioles situated near the divison plane after mitosis; cytokinesis performed by cleavage furrow or centrifugal cleavage; trochoplast or radichoplast; plasmodesmata absent; isogamy, anisogamy or oogamy; algae grow in freshwater, marine or soil habitats.

Classification at family level is uncertain.

Genera: *Chlamydomonas* Ehrenberg 1833; *Chloromonas* Gobi 1899; *Polytoma* Ehrenberg 1832; *Heterochlamydomonas* Cox et Deason 1969; *Gloeomonas* Klebs 1888; *Sphaerellopsis* Korschikoff 1925; *Lobomonas* Dangeard 1898; *Selenochloris* Pascher 1927; *Brachiomonas* Bohlin 1897; *Diplostauron* Korschikoff 1925; *Carteria* Diesing 1866 (incl. group I and II *sensu* Lembi);

Pseudocarteria Ettl 1958; *Provasoliella* Loeblich 1967; *Tetratoma* Bütschli 1884; *Chlaiomonas* Christen 1959; *Chlorobrachis* Korschikoff 1925; *Haematococcus* Agardh 1828; *Chlorogonium* Ehrenberg 1830; *Stephanosphaera* Cohn 1852; *Granulochloris* Pascher et Jahoda 1928; *Thorakomonas* Korshikoff 1925; *Coccomonas* Stein 1878; *Dysmorphococcus* Takeda 1916; *Pedinoperopsis* Korshikoff 1938; *Cephalomonas* Higinbotham 1942; *Wislouchiella* Skvortzow 1925; *Chlamydoblepharis* Francé 1894; *Fortiella* Pascher 1927; *Hemitoma* Skuja 1939; *Pedinopera* Pascher 1925; *Phacotus* Perty 1852; *Tingitanella* Bourrelly et Gayral 1950; *Pteromonas* Seligo 1887; *Iyengariomonas* Desikachary 1965; *Pyrobotrys* Arnoldi 1916; *Chlorcorone* Fott 1967; *Basichlamys* Skuja 1956; *Tetrabaena* (Dujardin) Fromentel 1874; *Asterophomene* Pocock 1954; *Gonium* Müller 1773; *Pandorina* Bory 1824; *Yamagisiella* Nozaki 1992; *Platydorina* Kofoid 1899; *Eudorina* Ehrenberg 1831; *Pleodorina* Shaw 1894; *Volvulina* Playfair 1915; *Volvox* Ehrenberg 1830; *Polytomella* Aragao 1910; *Spermatozopsis* Korchikoff 1913; ?*Hafniomonas* Ettl et Moestrup 1980; *Dunaliella* Teodorescu 1905; *Asteromonas* Artari 1913; *Chlorangiella* De Toni 1889; *Chlorangiopsis* Korshikoff 1932; *Chlamydomonadopsis* Fott 1972; *Physocytium* Borzi 1883; *Stylosphaeridium* Geitler et Gimesi in Geitler 1925; *Pseudochlorangium* Bourrelly 1966; *Chlorophysema* Pascher 1927; *Cecidochloris* Skuja 1948; *Metapolytoma* Skuja 1958; *Palmellopsis* Korschikoff 1953; *Tetrasporidium* Möbius 1893; *Gloeococcus* A. Braun 1851; *Pseudosphaerocystis* Woronichin 1931; *Chlamydocapsa* Fott 1972; *Ploeotila* Mrozi´nska-Webb 1972; *Asterococcus* Scherffel 1909; *Sphaerellocystis* Ettl 1960; *Nautocapsa* Ettl et Ettl 1959; *Tetraspora* Link 1809; *Apiocystis* Nägeli in Kützing 1849; *Paulschulzia* Skuja 1948; *Octosporiella* Kugrens 1984; *Chaetochloris* Pascher et Korschikoff in Korschikoff 1932;*Cystomonas* Ettl et Gärtner 1987; *Chlorococcum* Meneghini 1842; *Neospongiococcum* Deason 1971; *Radiosphaera* Snow 1918 ex Herndon 1958; *Apodochloris* Komárek 1959; *Nautococcus* Korschikoff 1926; *Planochloris* Komárek 1979; *Fasciculochloris* McLean et Trainor 1965; *Heterotetracystis* Cox et Deason 1968; *Tetracystis* Brown et Bold 1964; *Spongiococcum* Deason 1959; *Bordinellopsis* Dykstra 1971; *Axilosphaera* Cox et Deason 1968; *Actinochloris* Korschikoff 1953; *Macrochloris* Korschikoff 1926; *Deasonia* Ettl et Komárek 1982; *Pseudodictyochloris* Vinatzer 1975; *Characiochloris* Pascher 1927;

Chlamydopodium Ettl et Komárek 1982; *Hormotila* Borzi 1883; *Spongiochloris* Starr 1955; *Ascochloris* Bold et McEntee 1974; *Dictyochloris* Vischer 1945; *Bracteacoccus* Tereg 1923; *Ettlia* Komárek 1989; *Protosiphon* Klebs 1896; *Urnella* Playfair 1918; *Chlorosarcina* Gerneck 1907; *Chlorosarcinopsis* Herndon 1958; *Pseudotetracystis* Arneson 1973; *Chlorosphaeropsis* Vischer 1933; *Neochlorosarcina* Watanabe 1983;*Chlorochytrium* Cohn 1872; *Gongrosira* Kützing 1843; *Pleurastrum* Chodat 1894; ?*Botryococcus* Kützing 1849.

Order: Oedogoniales

Branched or unbranched thalli; flagellate cells (zoospore and male gamate) stephanokont; mitosis closed and acentric; cytokinesis performed by mesoplast and cell plate produced by ER; plasmodesmata absent; characteristic ring of cell wall produced by growth and division of cell; reticulate chloroplast has pyrenoids with cytoplasmic invaginations; life cycle haplontic involving oogamy; freshwater.

Family: Oedogoniaceae de Bary 1854

Genera: *Oedogonium* Link 1820; *Bulbochaete* Agardh 1817; *Oedocladium* Stahl 1891.

CHLOROPHYTA *incertae cedis*

Order: Oltmannsiellopsidales Nakayama *et al.* 1996

Unicellular or colonial quadriflagellates; cells naked or covered by square scales; flagellar apparatus cruciate with counterclockwise basal bodies; flagellum no.1 and 4 extending to the same direction; electron dense fiber overlays on distal fiber and s roots; sexual reproduction unknown.

Family: Oltmannsiellopsidaceae Nakayama *et al.* 1996

Genus: *Oltmannsiellopsis* Chihara et Inouye 1986.

Order: Cylindrocapsales Prescott 1951

Unbranched filaments; quadriflagellate zoospores surrounded by vesicle; quadriflagellate zoospre covered by thin cell wall (?); cruciate flagellar apparatus with d root sandwiched by electron dense components (system I fiber?) and s root composed of many microtubules; mitosis closed, centric and collapses at telophase; centrioles migrate near the divison plane after mitosis; cytokinesis performed by vesicles derived from ER and phycoplast (trochoplast?); doughter cells completely covered by new cell wall; prasmodesmata absent; asteroid chloroplast possessing pyrenoids with cytoplasmic penetrations; thick stratified cell walls; oogamy by oogonia and biflagellate sperm; freshwater.

Family: Cylindrocapsaceae Wille in Warming 1884

Genus: *Cyrindrocapsa* Reinsch 1867.

"***Ignatius* group"** *sensu* S. Watanabe (person. comm.)

Coccoid green algae; quadriflagellate zoospores produced; flagellar apparatus cruciate; counterclockwise upper basal bodies without overlap and clockwise lower basal bodies; pyrenoid with thylakoid transverse; freshwater.

Genera: *Ignatius* Bold et McEntee 1974; *Pseudocharacium* Korschikoff 1953.

Division: ***incertae cedis***

Class: Mesostigmatophyceae classis nov.

Algae unicellulares chlorophyllis a et b praeditae. Cellulae vegetativae monadoides foveis flagellarbus. Cellulae monadoides duobus flagellis aequalibus; squamis campanulatis quadratis, naviculiformis et squamis interioribus quadrataris tectae; micro-corpusculo elongato consociato cum "1d" radice microtubulari. Flagella squamis similibus folio Aceris tecta, sine serie Melkonianii et pilis minuta. Apparatus flageorum cruciatus, duo structuris multistratosis. Duo rhizoplasti extensi super chloroplasto. Omnes duplicatus-microtubi sine branchio dynini externo in parte distali flagelli; Hab. in aqua durci.

Genus typificum : Mesostigma *Lauterborm 1894.*

Unicellular algae with chlorophylls a and b. Vegetative cells monadoid with flagellar pit. Flagellate cells with two equal flagella; covered by basket-shaped, naviculoid and small square scales; with elongate microbody associated with 1d root. Flagella covered by maple-leaf shaped small scales, without Melkonian's row and hair scales. Flagellar apparatus cruciate, with two MLS. Two rhizoplasts extending above the chloroplast. All doublet lack outer dynin arm at distal part of flagellum. Habitat in freshwater.

Type genus: *Mesostigma* Lauterborn 1894.

Order: Mesostigmatales ord. nov.

Characteribus classis.

Genus typificum: Mesostigma *Lauterborm 1894.*

Characters are the same as the class Mesostigmatophyceae.

Type genus: *Mesostigma* Lauterborm 1894.

Family: Mesostigmataceae Fott 1974 ex Moestrup et Throndsen 1988

Genus: *Mesostigma* Lauterborm 1894

Division: ***incertae cedis***

Class: Pyramimonadophyceae classis nov.

Algae unicellulares chlorophyllis a *et* b *praeditae. Cellulae vegetativae monadoides.*

*Cellulae monadoides quattuor (raro 8 vel 16) flagellis aequalibus; squamis arachnoideis vel oriundis squamis arachnoideis tectae, et squamis interioribus quadrataris vel rhombicaris; ductu squamae et duobus apparatibus Golgis. Flagella fibra circinali, serie Melkonianii et pilis minuta. Apparatus flageorum vitta fibrillosa et fibra ductus; structura multistratosa et structura tabulari super "1s" radice microtubulari (*Pyramimonas *excepta). Rhizoplastus conjunctivus inter corpusculum basalem et regionem pyrnoidali chloroplasti. Unicus duplicatus-microtubus (#1) branchio dynini externo reducto; A-tubus duplicati-microtubi diversus (#6) structura cuneata. Hab. plerumque in mari, raro in aqua durci. Genus typificum:* Pyramimonas *Schmarda 1850.*

Unicellular algae with chlorophylls a and b. Vegetative cells monadoid. Flagellate cells with four (rarely 8 or 16) equal flagella; with covering of spider-web or its derived (limuloid, box, crown) scales, and square or diamond shaped inner scales; with scale duct and two Golgi apparatuses. Flagella with coiled fiber, Melkonian's row and hair scales. Flagellar apparatus with fibrillar band and duct fiber; with MLS and plate structure on 1s root (except *Pyramimonas*). Rhizoplast connecting between basal body and the pyrenoid region of the chloroplast. A doublet (#1) with reduced outer dynin arm; A-tubule of other doublet (#6) with V-shaped structure. Habitat usually marine, rarely in freshwater.

Type genus: *Pyramimonas* Schmarda 1850.

Order: Pyramimonadales Chadefaud 1950

syn. Halosphaerales T. Christensen 1960 (nom. nud.)

Family: Pterospermataceae Ostenfeld 1903

Flagella extend posteriorly; box scale and eyspot absent; pyrenoid with cytoprasmic penetration.

Genera: *Pterosperma* Pouchet 1893, *Tasmanites* Newton 1875.

Family: Pyramimonadaceae Korshikov 1938

syn. Halosphaeraceae Haeckel 1894; Pyramimonadaceae Ettl 1958

Flagella extend anteriorly at first; box scale and eyspot present; pyrenoid with crossing of tylakoid.

Genera: *Pyramimonas* Schmarda 1850, *Halosphaera* Schmitz 1879, *Cymbomonas* Schiller 1913.

Division: ***incertae cedis***

Class: Mamiellophyceae classis nov.

Syn. Micromonadophyceae Mattox et Stewart, Systematic of the Green Algae, p. 66. 1984. (nom. descriptivum)

Algae unicellulares chlorophyllis a *et* b, *prasinoxanthino et uriolide praeditae. Cellulae*

vegetativae monadoides vel coccoides. Cellulae motiles flagellis uno vel duobus subaequalibus vel inaequalibus; plerumque squamis arachnoideis tectae; strato interiore squamarum quadratarum vel rhombicarum nullo. Flagella plerumque pilis minuta. Apparatus flageorum sine radice microtubulari consociata cum corpusculo basali secundo; rhizoplastum unicum conjunctivo inter corpusculum basalem secoundum et regionem pyrnoidali chloroplasti; cum et sine structura multistratosa, fibra consociata cum "1s" radice microtubulari et fibra ductus. Algae marinus.

Genus typificum: Mamiella *Moestrup 1984.*

Unicellular algae with chlorophylls a and b, prasinoxanthin and urioride. Vegetative cells are monadoid or coccoid. Flagellate cells with two subequal or unequal flagella, or single flagelllum; usually with covering of spider-web scales; but lack an inner layer of square or diamond shaped scales. Flagella usually bearing hair scales. Flagellar apparatus without microtubular root associated with no.2 basal body; with single rhizoplast connecting no.2 basal bodies and the pyrenoid region of the chloroplast; with or without MLS, 1s-associated fiber and duct fiber. Algae growing in marine.

Type genus: *Mamiella* Moestrup 1984.

Order: Mamiellales Moestrup 1984

Classification at family level is uncertain.

Genera: *Mamiella* Moestrup 1984, *Dolichomastix* Manton 1977, *Crustomastix* Nakayama *et al.* 1996, *Mantoniella* Desikachary 1972, *Micromonas* Manton et Parke 1960, *Batycoccus* Eikrem et Throndsen 1990, ?*Prasinococcus* Miyashita et Chihara 1993, ?*Prasinoderma* Hasegawa et Chihara 1996, ??*Osterococcus* Courties et Chrétiennot-Dinet 1995

Division: ***incertae cedis***

Class: Nephroselmidophyceae classis nov.

Algae unicellulares chlorophyllis a *et* b *praeditae. Cellulae vegetativae monadoides vel coccoides. Cellulae motiles flagellis duobus subaequalibus vel inaequalibus; squamis distromaticis parvis tectae; cum vel sine squamis grandibus spineis. Flagella serie Melkonianii et pilis minuta. Apparatus flageorum sine "2s" radice microtubulari et structura multistratosa; carinis insidentibus "1d" radicis microtubularis; rhizoplastis duobus transientibus, regioni pyrnoidali chloroplasti extensis. Duplicatus-microtubus unicus sine branchio dynini externo. Hab. plerumque in mari, raro in aqua durci.*

Genus typificum: Nephroselmis *Stein 1878.*

Unicellular algae with chlorophylls a and b. Vegetative cells monadoid or coccoid. Flagellate cells with two subequal or unequal flagella; with covering of two-layered small

scales; with or without large spiny scales. Flagella with Melkonian's row and hair scales. Flagellar apparatus without 2s root and MLS; with keels sitting on d root; with merged rhizoplast from two basal bodies, extending to the pyrenoid region of the chloroplast. Single doublet without dynin arm (?). Habitat usually marine, rarely in freshwater.
Type genus: *Nephroselmis* Stein 1878.

Order: Nephroselmidales ord. nov.

Syn. Pseudoscourfieldiales Melkonian 1990 (nom. nud.)

Characteribus classis.

Genus typificum: Nephroselmis *Stein 1878.*

Characters are the same as the class Nephroselmidophyceae.

Type genus: *Nephroselmis* Stein 1878.

Family: Nephroselmidaceae Pascher 1913

Flagellate with laterally inserted flagella; prasinoxanthin absent.

Genus: *Nephroselmis* Stein 1878

Family: Pycnococcaceae Guillard 1991

Flagellate with posteriorly inserted flagella or coccoid; prasinoxanthin present.

Genera: *Pseudoscourfieldia* Manton 1975, ?*Pycnococcus* Guillard 1991

Division: *incertae cedis*

Class Pedinophyceae Moestrup 1991

Unicellular flagellates; cells naked or covered by thaca; cell have single flagellum (no. 1) and second reduced basal body; single doublet (#1) lacks dynin arm; basal bodies short (<500nm) and arranged end to end but offset counterclockwise without overlapping; distal fiber absent (?); s root consists of three microtubules (two over one); rhizoplast extends under s root and has no association with pyrenoid; mitosis closed; phycoplast or phragmoplast absent; marine or freshwater.

Order: Pedinomonadales Moestrup 1991

Family: Pedinomonadaceae Korshikoff 1938

Genera: *Pedinomonas* Korshikoff 1923; *Resultor* Moestrup 1991; *Marsupinomonas* Jones *et al.* 1994.

Division: *incertae cedis*

Class: *incertae cedis*

Order: Scourfieldiales Moestrup 1991

Naked green algae with two flagella; single doublet (#1) lacks dynin arm; long basal

bodies (>600nm); microtubular root absent; rhizoplast interconnects the basal bodies and pyrenoid region of the chloroplast; freshwater.

Family: Scourfieldiaceae Moestrup 1991

Genus: *Scourfieldia* G. S. West 1912

Division: ***incertae cedis***

Class: ***incertae cedis***

Order: Monomastigales ord. nov.

Algae unicellulares monadoides uno flagello (secundus), squamis planis sine acidis 2-keto-sacchari tectae. Corpuscula basalia minus quam 500nm longa, positis quasi rotatine contra horologii motum, imbricatis. Apparatus flageorum fibra distali et fibra proximali, cum duabus "d" radicibus microtubulorum. Hab. in aqua durci.

Genus typificum: Monomastix *Scherffel 1912.*

Unicellular flagellates with single flagellum (no. 2), covered by flat unmineralized scales without 2-keto-sugar acids; basal bodies short (ca. 500nm) and offset counterclockwise orientation with overlapping; distal and proximal fibers present (?); both basal bodies have d root consists of two microtubules; freshwater.

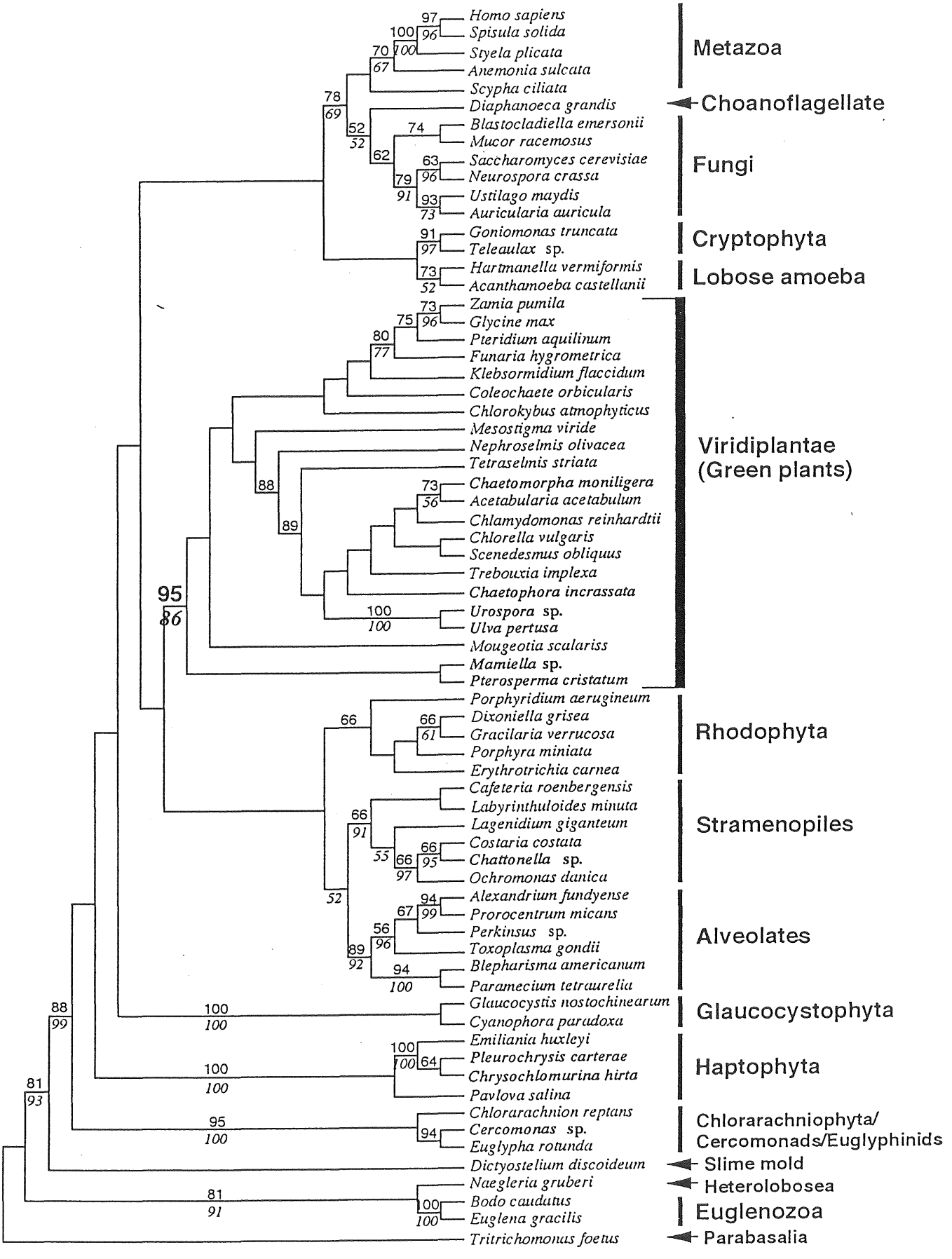
Type genus: *Monomastix* Scherffel 1912.

Family: Monomastigaceae Huber-Pestalozzi 1950

Genus: *Monomastix* Scherffel 1912

Figure 1. One of six most parsimonious trees generated from 18SrDNA sequences of various eukaryotes. Trees were rooted with *Tritrichomonas foetus* (Parabasalia). Numbers above the internal nodes indicate the bootstrap values (100 replications) using the MP method. The bootstrap values (100 replications) by the NJ method are also shown below the internal nodes in italicized script. Only bootstrap values of more than 50% are shown.

Figure 1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Spisula solida
Styela plicata
Anemonia sulcata
Scypha ciliata
Diaphanoeca grandis
Blastocladiella emersonii
Mucor racemosus
Saccharomyces cerevisiae
Neurospora crassa
Ustilago maydis
Auricularia auricula
Goniomonas truncata
Teleaulax sp.
Hartmanella vermiformis
Acanthamoeba castellanii
Zamia pumila
Glycine max
Pteridium aquilinum
Funaria hygrometrica
Klebsormidium flaccidum
Coleochaete orbicularis
Chlorokybus atmophyticus
Mesostigma viride
Nephroselmis olivacea
Tetraselmis striata
Chaetomorpha moniligera
Acetabularia acetabulum
Chlamydomonas reinhardtii
Chlorella vulgaris
Scenedesmus obliquus
Trebouxia implexa
Chaetophora incrassata
Urospora sp.
Ulva pertusa
Mougeotia scalariss
Mamiella sp.
Pterosperma cristatum
Porphyridium aerugineum
Dixoniella grisea
Gracilaria verrucosa
Porphyra miniata
Erythrotrichia carnea
Cafeteria roenbergensis
Labyrinthuloides minuta
Lagenidium giganteum
Costaria costata
Chattonella sp.
Ochromonas danica
Alexandrium fundyense
Prorocentrum micans
Perkinsus sp.
Toxoplasma gondii
Blepharisma americanum
Paramecium tetraurelia
Glaucocystis nostochinearum
Cyanophora paradoxa
Emiliania huxleyi
Pleurochrysis carterae
Chrysochlomurina hirta
Pavlova salina
Chlorarachnion reptans
Cercomonas sp.
Euglypha rotunda
Dictyostelium discoideum
Naegleria gruberi
Bodo caudatus
Euglena gracilis
Tritrichomonas foetus
Metazoa
Choanoflagellate
Fungi
Cryptophyta
Lobose amoeba
Viridiplantae (Green plants)
Rhodophyta
Stramenopiles
Alveolates
Glaucocystophyta
Haptophyta
Chlorarachniophyta/ Cercomonads/Euglyphinids
Slime mold
Heterolobosea
Euglenozoa
Parabasalia

Figure 2. Strict consensus tree of six most parsimonious trees generated from 18SrDNA sequences from various green plants. Trees were rooted with some other eukaryotes. Numbers above the internal nodes indicate the bootstrap values (100 replications) using the MP method. Only bootstrap values of more than 50% are shown.

Figure 2

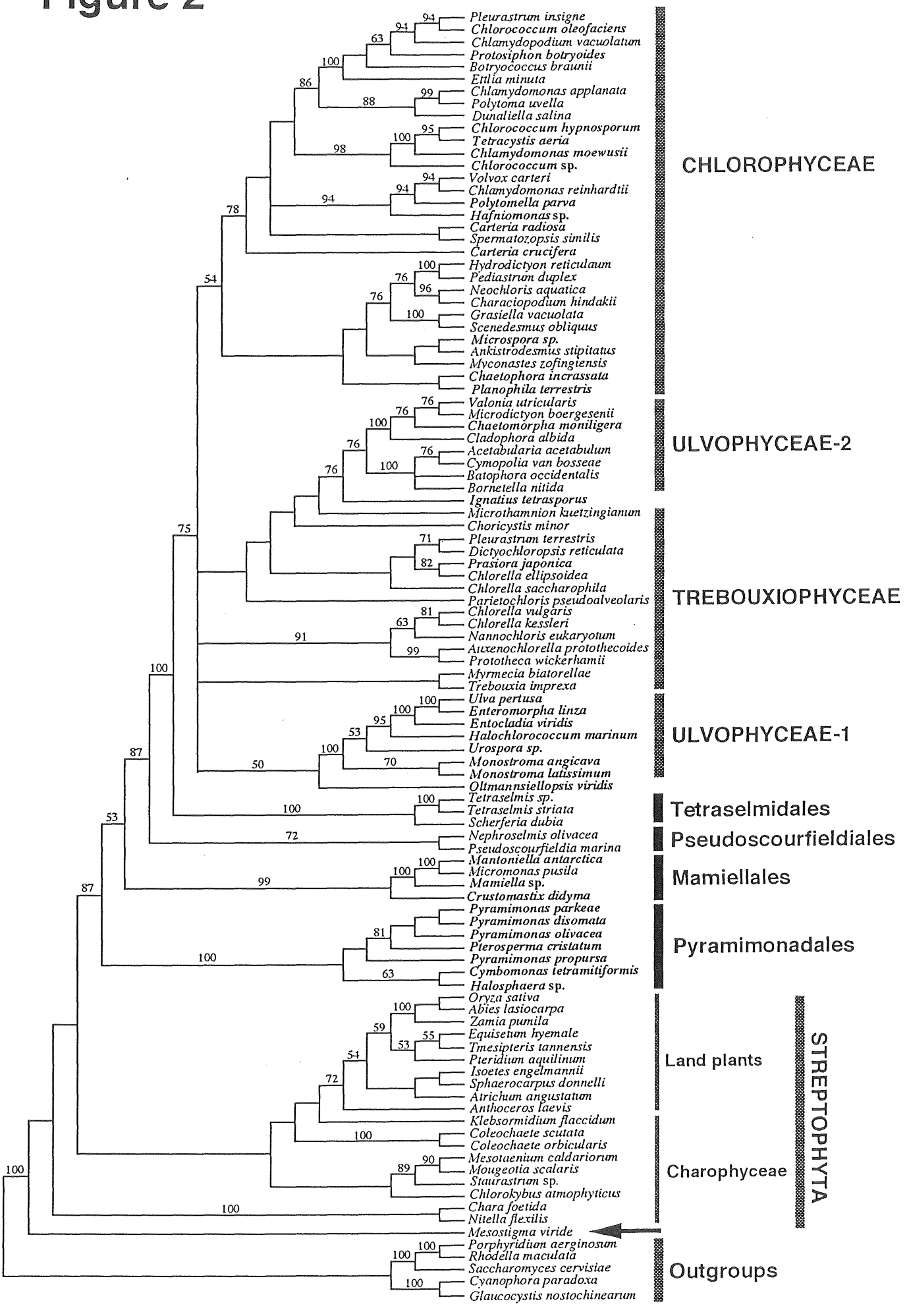

Pleurastrum insigne
Chlorococcum oleofaciens
Chlamydopodium vacuolatum
Protosiphon botryoides
Botryococcus braunii
Ettlia minuta
Chlamydomonas applanata
Polytoma uvella
Dunaliella salina
Chlorococcum hypnosporum
Tetracystis aeria
Chlamydomonas moewusii
Chlorococcum sp.
Volvox carteri
Chlamydomonas reinhardtii
Polytomella parva
Hafniomonas sp.
Carteria radiosa
Spermatozopsis similis
Carteria crucifera
Hydrodictyon reticulaum
Pediastrum duplex
Neochloris aquatica
Characiopodium hindakii
Grasiella vacuolata
Scenedesmus obliquus
Microspora sp.
Ankistrodesmus stipitatus
Myconastes zofingiensis
Chaetophora incrassata
Planophila terrestris
CHLOROPHYCEAE
Valonia utricularis
Microdictyon boergesenii
Chaetomorpha moniligera
Cladophora albida
Acetabularia acetabulum
Cymopolia van bosseae
Batophora occidentalis
Bornetella nitida
ULVOPHYCEAE-2
Ignatius tetrasporus
Microthamnion kuetzingianum
Choricystis minor
Pleurastrum terrestris
Dictyochloropsis reticulata
Prasiora japonica
Chlorella ellipsoidea
Chlorella saccharophila
Parietochloris pseudoalveolaris
Chlorella vulgaris
Chlorella kessleri
Nannochloris eukaryotum
Auxenochlorella protothecoides
Prototheca wickerhamii
Myrmecia biatorellae
Trebouxia imprexa
TREBOUXIOPHYCEAE
Ulva pertusa
Enteromorpha linza
Entocladia viridis
Halochlorococcum marinum
Urospora sp.
Monostroma angicava
Monostroma latissimum
Oltmannsiellopsis viridis
ULVOPHYCEAE-1
Tetraselmis sp.
Tetraselmis striata
Scherferia dubia
Tetraselmidales
Nephroselmis olivacea
Pseudoscourfieldia marina
Pseudoscourfieldiales
Mantoniella antarctica
Micromonas pusila
Mamiella sp.
Crustomastix didyma
Mamiellales
Pyramimonas parkeae
Pyramimonas disomata
Pyramimonas olivacea
Pterosperma cristatum
Pyramimonas propursa
Cymbomonas tetramitiformis
Halosphaera sp.
Pyramimonadales
Oryza sativa
Abies lasiocarpa
Zamia pumila
Equisetum hyemale
Tmesipteris tannensis
Pteridium aquilinum
Isoetes engelmannii
Sphaerocarpus donnelli
Atrichum angustatum
Anthoceros laevis
Land plants
Klebsormidium flaccidum
Coleochaete scutata
Coleochaete orbicularis
Mesotaenium caldariorum
Mougeotia scalaris
Staurastrum sp.
Chlorokybus atmophyticus
Charophyceae
STREPTOPHYTA
Chara foetida
Nitella flexilis
Mesostigma viride
Porphyridium aerginosum
Rhodella maculata
Saccharomyces cervisiae
Cyanophora paradoxa
Glaucocystis nostochinearum
Outgroups

Figure 3. Phylogenetic tree inferred from 18SrDNA sequences of various green plants (data set A). This tree constructed with neighbor-joining (NJ) method based on Kimura's correction using Clustal W computer program. Branch lengths are proportional to the evolutionary distances. Trees were rooted with some other eukaryotes. Numbers above the internal nodes indicate the bootstrap values (100 replications) using the NJ method. Only bootstrap values of more than 50% are shown.

Figure 3

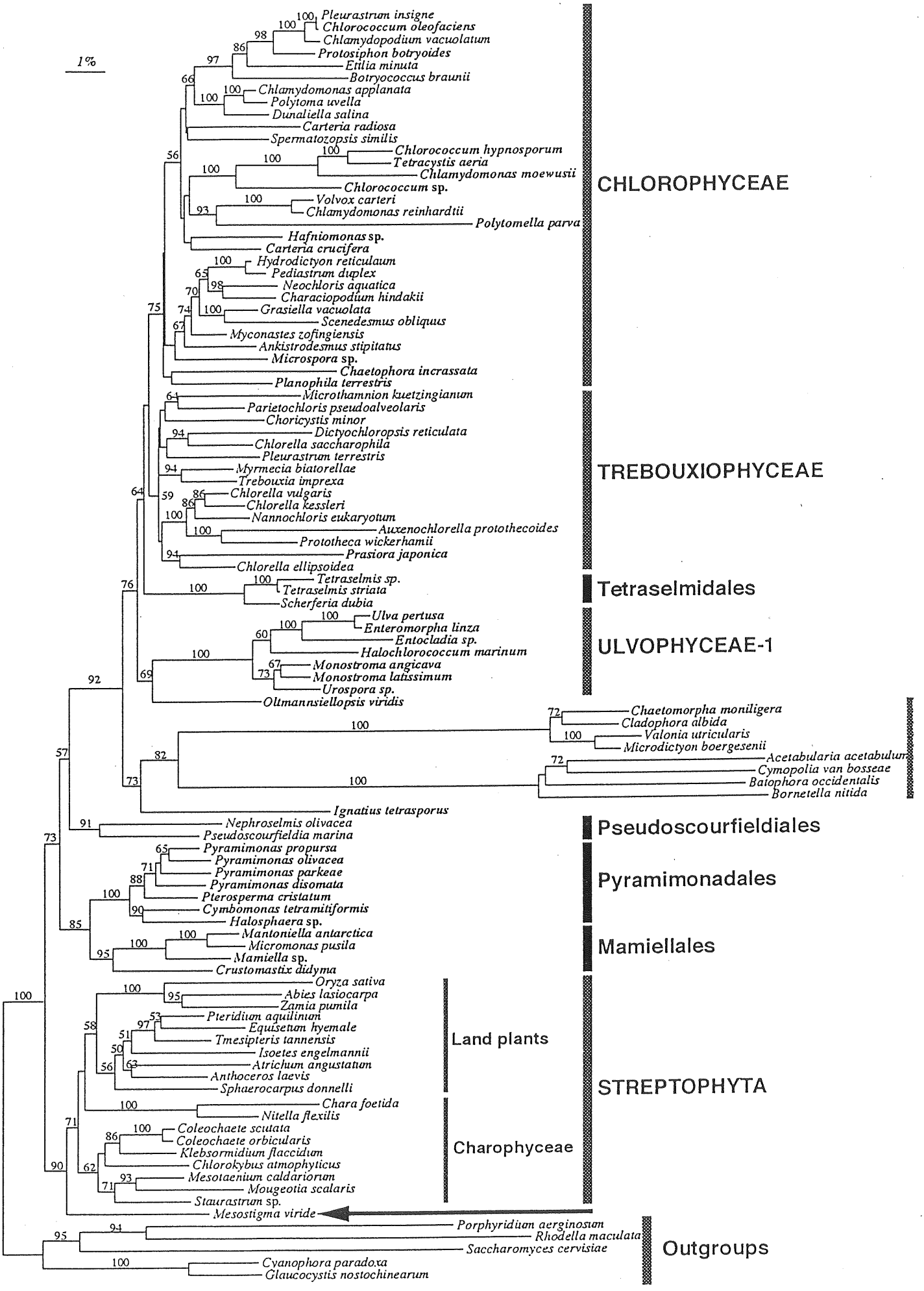

1%
Pleurastrum insigne
Chlorococcum oleofaciens
Chlamydopodium vacuolatum
Protosiphon botryoides
Etilia minuta
Botryococcus braunii
Chlamydomonas applanata
Polytoma uvella
Dunaliella salina
Carteria radiosa
Spermatozopsis similis
Chlorococcum hypnosporum
Tetracystis aeria
Chlamydomonas moewusii
Chlorococcum sp.
Volvox carteri
Chlamydomonas reinhardtii
Polytomella parva
Hafniomonas sp.
Carteria crucifera
Hydrodictyon reticulaum
Pediastrum duplex
Neochloris aquatica
Characiopodium hindakii
Grasiella vacuolata
Scenedesmus obliquus
Myconastes zofingiensis
Ankistrodesmus stipitatus
Microspora sp.
Chaetophora incrassata
Planophila terrestris
CHLOROPHYCEAE
Microthamnion kuetzingianum
Parietochloris pseudoalveolaris
Choricystis minor
Dictyochloropsis reticulata
Chlorella saccharophila
Pleurastrum terrestris
Myrmecia biatorellae
Trebouxia imprexa
Chlorella vulgaris
Chlorella kessleri
Nannochloris eukaryotum
Auxenochlorella protothecoides
Prototheca wickerhamii
Prasiora japonica
Chlorella ellipsoidea
TREBOUXIOPHYCEAE
Tetraselmis sp.
Tetraselmis striata
Scherferia dubia
Tetraselmidales
Ulva pertusa
Enteromorpha linza
Entocladia sp.
Halochlorococcum marinum
Monostroma angicava
Monostroma latissimum
Urospora sp.
Oltmannsiellopsis viridis
ULVOPHYCEAE-1
Chaetomorpha moniligera
Cladophora albida
Valonia utricularis
Microdictyon boergesenii
Acetabularia acetabulum
Cymopolia van bosseae
Batophora occidentalis
Bornetella nitida
Ignatius tetrasporus
Nephroselmis olivacea
Pseudoscourfieldia marina
Pseudoscourfieldiales
Pyramimonas propursa
Pyramimonas olivacea
Pyramimonas parkeae
Pyramimonas disomata
Pterosperma cristatum
Cymbomonas tetramitiformis
Halosphaera sp.
Pyramimonadales
Mantoniella antarctica
Micromonas pusila
Mamiella sp.
Crustomastix didyma
Mamiellales
Oryza sativa
Abies lasiocarpa
Zamia pumila
Pteridium aquilinum
Equisetum hyemale
Tmesipteris tannensis
Isoetes engelmannii
Atrichum angustatum
Anthoceros laevis
Sphaerocarpus donnelli
Land plants
Chara foetida
Nitella flexilis
Coleochaete scutata
Coleochaete orbicularis
Klebsormidium flaccidum
Chlorokybus atmophyticus
Mesotaenium caldariorum
Mougeotia scalaris
Staurastrum sp.
Charophyceae
Mesostigma viride
STREPTOPHYTA
Porphyridium aerginosum
Rhodella maculata
Saccharomyces cervisiae
Cyanophora paradoxa
Glaucocystis nostochinearum
Outgroups

Figure 4. Single most parsimonious tree found in heuristic search using PAUP computer program. This tree constructed from data set B under the weighted condition. The horizontal lengths are proportional to the number of changes. Numbers above the internal nodes indicate the bootstrap values (100 replications) using the MP method. Only bootstrap values of more than 50% are shown.

Figure 4

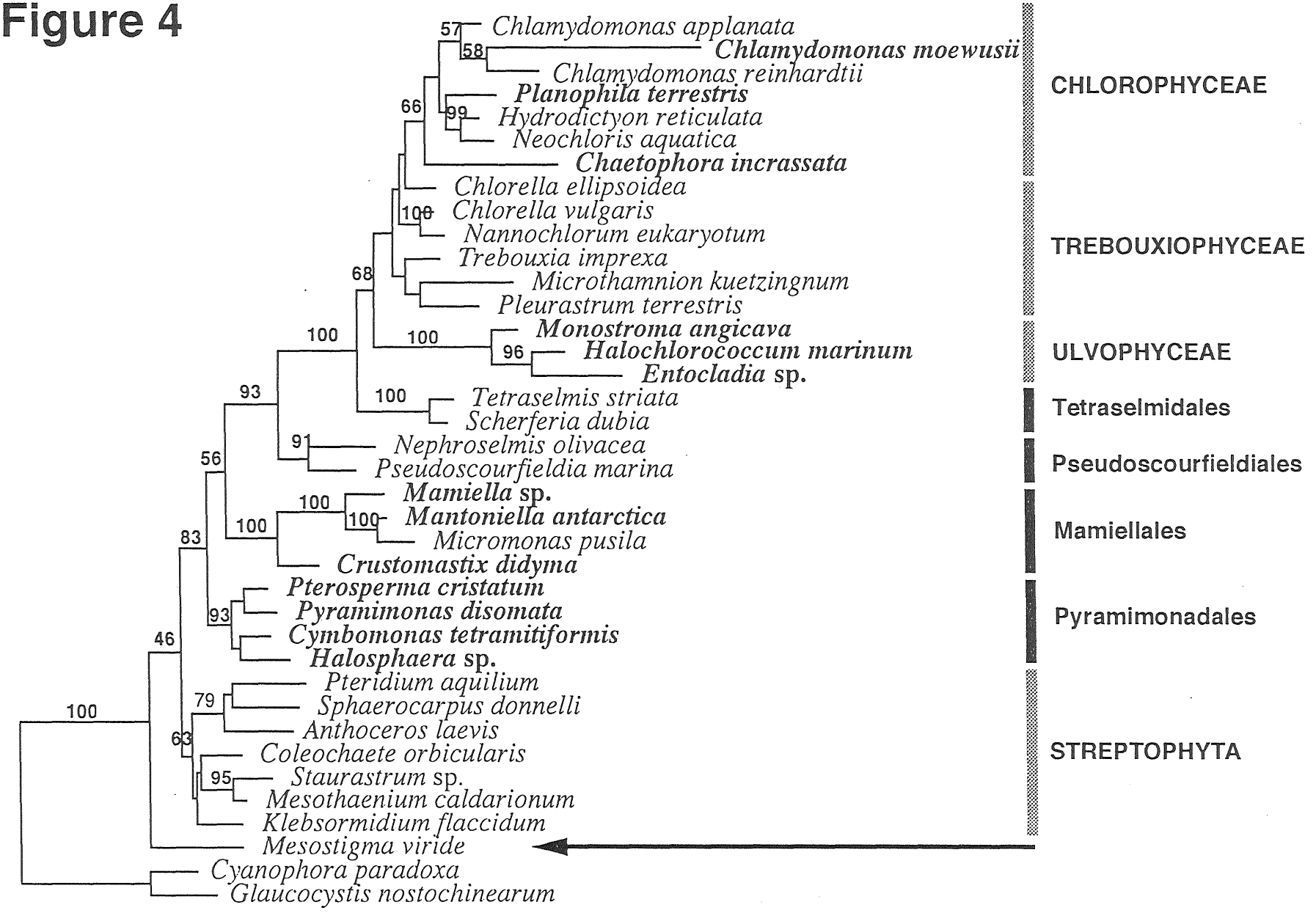

Chlamydomonas applanata
Chlamydomonas moewusii
Chlamydomonas reinhardtii
Planophila terrestris
Hydrodictyon reticulata
Neochloris aquatica
Chaetophora incrassata
Chlorella ellipsoidea
Chlorella vulgaris
Nannochlorum eukaryotum
Trebouxia imprexa
Microthamnion kuetzingnum
Pleurastrum terrestris
Monostroma angicava
Halochlorococcum marinum
Entocladia sp.
Tetraselmis striata
Scherferia dubia
Nephroselmis olivacea
Pseudoscourfieldia marina
Mamiella sp.
Mantoniella antarctica
Micromonas pusila
Crustomastix didyma
Pterosperma cristatum
Pyraminonas disomata
Cymbomonas tetramitiformis
Halosphaera sp.
Pteridium aquilium
Sphaerocarpus donnelli
Anthoceros laevis
Coleochaete orbicularis
Staurastrum sp.
Mesothaenium caldarionum
Klebsormidium flaccidum
Mesostigma viride
Cyanophora paradoxa
Glaucocystis nostochinearum
CHLOROPHYCEAE
TREBOUXIOPHYCEAE
ULVOPHYCEAE
Tetraselmidales
Pseudoscourfieldiales
Mamiellales
Pyramimonadales
STREPTOPHYTA

Figure 5. Phylogenetic tree inferred from 18SrDNA sequences of green plants (data set B). This tree constructed with neighbor-joining (NJ) method based on Kimura's correction using Clustal W computer program. Branch lengths are proportional to the evolutionary distances. Numbers above the internal nodes indicate the bootstrap values (100 replications) using the NJ method. Only bootstrap values of more than 50% are shown.

Figure 5

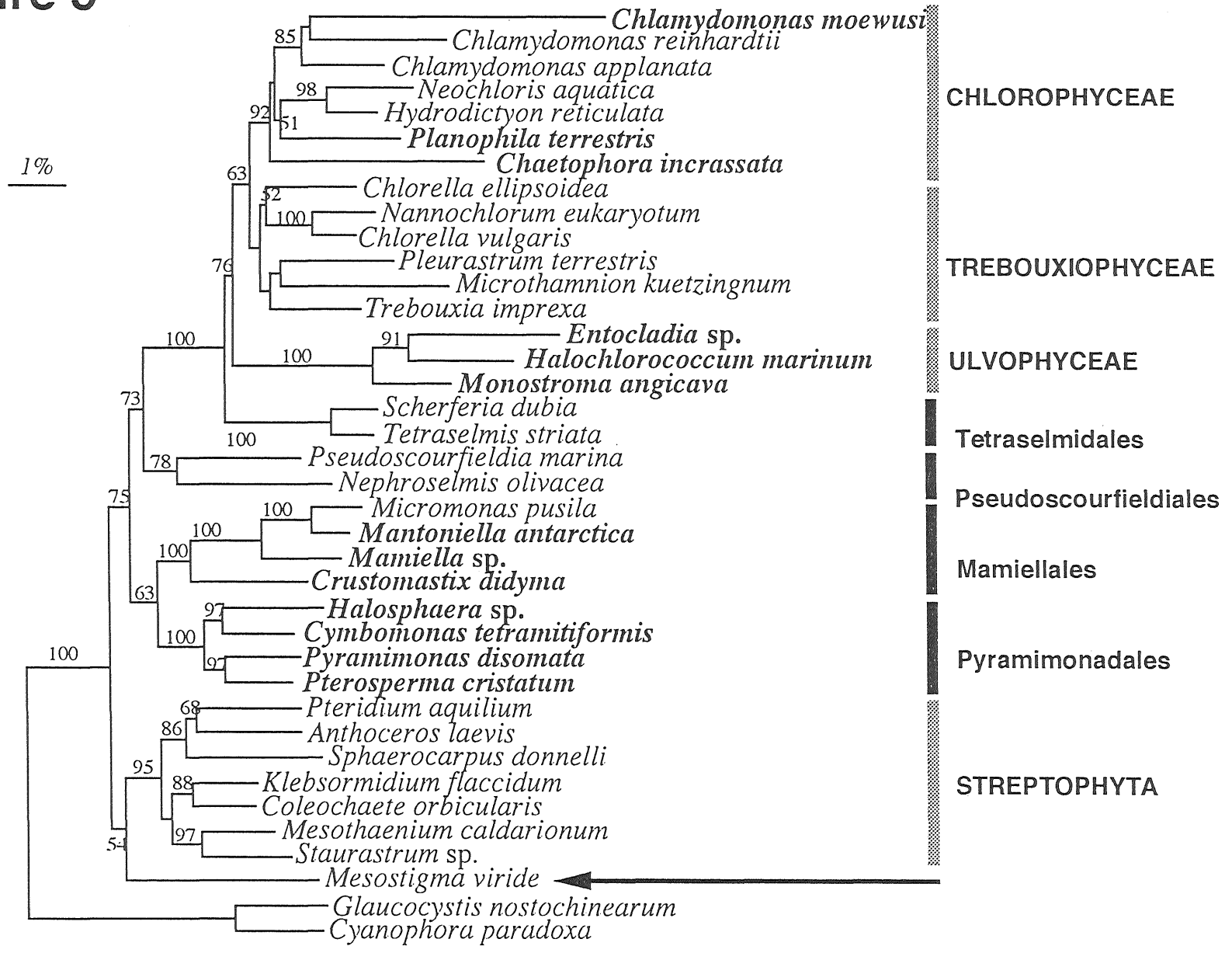

1%
Chlamydomonas moewusi
Chlamydomonas reinhardtii
Chlamydomonas applanata
Neochloris aquatica
Hydrodictyon reticulata
Planophila terrestris
Chaetophora incrassata
Chlorella ellipsoidea
Nannochlorum eukaryotum
Chlorella vulgaris
Pleurastrum terrestris
Microthamnion kuetzingnum
Trebouxia imprexa
Entocladia sp.
Halochlorococcum marinum
Monostroma angicava
Scherferia dubia
Tetraselmis striata
Pseudoscourfieldia marina
Nephroselmis olivacea
Micromonas pusila
Mantoniella antarctica
Mamiella sp.
Crustomastix didyma
Halosphaera sp.
Cymbomonas tetramitiformis
Pyramimonas disomata
Pterosperma cristatum
Pteridium aquilium
Anthoceros laevis
Sphaerocarpus donnelli
Klebsormidium flaccidum
Coleochaete orbicularis
Mesothaenium caldarionum
Staurastrum sp.
Mesostigma viride
Glaucocystis nostochinearum
Cyanophora paradoxa
CHLOROPHYCEAE
TREBOUXIOPHYCEAE
ULVOPHYCEAE
Tetraselmidales
Pseudoscourfieldiales
Mamiellales
Pyramimonadales
STREPTOPHYTA

Figure 6. *Crustomastix didyma.*
Cell viewed from the ventral side (right) and from the right side of the cell (left).

Abbreviations: **1**, no. 1 flugellum; **2**, no. 2 flugellum; **A**, anterior side of the cell; **C**, chloroplast; **D**, dorsal side of the cell; **E**, eyespot; **G**, Golgi apparatus; **L**, left side of the cell; **M**, mitochondrion; **m**, microbody; **N**, nucleus; **P**, posterior side of the cell; **R**, right side of the cell; **S**, starch grain; **Sd**, scale duct; **V**, ventral side of the cell.

Figure 6

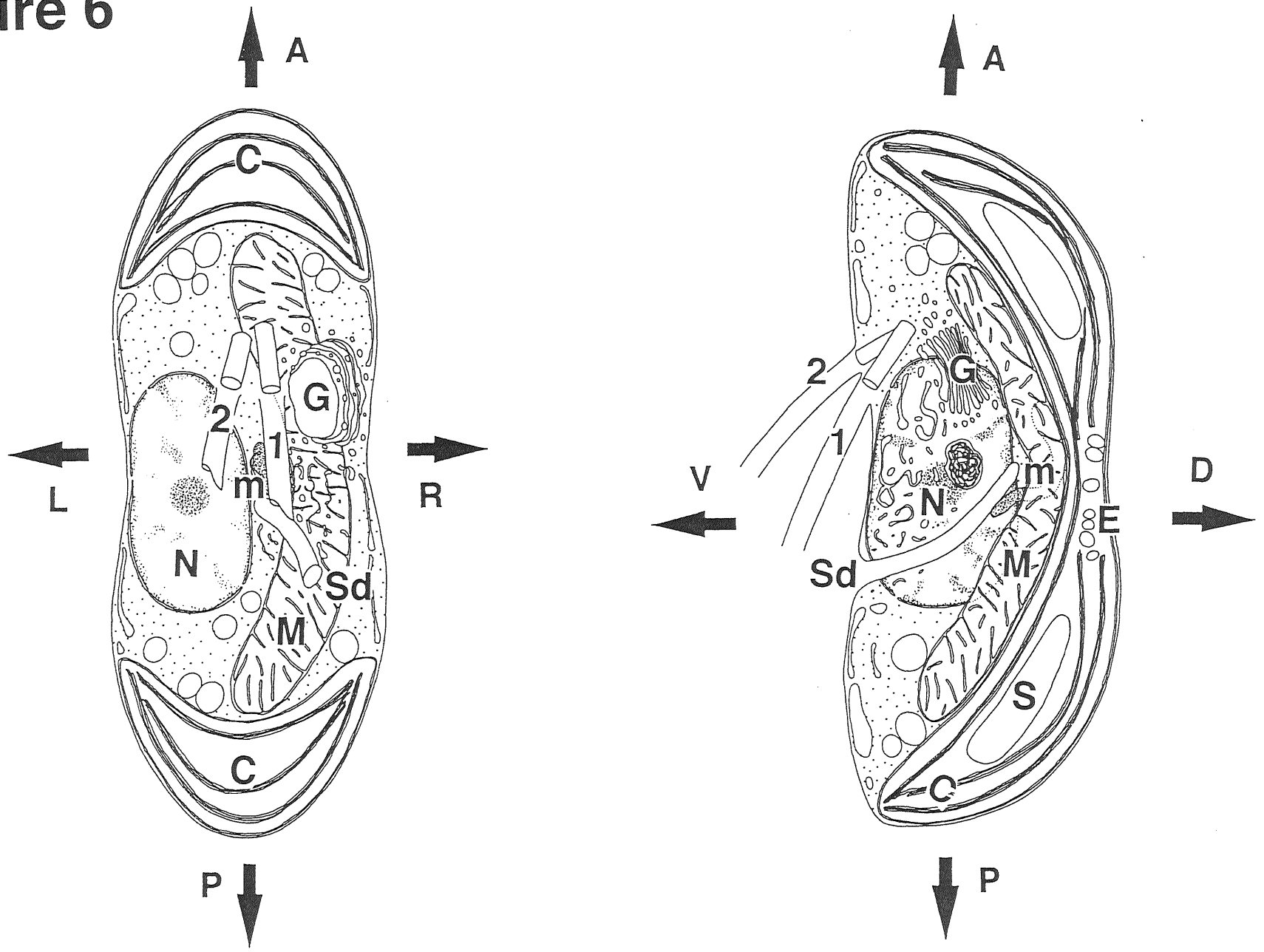


Figure 7. *Crustomastix didyma*
Diagram of flagellar apparatus viewed from the posterior side of the cell.

Abbreviations: **1**, no. 1 basal body; **1d**, 1d root; **1s**, 1s root, **1sF**, 1s-associated fiber; **2**, no. 2 basl body; **DF**, distal fiber; **dF**, duct fiber; **G**, Golgi apparatus; **L**, left side of the cell; **M**, mitochondrion; **m**, microbody; **MLS**, multilayered structure; **N**, nucleus; **PF**, proximal fiber; **Rh**, rhizoplast; **Sd**, scale duct.

Figure 7

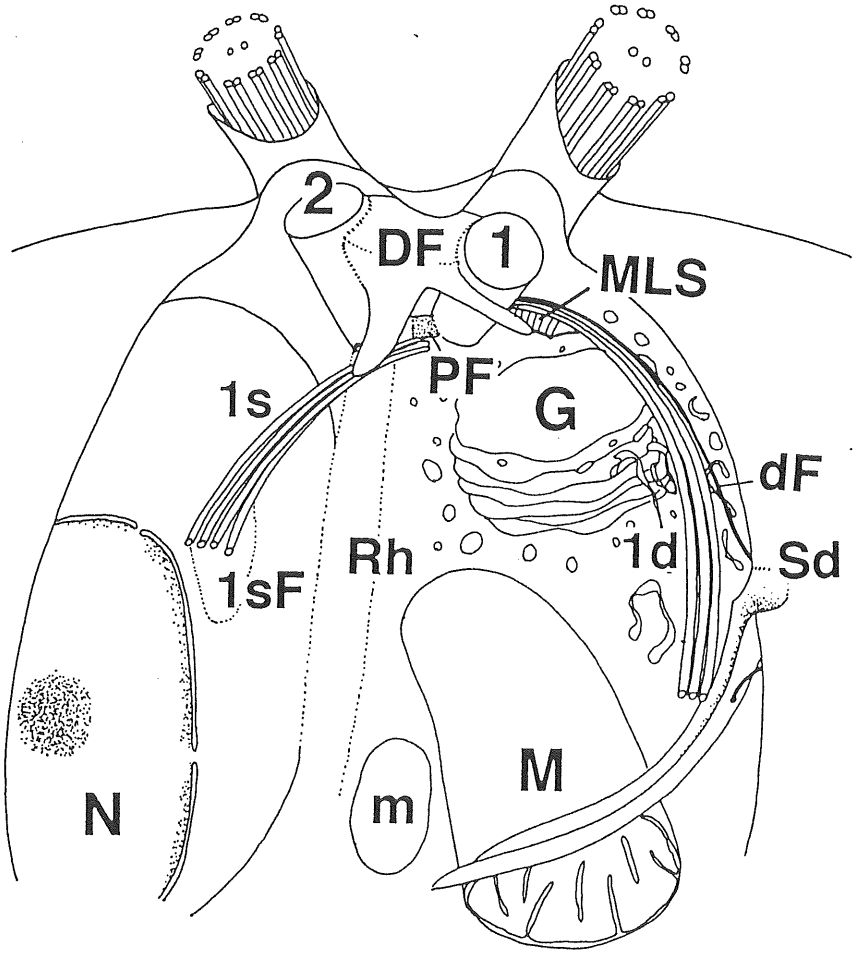


Figure 8. *Mantoniella antarctica.*
Cell viewed from the ventral side (right) and from the right side of the cell (left).

Abbreviations: 1, no. 1 flugellum; **2**, no. 2 flugellum; **A**, anterior side of the cell; **C**, chloroplast; **D**, dorsal side of the cell; **E**, eyespot; **es**, extrusome; **G**, Golgi apparatus; **L**, left side of the cell; **M**, mitochondrion; **m**, microbody; **N**, nucleus; **P**, posterior side of the cell; **R**, right side of the cell; **S**, starch grain; **V**, ventral side of the cell.

Figure 8

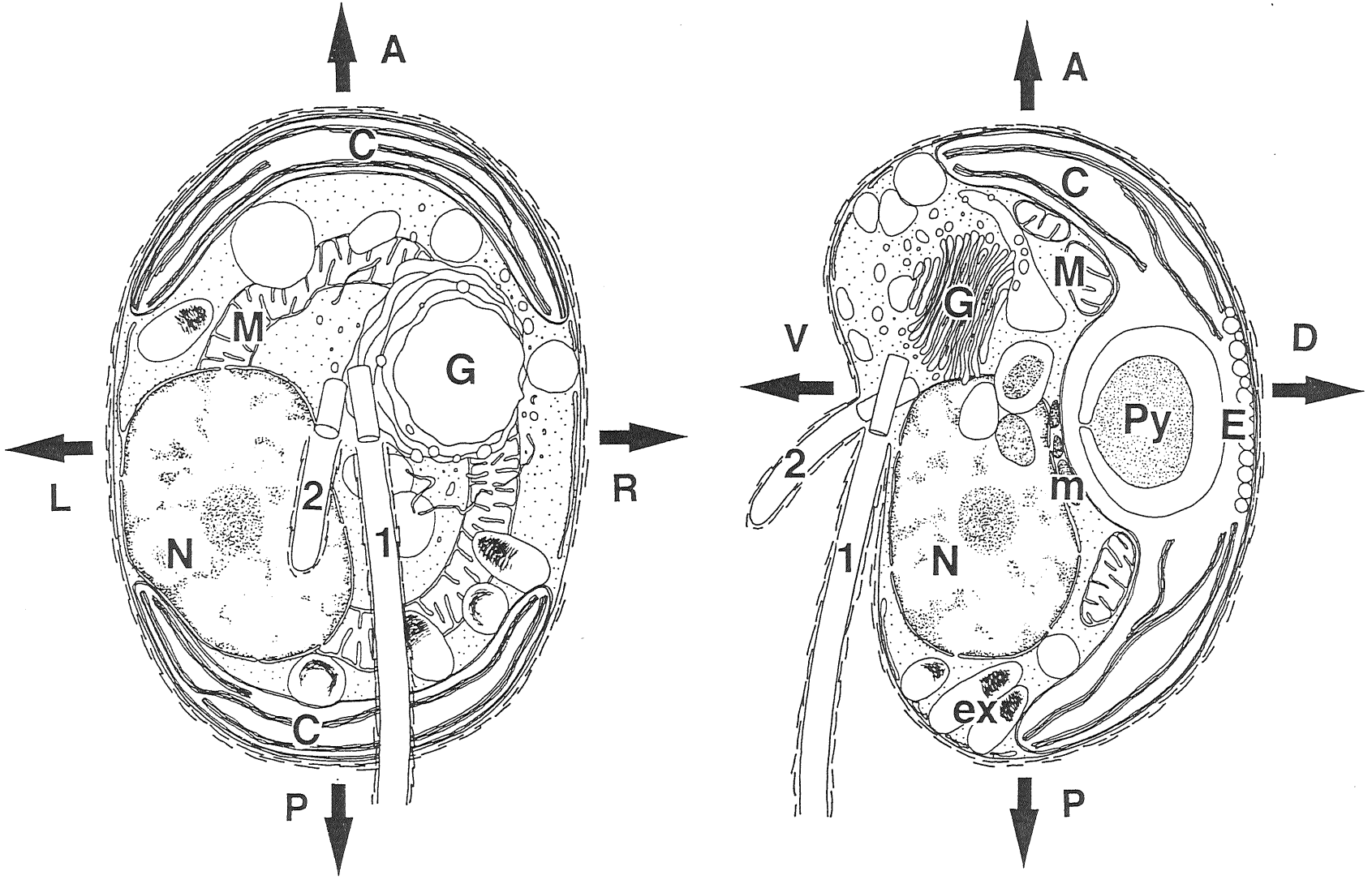


Figure 9. *Mantoniella antarctica*
Diagram of flagellar apparatus of viewed from the posterior side of the cell.

Abbreviations: 1, no. 1 basal body; **1d**, 1d root; **1s**, 1s root, **1sF**, 1s-associated fiber; **2**, no. 2 basl body; **C**, chloroplast; **DF**, distal fiber; **dF**, duct fiber; **G**, Golgi apparatus; **L**, left side of the cell; **M**, mitochondrion; **m**, microbody; **MLS**, multilayered structure; **mtb**, microtubular band; **N**, nucleus; **PF**, proximal fiber; **Rh**, rhizoplast.

Figure 9

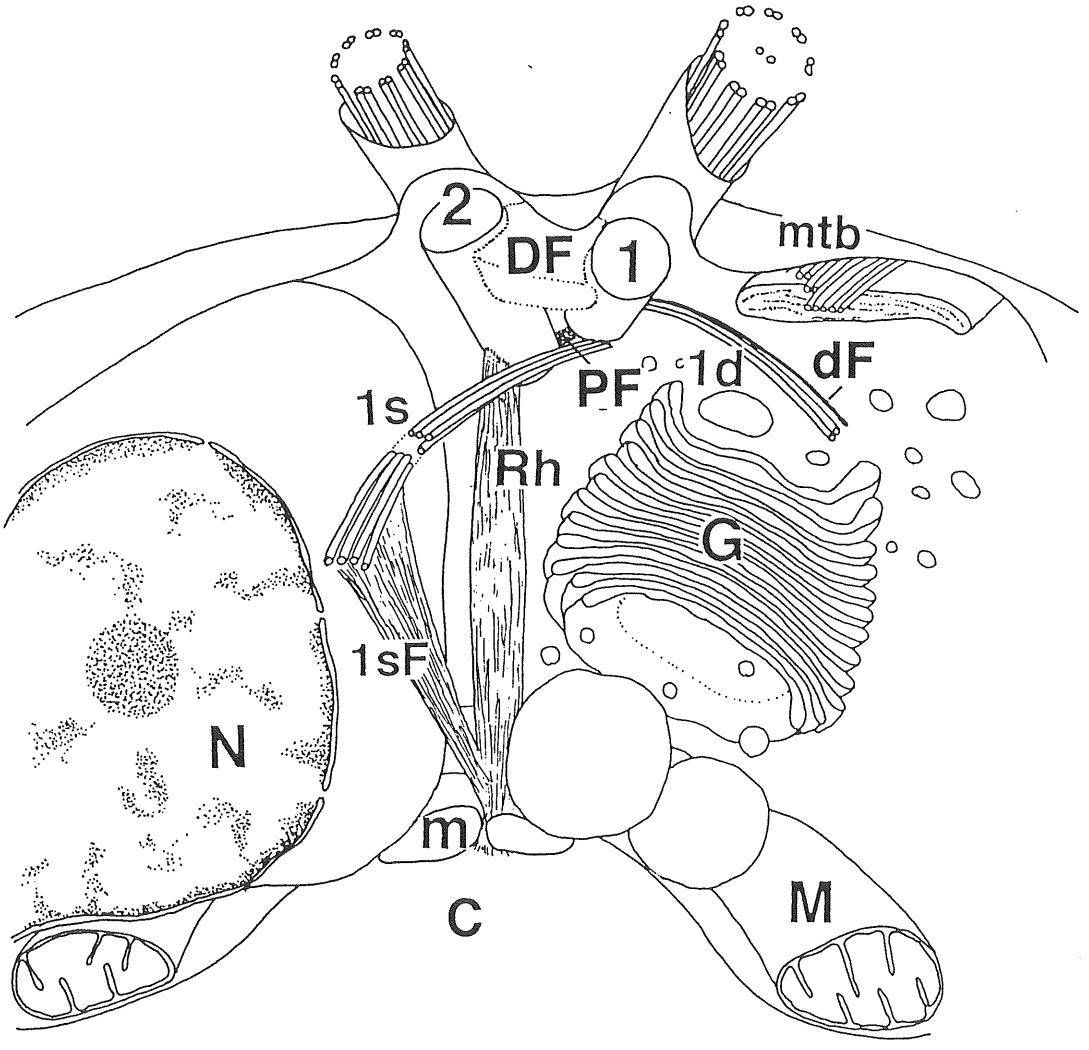
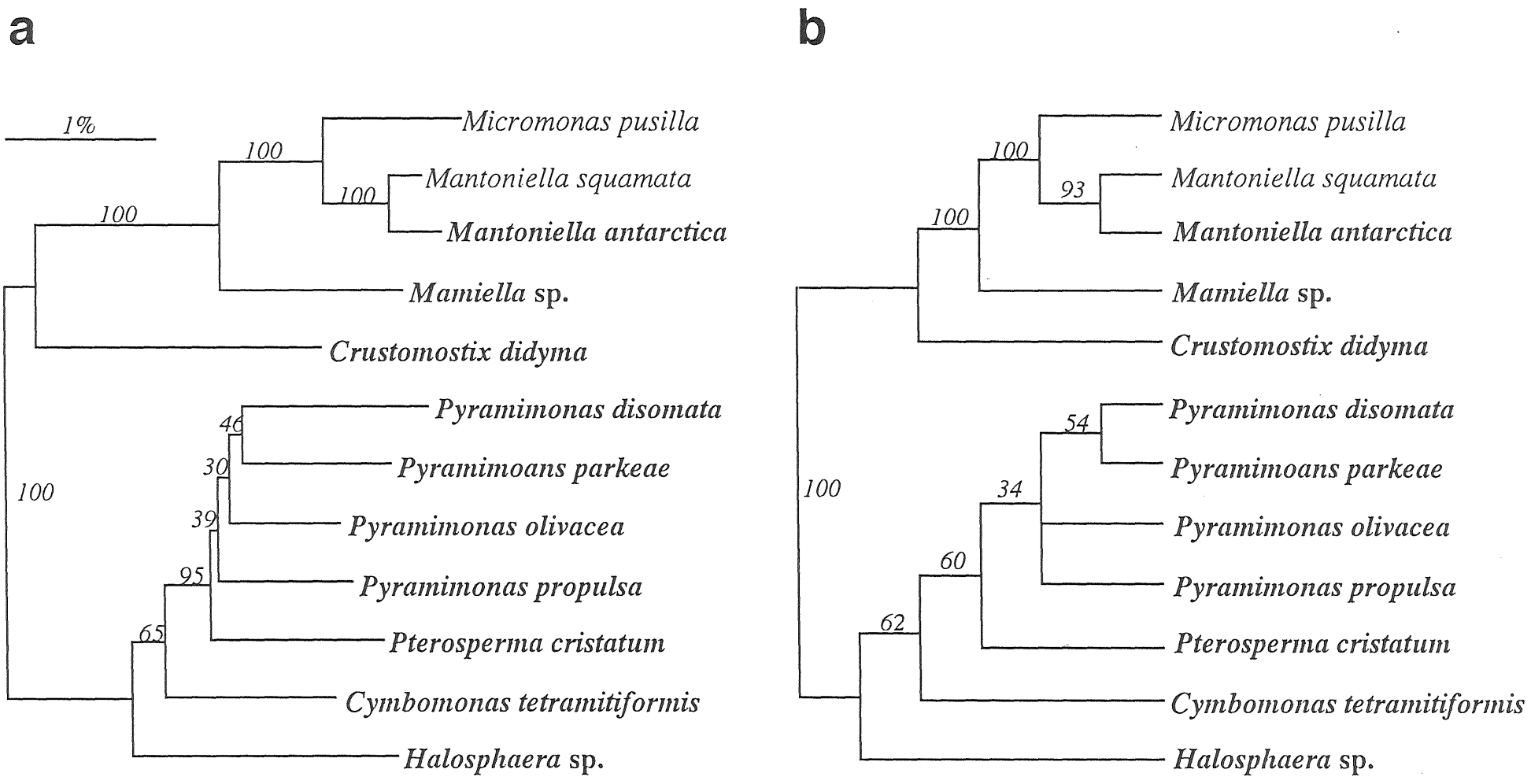


Figure 10. Phylogenetic trees inferred from 18SrDNA sequences from the Mamiellales and Pyramimonadales. Tree was rooted with each other order. Numbers above the internal nodes indicate the bootstrap values (100 replicates). Taxa shown in bold letters are the species examined in this study. **a.** Distance tree constructed with neighbor-joining (NJ) method based on Kimura distances. The horizontal lengths are proportional to evolutionary distance. **b.** Strict consensus tree of two most parsimonious trees found in heuristic search.

Figure 10


a
1%
100
100
100
100
46
30
39
95
65
100
Micromonas pusilla
Mantoniella squamata
Mantoniella antarctica
Mamiella sp.
Crustomostix didyma
Pyramimonas disomata
Pyramimoans parkeae
Pyramimonas olivacea
Pyramimonas propulsa
Pterosperma cristatum
Cymbomonas tetramitiformis
Halosphaera sp.
b
100
100
93
100
54
34
60
62
Micromonas pusilla
Mantoniella squamata
Mantoniella antarctica
Mamiella sp.
Crustomostix didyma
Pyramimonas disomata
Pyramimoans parkeae
Pyramimonas olivacea
Pyramimonas propulsa
Pterosperma cristatum
Cymbomonas tetramitiformis
Halosphaera sp.

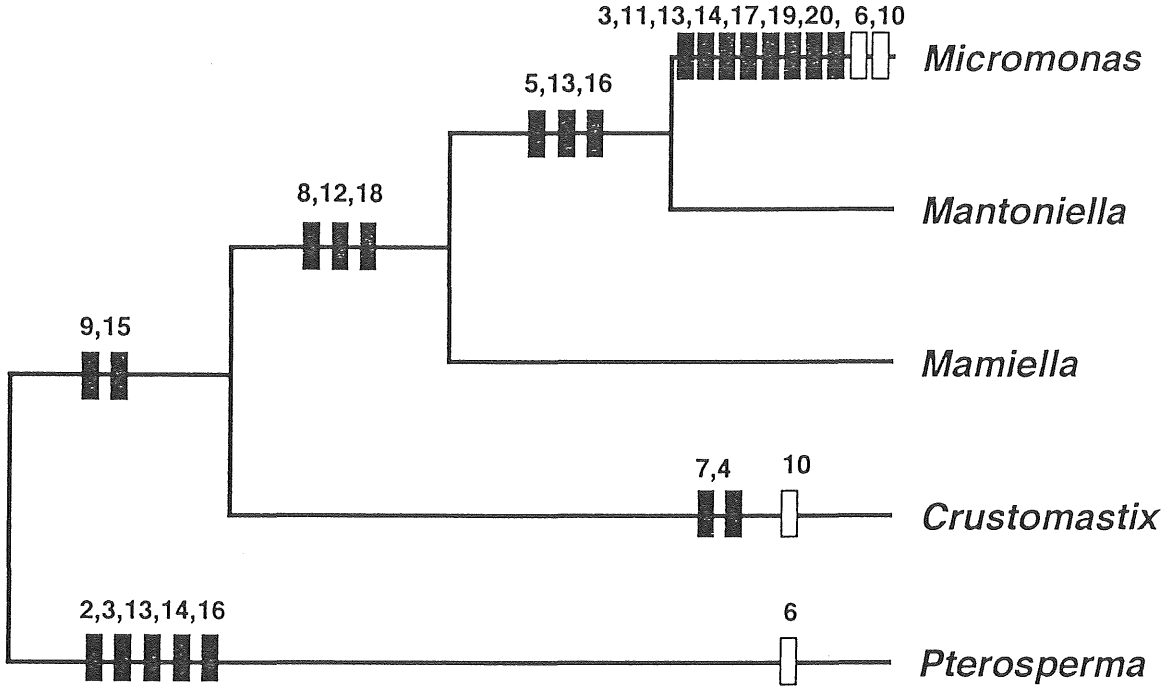


Figure 11. Cladogram of the Mamiellales inferred from morphological characters listed in Table 4. Tree length = 26 steps. Consistensy index = 0.923. Black bars are apomorphic characters, open bars indicate reversals or convergences. Numbers correspond to those in Table 4.

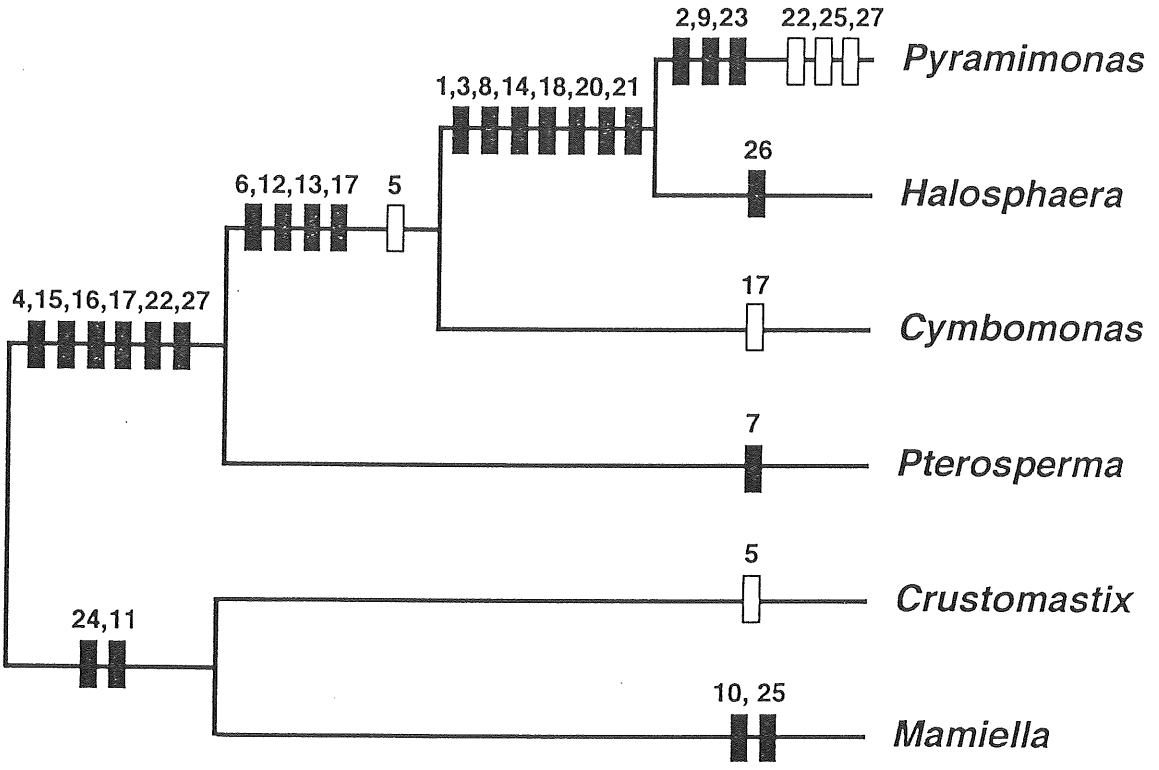


Figure 12. Cladogram of the Pyramimonadales inferred from morphological characters listed in Table 5. Tree length = 33 steps. Consistensy index = 0.818. Black bars are apomorphic characters, open bars indicate reversals or convergences. Numbers correspond to those in Table 5.

Figure 13. Phylogenetic trees inferred from 18SrDNA sequences of chlorophytes (data set A). A total of 1746 nucleotides was considered. Trees were rooted with pseudoscourfieldian prasinophytes (*Nephroselmis olivacea* and *Pseudoscourfieldia marina*). Numbers above the internal nodes indicate the bootstrap values (1000 replicates) more than 50% (b,c). Taxa shown in bold letters are the species examined in this study. **a.** Phylogenetic tree deduced from the maximum-likelihood (ML) method. The horizontal lengths are proportional to the estimated number of substitutions per site. **b.** Single most parsimonious tree found in heuristic search using weighted sequences. The horizontal lengths are proportional to the number of changes. Consistency index (CI) = 0.836. **c.** Distance tree constructed with neighbor-joining (NJ) method based on Kimura distances. The horizontal lengths are proportional to evolutionary distance.

Figure 13

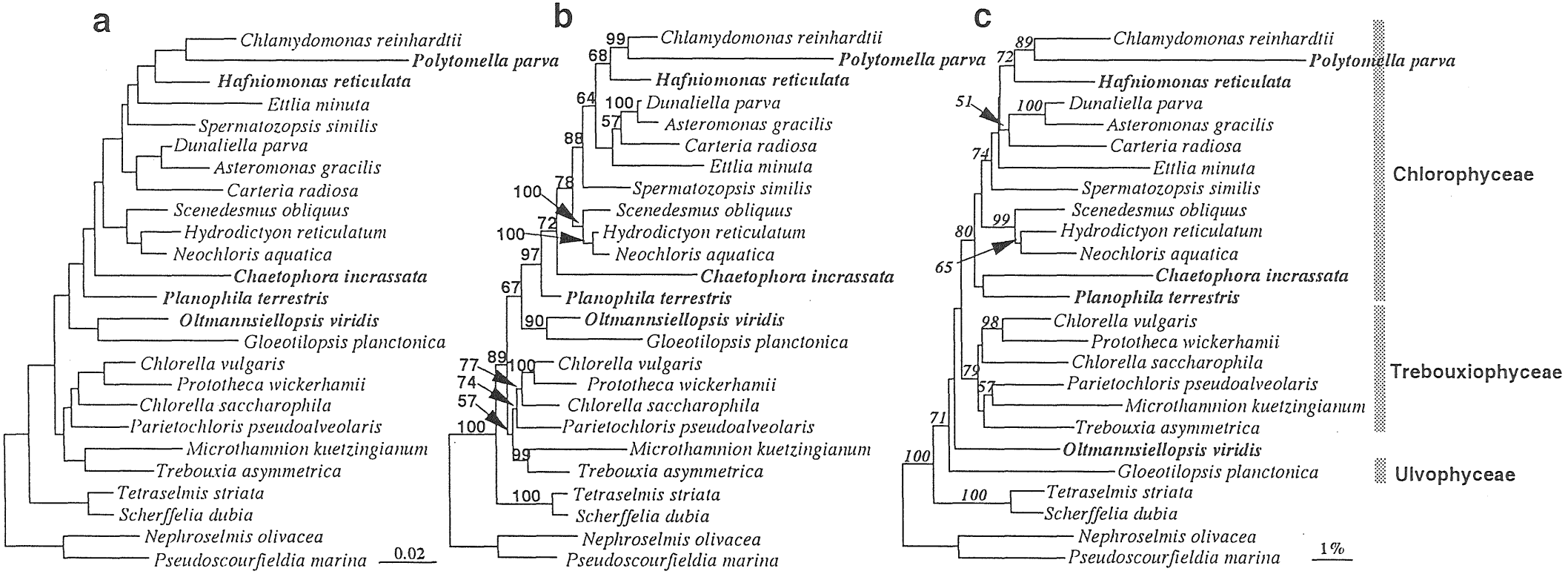

a
Chlamydomonas reinhardtii
Polytomella parva
Hafniomonas reticulata
Ettlia minuta
Spermatozopsis similis
Dunaliella parva
Asteromonas gracilis
Carteria radiosa
Scenedesmus obliquus
Hydrodictyon reticulatum
Neochloris aquatica
Chaetophora incrassata
Planophila terrestris
Oltmannsiellopsis viridis
Gloeotilopsis planctonica
Chlorella vulgaris
Prototheca wickerhamii
Chlorella saccharophila
Parietochloris pseudoalveolaris
Microthamnion kuetzingianum
Trebouxia asymmetrica
Tetraselmis striata
Scherffelia dubia
Nephroselmis olivacea
Pseudoscourfieldia marina
0.02
b
99
68
64
100
57
88
78
100
72
100
97
67
90
77
89
100
74
57
100
99
100
c
89
72
51
100
74
99
80
65
98
79
57
71
100
100
1%
Chlorophyceae
Trebouxiophyceae
Ulvophyceae

Figure 14. Phylogenetic trees inferred from 18SrDNA sequences of chlorophycean algae (data set B). A total of 1770 nucleotides was considered. Taxa shown in bold letters are the species examined in this study. The trees were rooted with *Chlorella vulgaris, Trebouxia asymmetrica* and *Oltmannsiellopsis viridis*. **a.** Phylogenetic tree deduced from the maximum-likelihood (ML) method. The horizontal lengths are proportional to the estimated number of substitutions per site. **b.** Single most parsimonious tree found in a heuristic search using weighted sequences. The horizontal lengths are proportional to the number of base changes. Consistency index (CI) = 0.826. Numbers above the internal nodes indicate the bootstrap values (1000 replications) using the MP method. The topology of distance tree constructed with NJ method based on Kimura distances is identical with this tree. The bootstrap values (1000 replications) by the NJ method are shown below the internal nodes in italicized script. Only bootstrap values of more than 50% are shown.

Figure 14

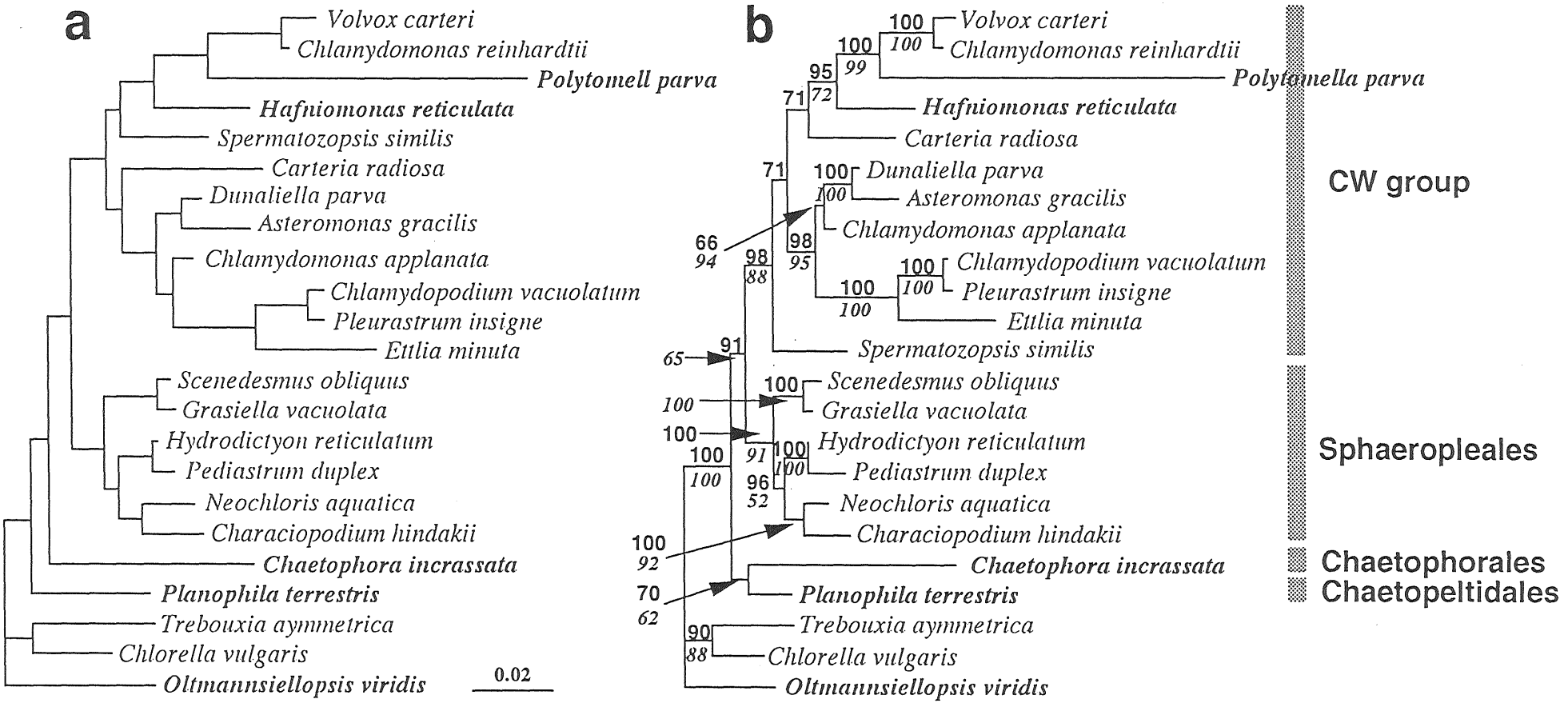

a
Volvox carteri
Chlamydomonas reinhardtii
Polytomell parva
Hafniomonas reticulata
Spermatozopsis similis
Carteria radiosa
Dunaliella parva
Asteromonas gracilis
Chlamydomonas applanata
Chlamydopodium vacuolatum
Pleurastrum insigne
Ettlia minuta
Scenedesmus obliquus
Grasiella vacuolata
Hydrodictyon reticulatum
Pediastrum duplex
Neochloris aquatica
Characiopodium hindakii
Chaetophora incrassata
Planophila terrestris
Trebouxia aymmetrica
Chlorella vulgaris
Oltmannsiellopsis viridis
0.02
b
Volvox carteri
Chlamydomonas reinhardtii
Polytomella parva
Hafniomonas reticulata
Carteria radiosa
Dunaliella parva
Asteromonas gracilis
Chlamydomonas applanata
Chlamydopodium vacuolatum
Pleurastrum insigne
Ettlia minuta
Spermatozopsis similis
Scenedesmus obliquus
Grasiella vacuolata
Hydrodictyon reticulatum
Pediastrum duplex
Neochloris aquatica
Characiopodium hindakii
Chaetophora incrassata
Planophila terrestris
Trebouxia aymmetrica
Chlorella vulgaris
Oltmannsiellopsis viridis
CW group
Sphaeropleales
Chaetophorales
Chaetopeltidales

Figure 15. Phylogenetic trees inferred from 18SrDNA sequences. This is a strict consensus tree for three equally most parsimonious trees (1922 steps, consistency index is 0.540) generated by heuristic searches in PAUP computer program. A total 1676 nucleotides was considered. Trees were rooted with *Pseudoscourfieldia marina* and *Nephroselmis olivacea.* Numbers on branches indicate bootstrap values (100 replicates) larger than 50%. Taxa shown in bold letters are the species for which 18SrDNA sequences were determined in this study.

Figure 15

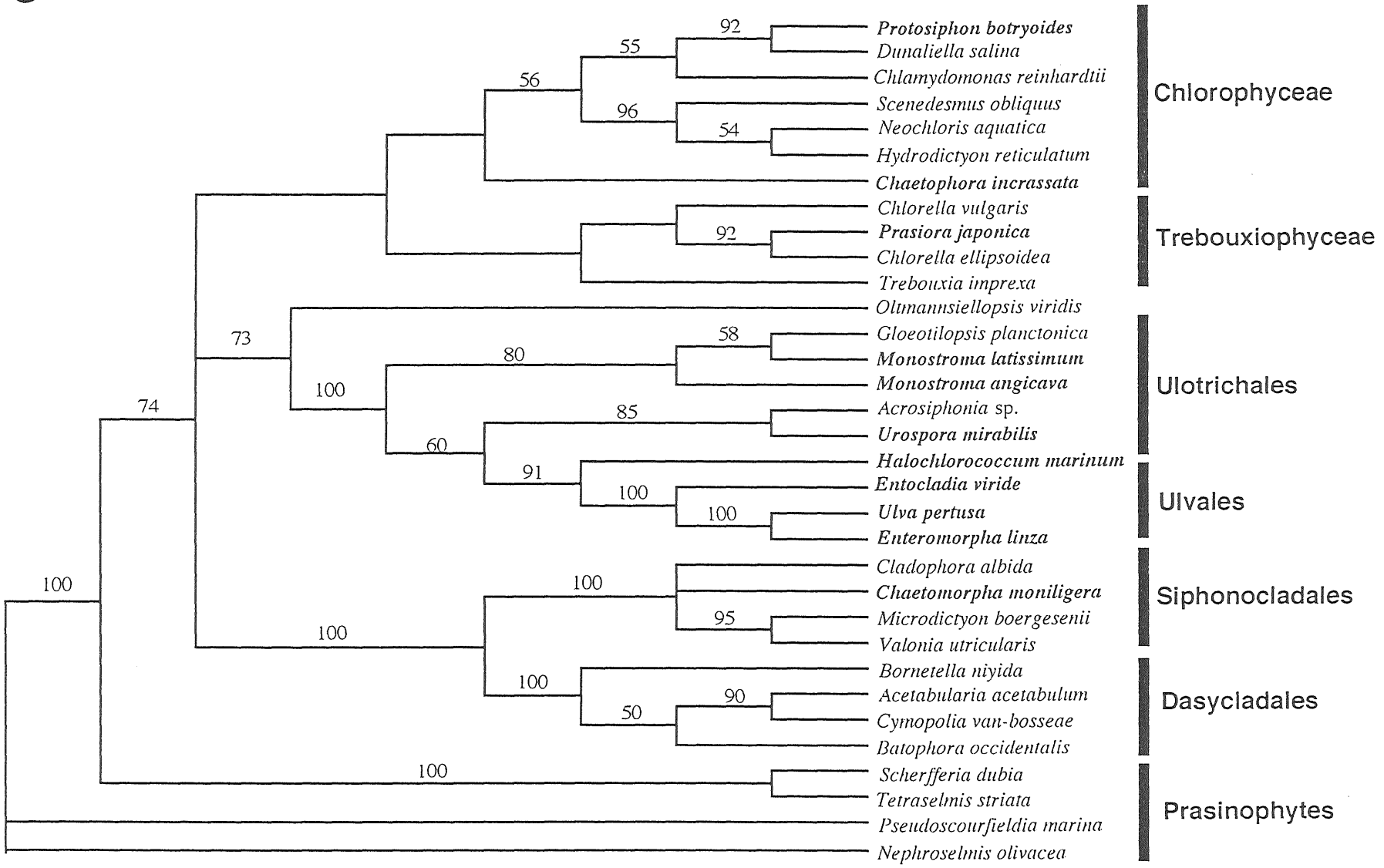

Protosiphon botryoides
Dunaliella salina
Chlamydomonas reinhardtii
Scenedesmus obliquus
Neochloris aquatica
Hydrodictyon reticulatum
Chaetophora incrassata
Chlorella vulgaris
Prasiora japonica
Chlorella ellipsoidea
Trebouxia inprexa
Oltmannsiellopsis viridis
Gloeotilopsis planctonica
Monostroma latissimum
Monostroma angicava
Acrosiphonia sp.
Urospora mirabilis
Halochlorococcum marinum
Entocladia viride
Ulva pertusa
Enteromorpha linza
Cladophora albida
Chaetomorpha moniligera
Microdictyon boergesenii
Valonia utricularis
Bornetella niyida
Acetabularia acetabulum
Cymopolia van-bosseae
Batophora occidentalis
Scherfferia dubia
Tetraselmis striata
Pseudoscourfieldia marina
Nephroselmis olivacea
92
55
56
96
54
92
73
58
80
100
74
85
60
91
100
100
100
100
100
95
100
100
90
50
100
Chlorophyceae
Trebouxiophyceae
Ulotrichales
Ulvales
Siphonocladales
Dasycladales
Prasinophytes

Figure 16. Distance tree constructed with neighbor-joining (NJ) method based on Kimura's correction using Clustal W computer program. A total 1676 nucleotides was considered. Branch lengths are proportional to the evolutionary distances. Numbers on branches indicate bootstrap values (100 replicates) larger than 50%. Taxa shown in bold letters are the species for which 18SrDNA sequences were determined in this study.

Figure 16

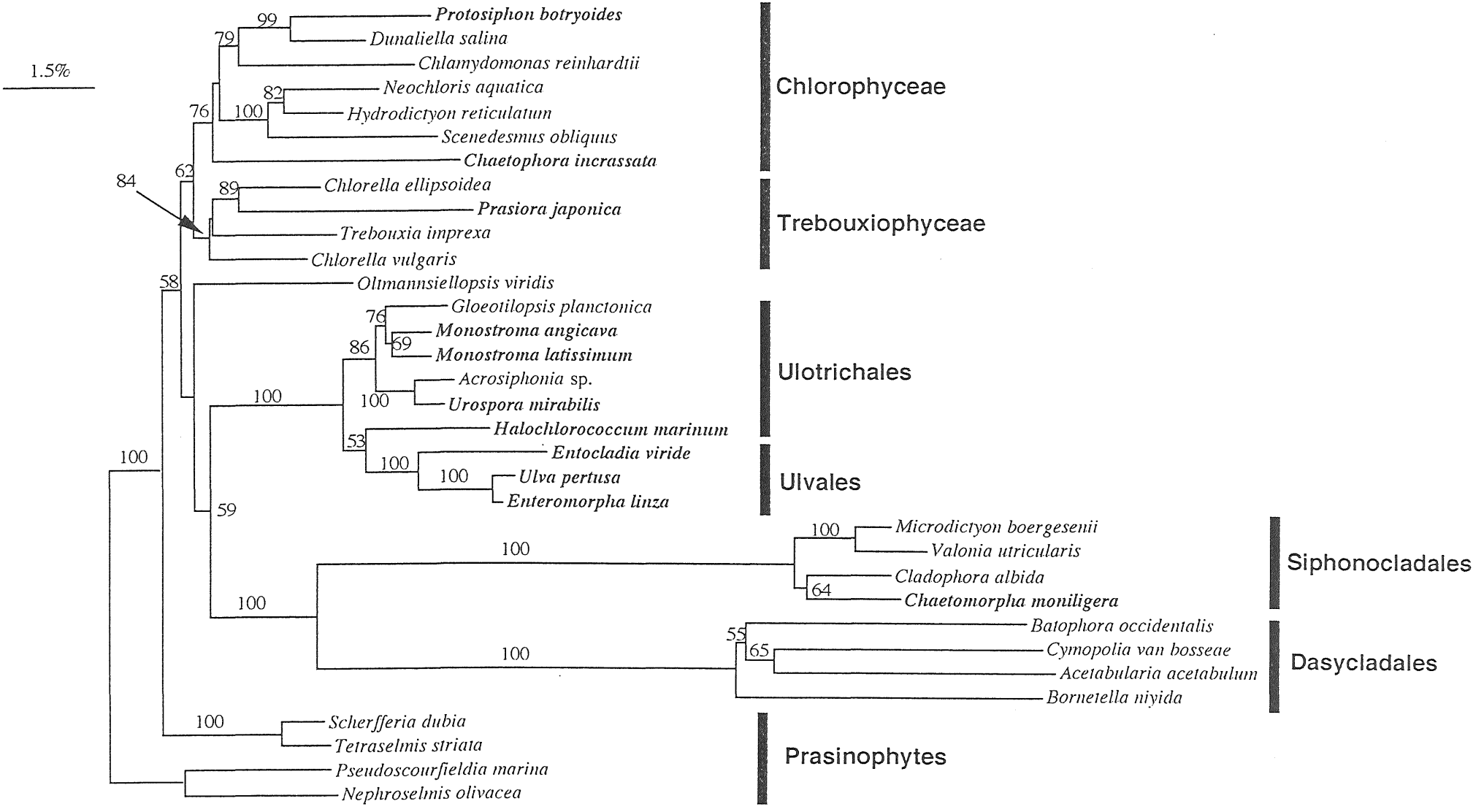

1.5%
Protosiphon botryoides
Dunaliella salina
Chlamydomonas reinhardtii
Neochloris aquatica
Hydrodictyon reticulatum
Scenedesmus obliquus
Chaetophora incrassata
Chlorophyceae
Chlorella ellipsoidea
Prasiora japonica
Trebouxia impressa
Chlorella vulgaris
Trebouxiophyceae
Oltmannsiellopsis viridis
Gloeotilopsis planctonica
Monostroma angicava
Monostroma latissimum
Acrosiphonia sp.
Urospora mirabilis
Halochlorococcum marinum
Ulotrichales
Entocladia viride
Ulva pertusa
Enteromorpha linza
Ulvales
Microdictyon boergesenii
Valonia utricularis
Cladophora albida
Chaetomorpha moniligera
Siphonocladales
Batophora occidentalis
Cymopolia van bosseae
Acetabularia acetabulum
Bornetella niyida
Dasycladales
Scherfferia dubia
Tetraselmis striata
Pseudoscourfieldia marina
Nephroselmis olivacea
Prasinophytes

Figure 17. Phylogenetic trees inferred from 18SrDNA sequences. A total 1772 nucleotides was considered. Trees were rooted with *Oltmannsiella, Tetarselmis* and trebouxiophycean algae. Numbers on branches indicate bootstrap values (100 replicates) larger than 50%. Taxa shown in bold letters are the species for which 18SrDNA sequences were determined in this study. **a.** Single most parsimonious tree (663 steps, consistency index is 0.698) found in a heuristic search using PAUP computer program. The horizontal lengths are proportional to the number of base changes. **b.** Distance tree constructed with neighbor-joining (NJ) method based on Kimura's correction using Clustal W computer program. Branch lengths are proportional to the evolutionary distances.

Figure 17

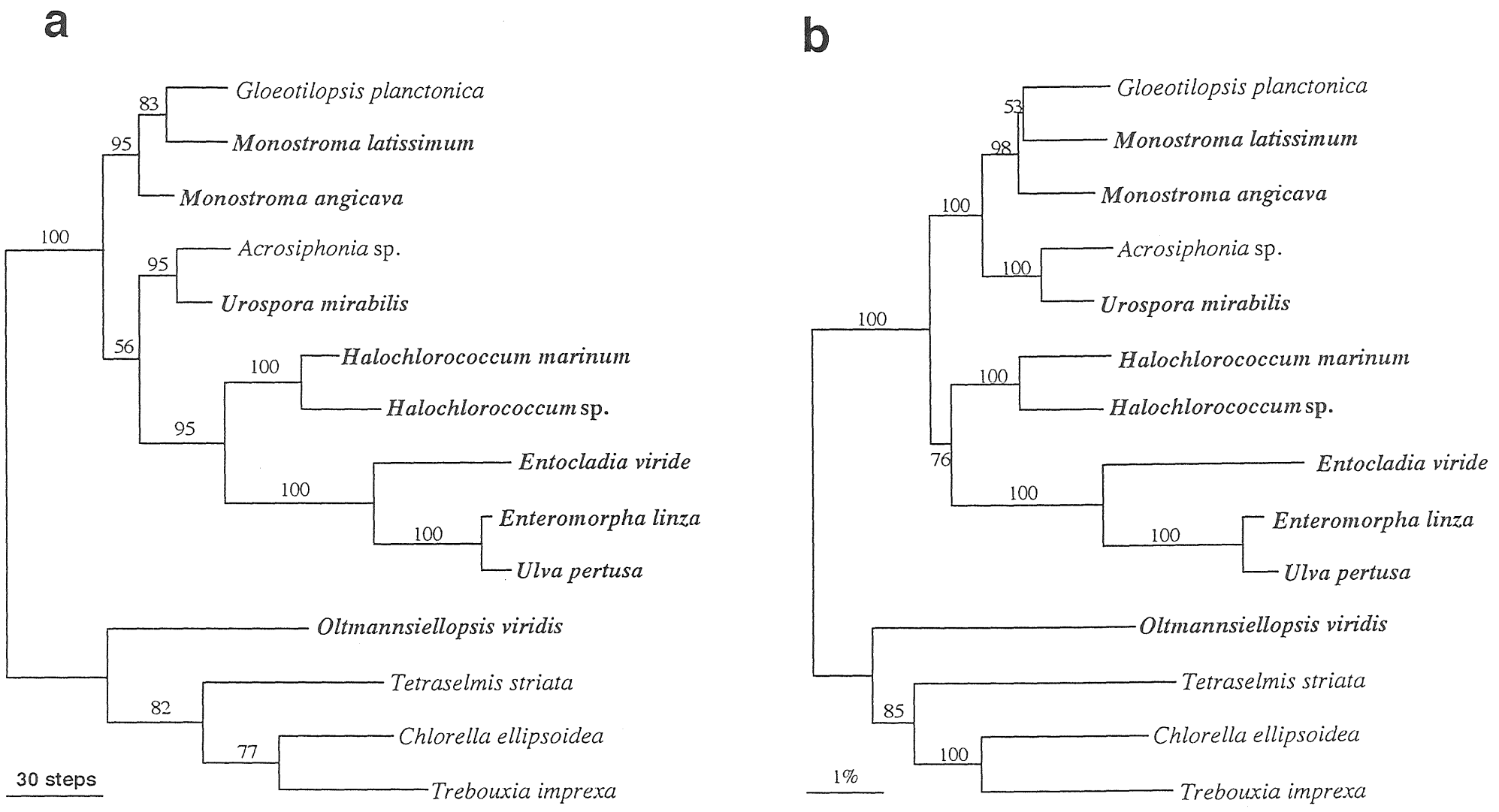

a
Gloeotilopsis planctonica
Monostroma latissimum
Monostroma angicava
Acrosiphonia sp.
Urospora mirabilis
Halochlorococcum marinum
Halochlorococcum sp.
Entocladia viride
Enteromorpha linza
Ulva pertusa
Oltmannsiellopsis viridis
Tetraselmis striata
Chlorella ellipsoidea
Trebouxia imprexa
83
95
100
95
56
100
95
100
100
82
77
30 steps
b
Gloeotilopsis planctonica
Monostroma latissimum
Monostroma angicava
Acrosiphonia sp.
Urospora mirabilis
Halochlorococcum marinum
Halochlorococcum sp.
Entocladia viride
Enteromorpha linza
Ulva pertusa
Oltmannsiellopsis viridis
Tetraselmis striata
Chlorella ellipsoidea
Trebouxia imprexa
53
98
100
100
100
100
76
100
100
85
100
1%

Figure 18. Diagrammatic representasions of the flagellar apparatus of ulvophycean algae.
a-b. Biflagellate zoospore of *Monostroma latissimum.*
a-b. Quadriflagellate zoospore of *Halochlorococcum* sp.

Abbreviations: d, d (right) microtubular root; **DF**, distal fiber; **F1**, system I fiber (= SMAC); **PB**, proximal band; **s**, s (left) microtubular root plast; **PS**, proximal sheath; **Rh**, rhizoplast (system II fiber); **SB1-3**, striated band 1-3; **TC**, terminal cap.

Figure 18

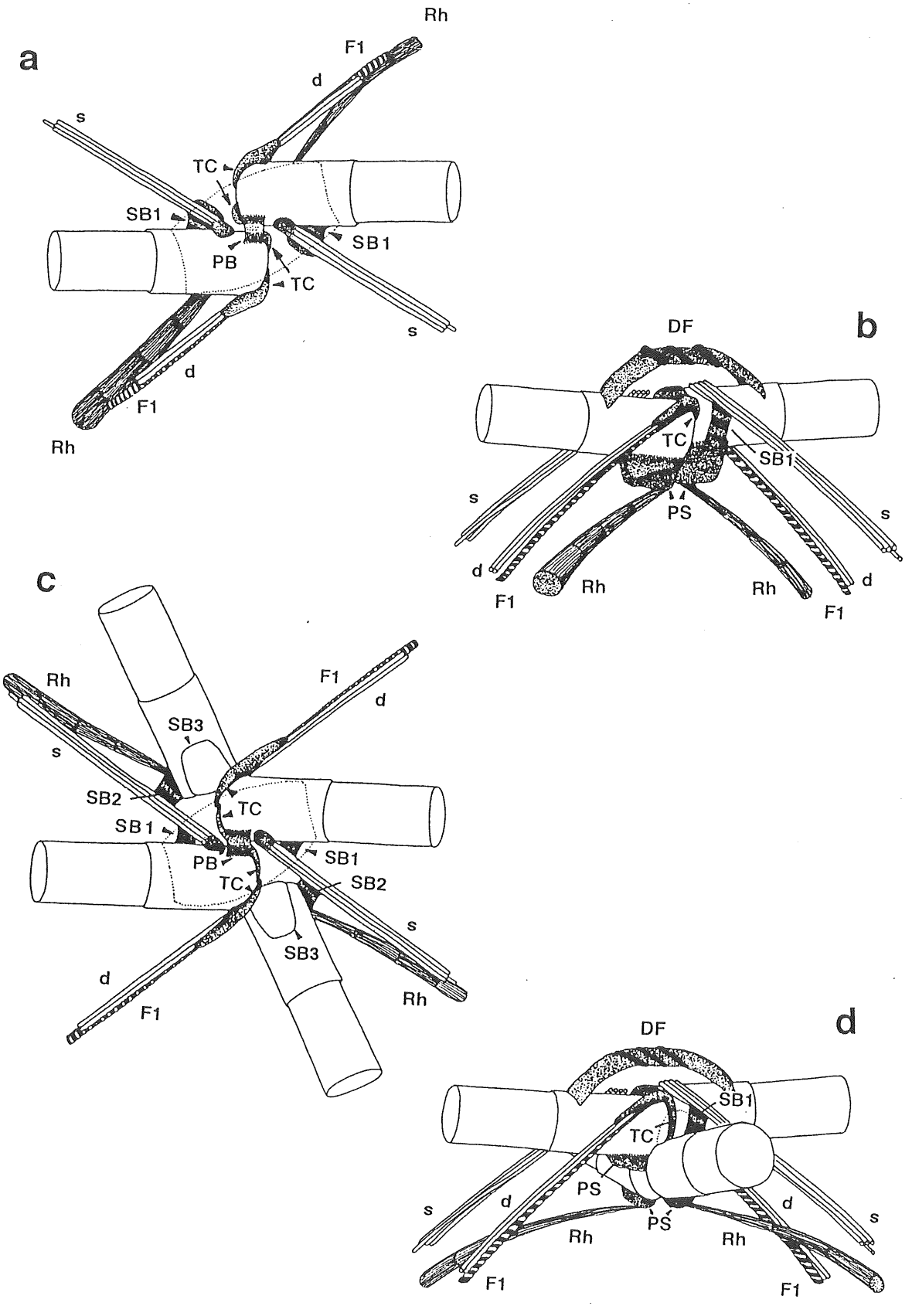

a
Rh
F1
d
s
TC
SB1
PB
SB1
TC
s
d
F1
Rh
b
DF
TC
SB1
s
PS
s
d
F1
Rh
Rh
d
F1
c
Rh
F1
d
SB3
s
SB2
TC
SB1
PB
SB1
TC
SB2
s
SB3
d
F1
Rh
d
DF
SB1
TC
PS
s
d
PS
d
s
Rh
Rh
F1
F1

Figure 19. Phylogenetic trees inferred from 18SrDNA sequences. A total 1706 nucleotides was considered. Trees were rooted with *Chlorella vulgaris* and *Trebouxia impressa.* Numbers on branches indicate bootstrap values (1000 replicates) larger than 50%. Taxa shown in bold letters are the species for which 18SrDNA sequences were determined in this study. **a.** Distance tree constructed with neighbor-joining (NJ) method based on Kimura's correction using Clustal W computer program. Branch lengths are proportional to the evolutionary distances. **b.** Strict consensus tree for twelve equally most parsimonious trees (1011 steps, consistency index is 0.575) generated by heuristic searches in PAUP computer program.

Figure 19

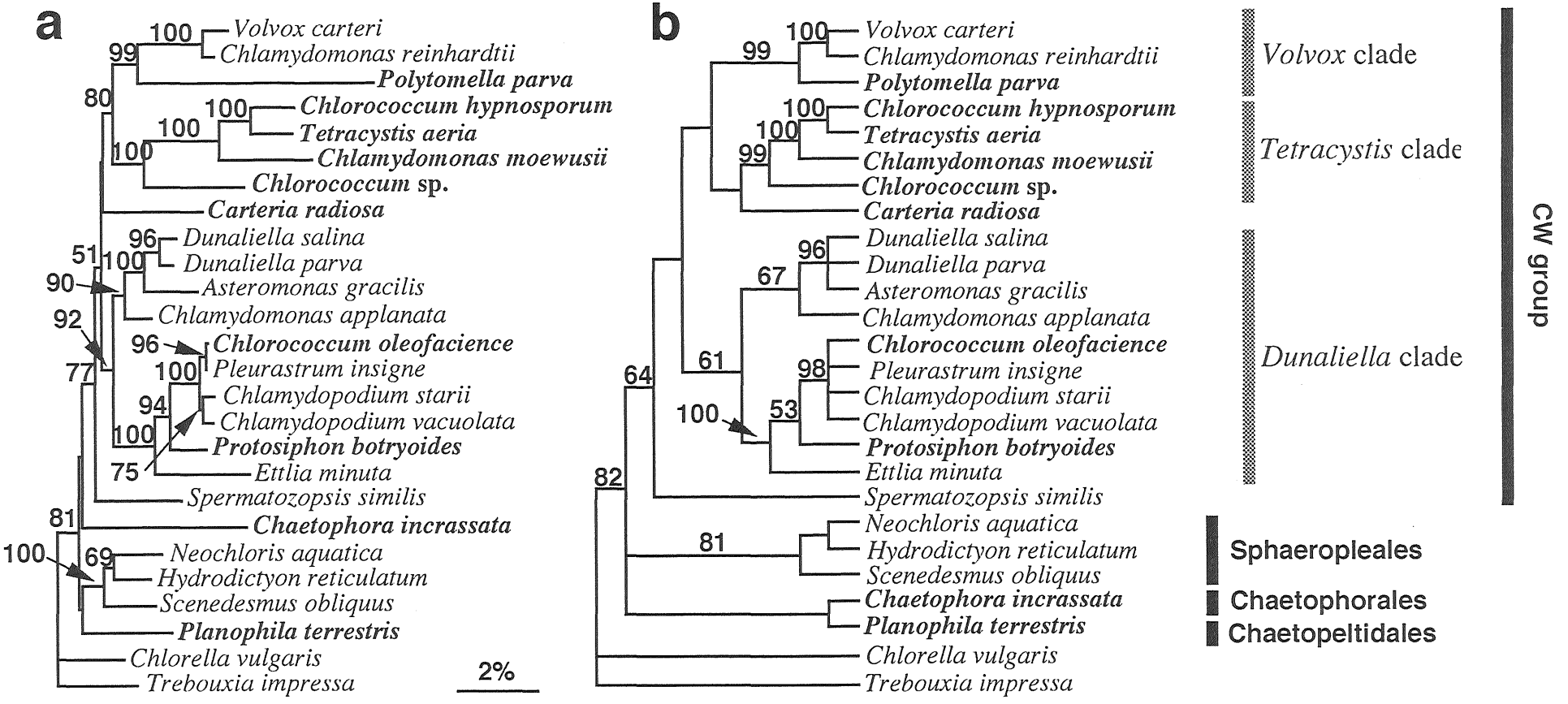

a
Volvox carteri
Chlamydomonas reinhardtii
Polytomella parva
Chlorococcum hypnosporum
Tetracystis aeria
Chlamydomonas moewusii
Chlorococcum sp.
Carteria radiosa
Dunaliella salina
Dunaliella parva
Asteromonas gracilis
Chlamydomonas applanata
Chlorococcum oleofacience
Pleurastrum insigne
Chlamydopodium starii
Chlamydopodium vacuolata
Protosiphon botryoides
Ettlia minuta
Spermatozopsis similis
Chaetophora incrassata
Neochloris aquatica
Hydrodictyon reticulatum
Scenedesmus obliquus
Planophila terrestris
Chlorella vulgaris
Trebouxia impressa
2%
b
Volvox carteri
Chlamydomonas reinhardtii
Polytomella parva
Chlorococcum hypnosporum
Tetracystis aeria
Chlamydomonas moewusii
Chlorococcum sp.
Carteria radiosa
Dunaliella salina
Dunaliella parva
Asteromonas gracilis
Chlamydomonas applanata
Chlorococcum oleofacience
Pleurastrum insigne
Chlamydopodium starii
Chlamydopodium vacuolata
Protosiphon botryoides
Ettlia minuta
Spermatozopsis similis
Neochloris aquatica
Hydrodictyon reticulatum
Scenedesmus obliquus
Chaetophora incrassata
Planophila terrestris
Chlorella vulgaris
Trebouxia impressa
Volvox clade
Tetracystis clade
Dunaliella clade
CW group
Sphaeropleales
Chaetophorales
Chaetopeltidales

Figure 20. A part (only CW group) of the strict consensus tree for NJ and MP trees (see Fig. 19). Possible character changes are indicated by arrows and bars. Arrows indicate putative gain of coccoid vegetative form. Gray and black bars indicate thinning and losses of cell walls on flagellate cells, respectively.

Figure 20

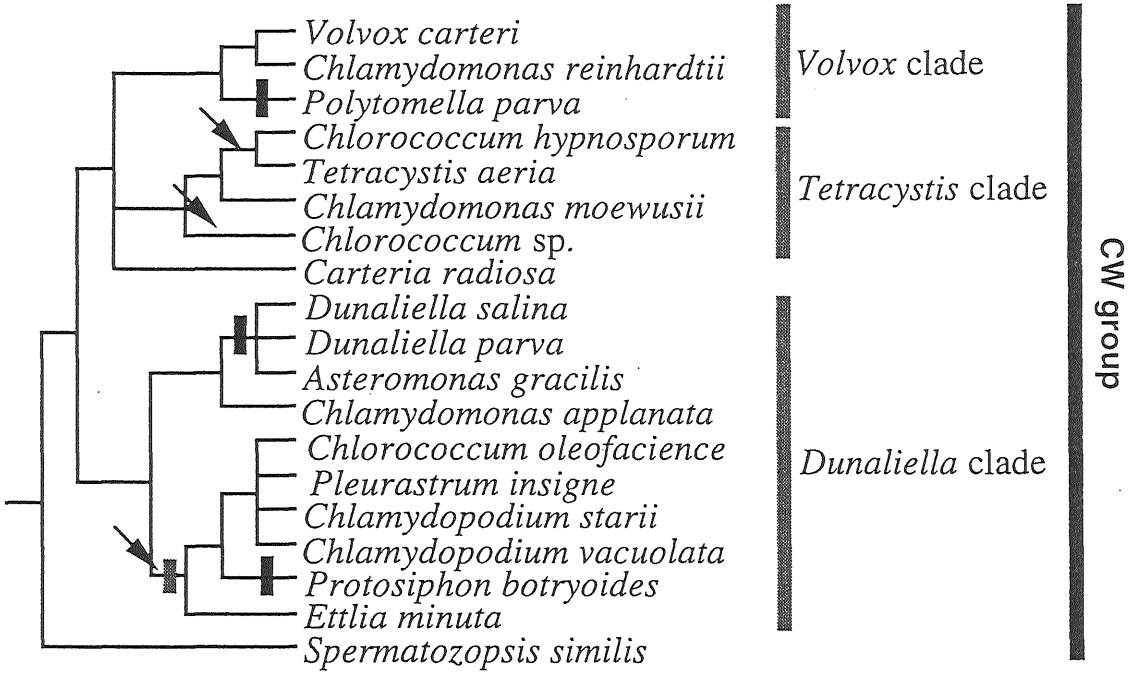

Volvox carteri
Chlamydomonas reinhardtii
Polytomella parva
Chlorococcum hypnosporum
Tetracystis aeria
Chlamydomonas moewusii
Chlorococcum sp.
Carteria radiosa
Dunaliella salina
Dunaliella parva
Asteromonas gracilis
Chlamydomonas applanata
Chlorococcum oleofacience
Pleurastrum insigne
Chlamydopodium starii
Chlamydopodium vacuolata
Protosiphon botryoides
Ettlia minuta
Spermatozopsis similis
Volvox clade
Tetracystis clade
Dunaliella clade
CW group

Plate 1. *Crustomastix didyma.*

1. Light microscopic image.

2. Whole cell with two subequal flagella and three types of hair scales. Both flagella are covered by T-hairs (small arrows) and on their tips displays tip hairs (arrowhead). P_1-hairs (large arrows) are confined to the shorter flagellum.Scale bar = 1 μm.

Plate 1

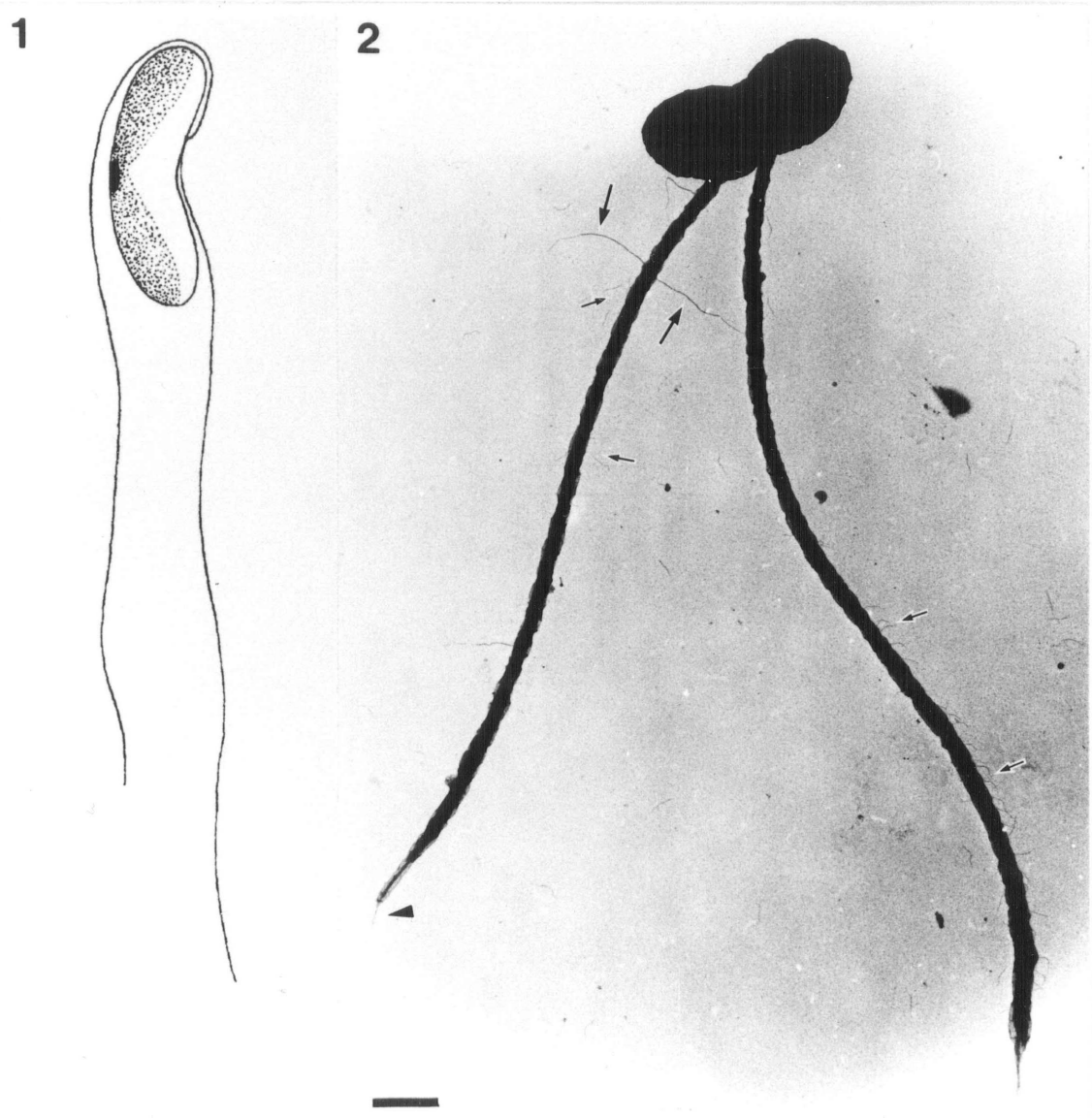


Plate 2. *Crustomastix didyma.*

1-3. Three types of hair scales of *Crustomastix didyma.*

1. P_1-hair. **2.** Tip hair. **3.** Negatively stained preparation of a T-hair. Scale bar = 200 nm.

Plate 2

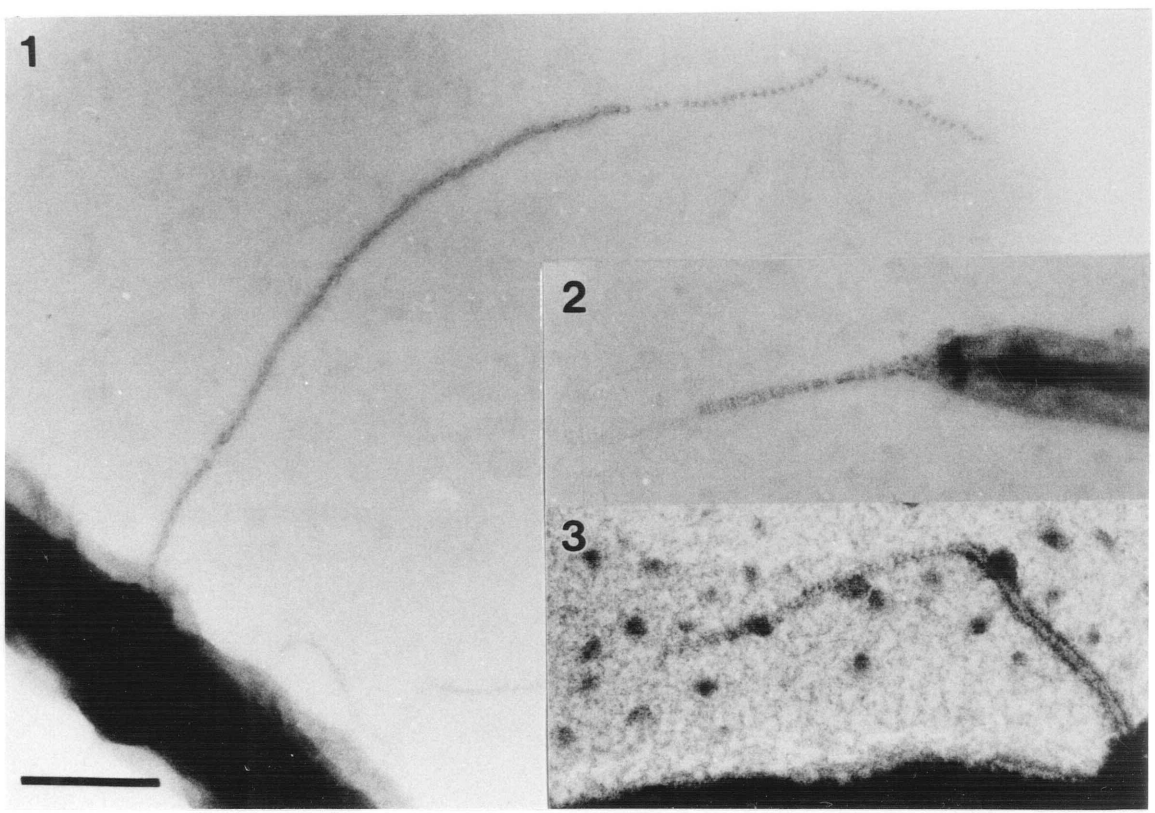


Plate 3. *Crustomastix didyma.*

1. Longitudal section of the cell cut perpendicullar to the dorsi-ventral axis of the cell. Note a conspicuaus starch grain situated in central part of each lobe of the chloroplast. Arrow indicates scale duct. Scale bar = 0.5 μm.

2. Transvers section of the cell cut nearly parallel to the left-right axis, as viewed from posterior side. Note long and narrow scale duct (arrow) extending to the ventral surface of the chloroplast. Scale bar = 0.5 μm.

3. Longitudal section cut parallel to the dorsi-ventral axis of the cell, as viewed from right side. Cell and flagella are covered by fibrous coat. At the proximal end of the 1d root (large arrows), lamellar structure (arrowhead) and plate (small arrow) are recognized. Note vesicular system with inner fibrous coat situated between Golgi apparatus and scale duct. Scale bar = 200 μm.

Abbreviations: **1**, no. 1 basal body; **C**, chloroplast; **D**, scale duct; **G**, Golgi apparatus; **M**, mitochondrion; **m**, microbody; **N**, nucleus.

Plate 3

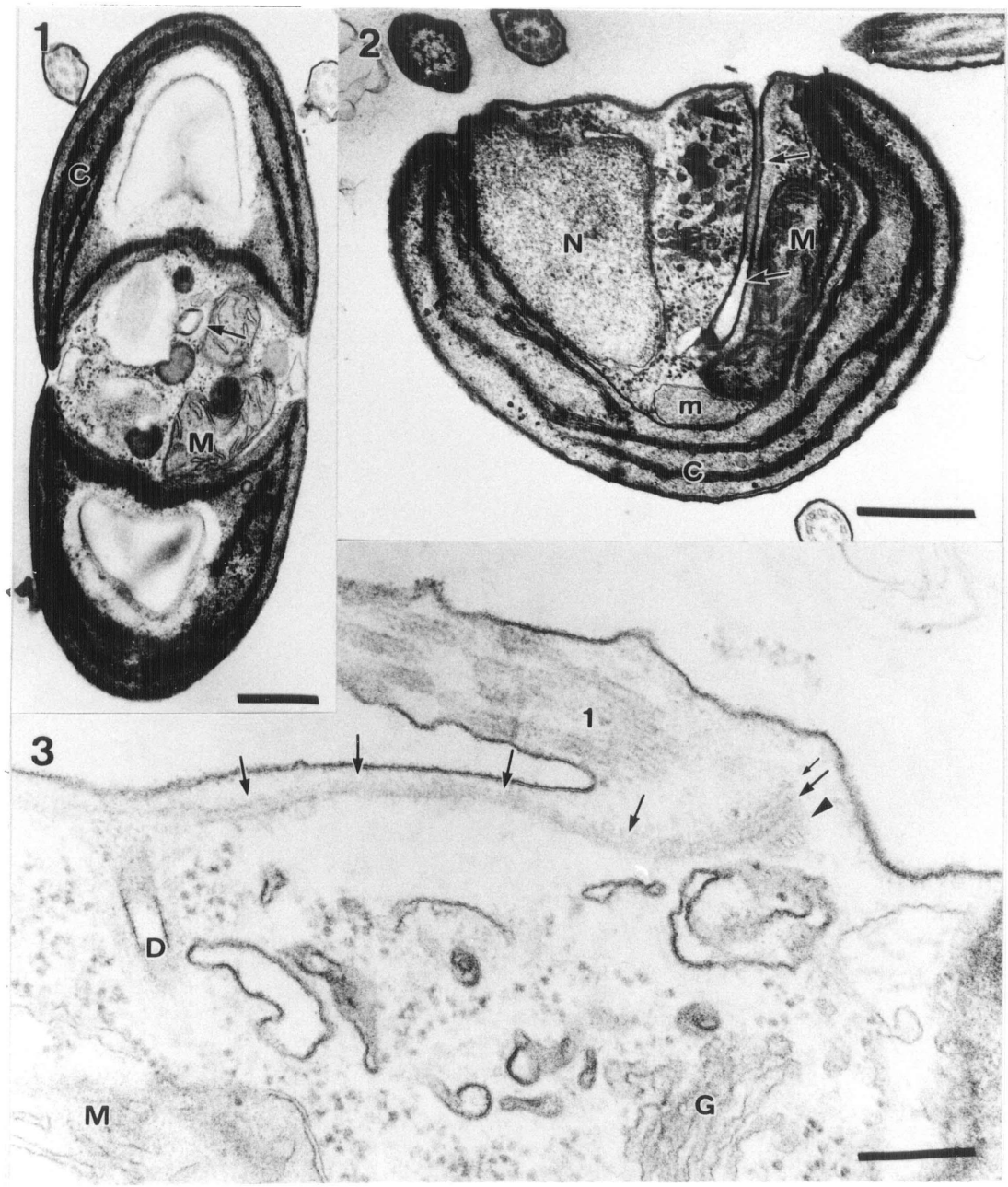


Plate 4. *Crustomastix didyma.*

1-6. Adjacent longitudal serial sections of the cell cut parallel to the dorsi-ventral axis (from left to rigt). 1s root (small arrows) originates from ventral side of the no. 1 basal body. ld root (large arrows) extends to the left side of the scale duct, while duct fiber (arrowheads) extends to its right side. Note large mitochondrion and weakly developed eyespot. Scale bar = 0.5 μm.

Abbreviations: 1, no. 1 basal body; **C**, chloroplast; **D**, scale duct; **E**, eyespot; **M**, mitochondrion; **m**, microbody.

Plate 4

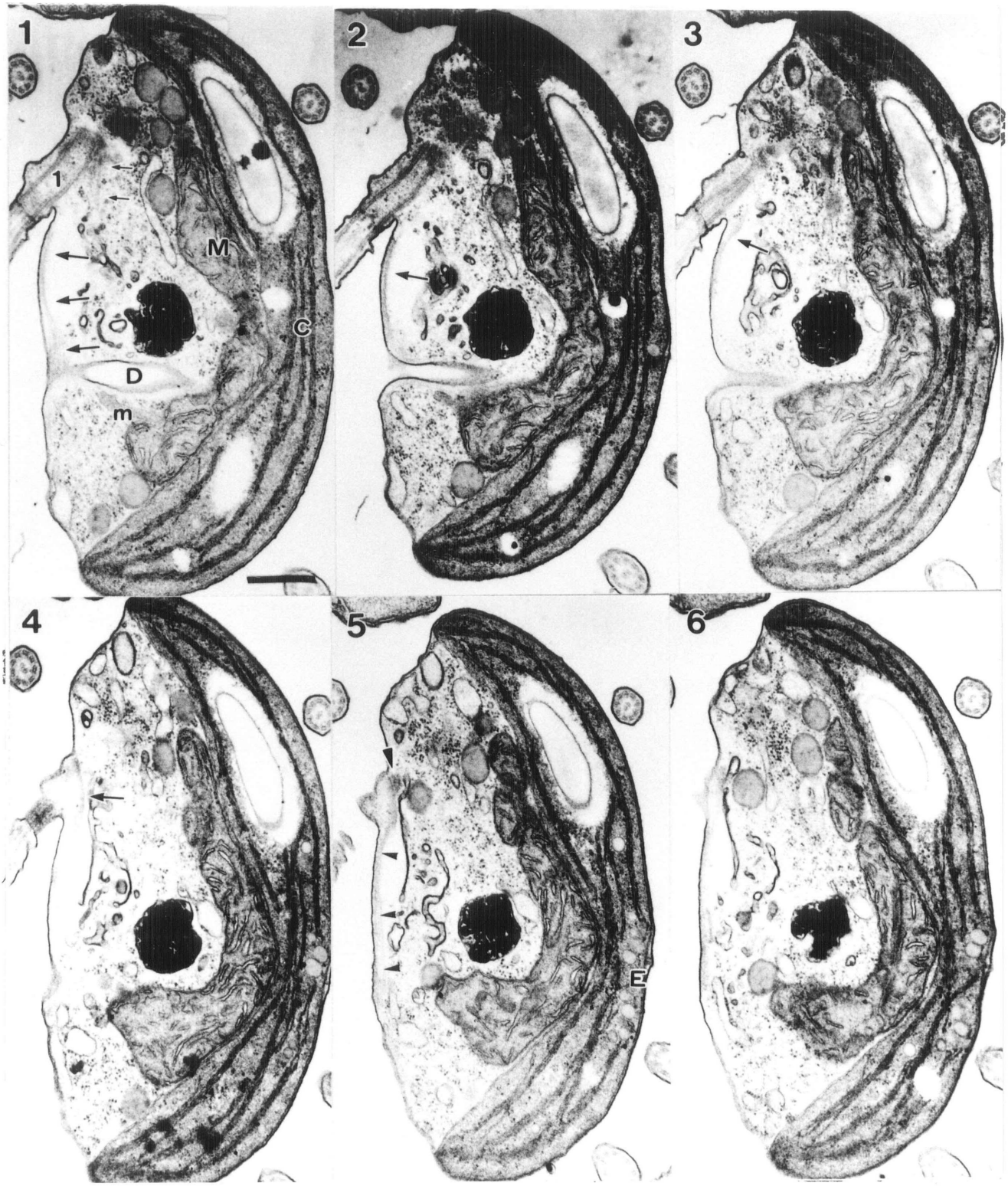


Plate 5. *Crustomastix didyma.*

1-4. Selected serial sections through basal body region viewed from the posterior side (anteror to posterior). 1d root consists of three microtubules (small arrows), while 1s root consists of four microtubules arranged in three over one configulation (small arrowheads). A fibrous structure extends along 1d root (large arrows). Note an extension from distal fiber to 1d root (open arrows) and an inconspicuous fibrous structure (large arrowheads). Scale bar = 0.5 μm.

5. More posterior part in comparison with Figs. 1-4. Number of microtubules in each root have decreased (small arrows and arrowheads indicate 1d and 1s root, respectively). Note an electron dense material situated beneath the 1s root (large arrow). Scale bar = 0.5 μm.

6. Longitudal section of the basal body, showing a single stellate structure underlain the transitional plate. Scale bar = 0.4 μm.

Abbreviations: 1, no. 1 basal body or flagellum; **2**, no. 2 basal body or flagellum; **DF**, distal fiber; **G**, Golgi apparatus; **M**, mitochondrion; **N**, nucleus.

Plate 5

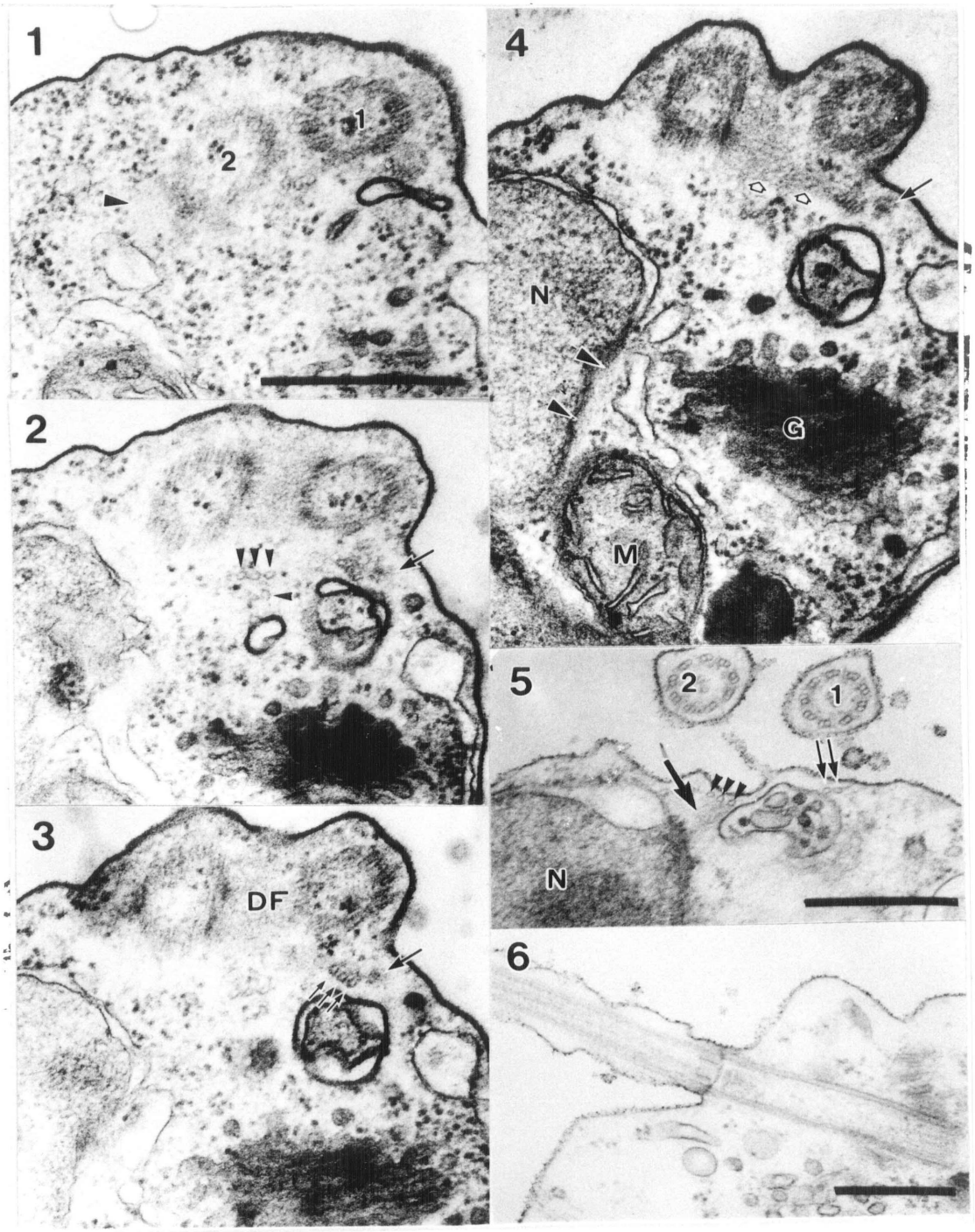


Plate 6. *Mantoniella antarctica.*

1. Longitudal section of the cell cut perpendicular to the dorsi-ventral axis. Note the position of a nucleus and Golgi apparatus.

2. Longitudal section of the cell cut parallel to the dorsi-ventral axis as viewed from right side.

3. Transverse section of the cell cut parallel to the left-right axis, as viewed from posterior side. Scale bar = 0.5 μm (common to Fig. 1-4).

4. Many extrusomes lying under the plasmalemma.

Abbreviations: **C**, chloroplast; **E**, eyespot; **ex**, extrusome; **G**, Golgi apparatus; **M**, mitochondrion; m, microbody; **N**, nucleus.

Plate 6

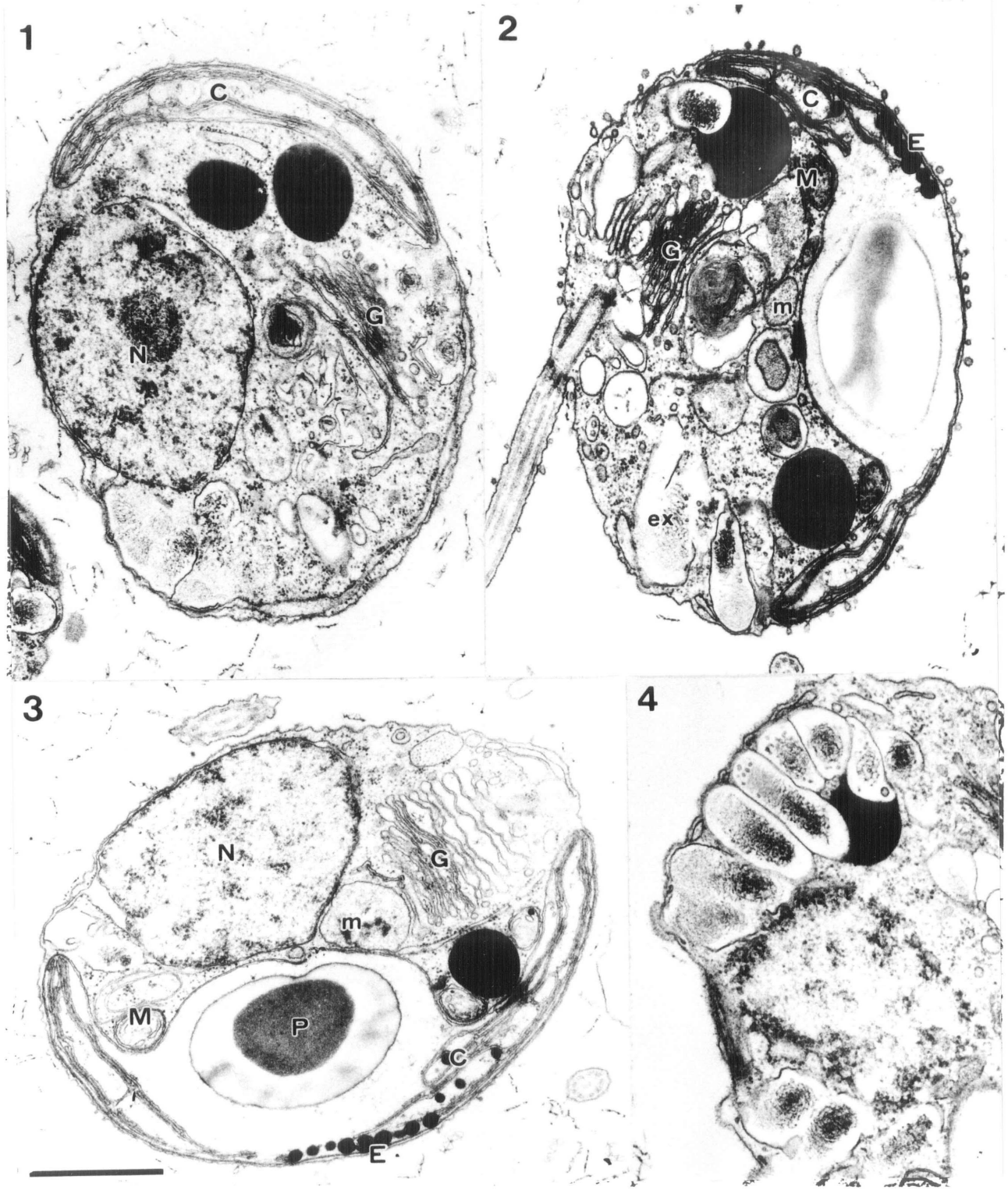


Plate 7. *Mantoniella antarctica.*

1-3. Adjacent serial sections of the flagellar apparatus viewed from posterior side of the cell. 1d root (arrow) composed 2 microtubules originate from ventral side of the no. 1 basal body. A fibrous structure extends along the right side of the 1d root. Two basal bodies are connected by proximal fiber (arrowhead) and distal fiber. Scale bar = 0.5 μm.

4-6. Adjacent serial sections of the flagellar apparatus viewed from posterior side of the cell. 1s root (arrowheads) composed of four microtubules (three over one configulation) originates from the dorsal side of the no. 1 basal body. Scale bar = 0.5 μm.

7-8. Adjacent serial sections of the fibrous structures. 1s root (arrowheads) extends to the nucleus. Note 1s-associated fiber (small arrows) and rhizoplast (large arrows) extends to the chloroplast. Scale bar = 0.5 μm.

Abbreviations: 1, no. 1 basal body; **2**, no. 2 basal body; **C**, chloroplast; **DF**, distal fiber; **G**, Golgi apparatus; **m**, microbody; **N**, nucleus; **P**, pyrenoid.

Plate 7

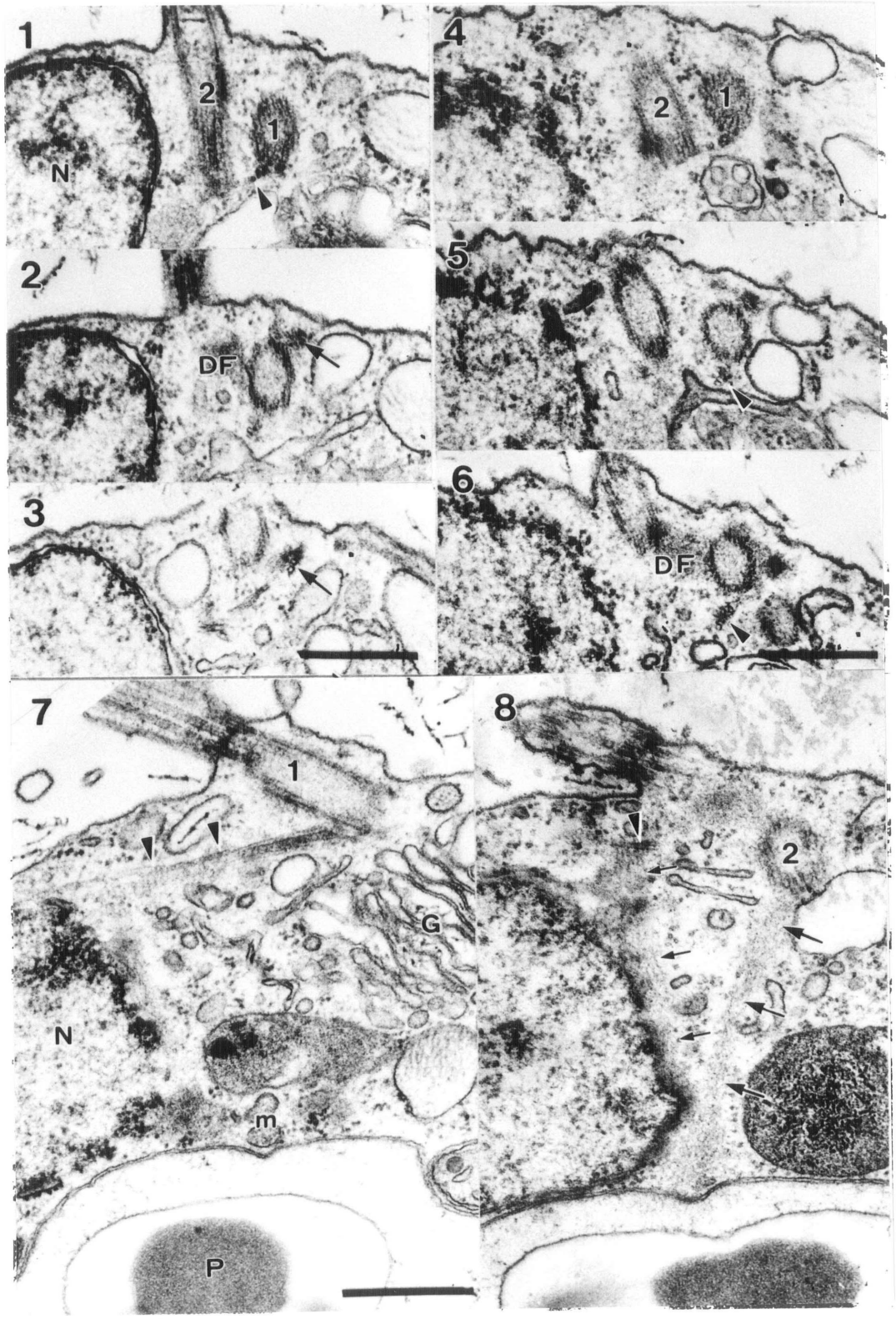


Plate 8. *Mantoniella antarctica* and *Micromonas pusilla.*

1-2. *Mantoniella antarctica.* Arrowheads indicate microtubular band which has no association to the basal bodies. This band extends to right-anterior side of the cell. Scale bars = 0.5 μm.

3-8. *Micromonas pusilla.*

3. Longitudal section of the cell cut parallel to the dorsi-ventral axis as viewed from right side. Note lens-shaped chloroplast and nucleus situated anteriorly. Scale bars = 0.5 μm. **4.** Longitudal section of the cell cut perpendicular to the dorsi-ventral axis. Note large extrusome. Scale bars = 0.5 μm. **5-8.** Non-adjacent serial sections of the basal body. Two microtubular roots (?) composed of two microtubules situated right (arrows) and left (arrowheads) side of the basal body. Scale bars = 0.5 μm.

Abbreviations: **C**, chloroplast; **ex**, extrusome; **G**, Golgi apparatus; **M**, mitochondrion; **N**, nucleus; **P**, pyrenoid.

Plate 8

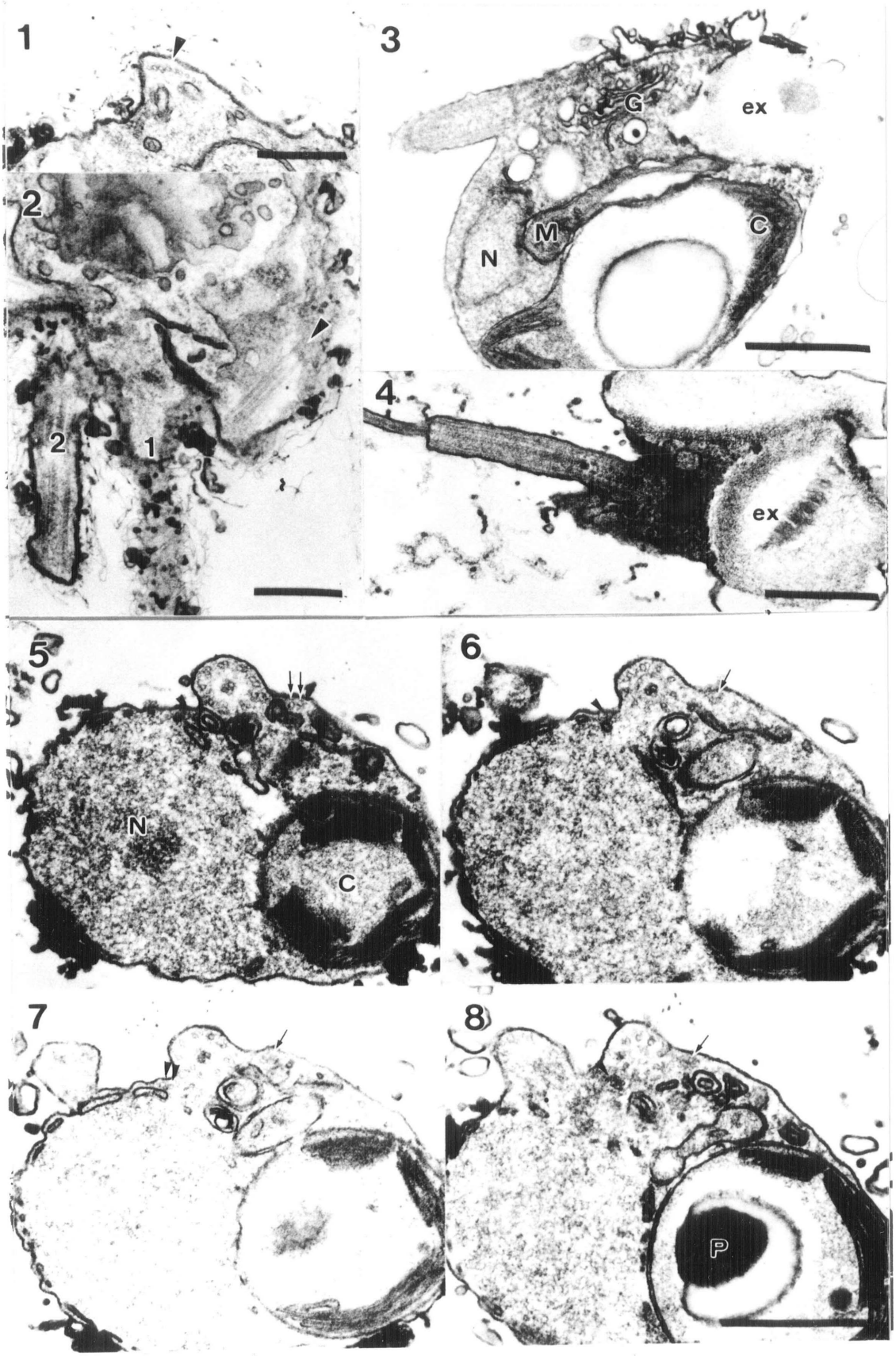


Plate 9. *Monostroma latissimum* and *Halochlorococcum* sp.
1-6. Flagellar apparatus of *Monostroma latissimum* biflagellate zoospore. **1.** Cross section through the apical papilla showing the circular structures (arrowheads). Note CCW basal bodies and cruciate microtubular roots (arrows). Scale bar = 0.2 μm. **2.** Longitudinal section through the apical papilla showing the circular structure (small arrowheads) and proximal sheath (arrow). Note small terminal cap (large arrowhead). Scale bar = 0.2 μm. **3-4.** Adjacent serial sections of the basal bodies which are connected by proximal fiber (small arrowheads) and connection between the proximal sheath (arrow). Large arrowheads indicate rhizoplast. Scale bar = 0.5 μm. **5-6.** Adjacent serial sections of the flagellar roots. The d microtubular root (arrows) is associated with system I fiber (small arrowheads) and rhizoplast (large arrowheads). Note membrane-associated plaque (mp). Scale bar = 0.5 μm. **7-12.** Flagellar apparatus of *Halochlorococcum* sp. quadriflagellate zoospore. **7.** Longitudinal section of the cell showing the upper basal body completely covered by terminal cap. Scale bar = 1 μm. **8.** Indistinct body scales in tangential section. Scale bar = 0.5 μm. **9.** The s root consisting of four microtubules (small arrows). Note striated band (large arrow) connecting this root to the lower basal body (L). Scale bar = 0.5 μm. **10.** The d root consisting of two microtubules (small arrows). Note electron dense core in the basal bodies. Scale bar = 0.5 μm. **11-12.** Adjacent serial sections of the flagellar apparatus. The s microtubular root (large arrows) is associated with rhizoplast (large arrowheads). Note terminal cap (small arrowheads), proximal sheath (PS) and striated band (small arrow) connecting upper (U) and lower basal (L) bodies. Scale bar = 0.5 μm.

Abbreviations: **E**, eyespot; **L**, lower basal body; **mp**, membrane-associated plaque; **N**, nucleus; **PS**, proximal sheath; **U**, upper basal body.

Plate 9