7 Systematics of the green algae: conflict of classic and modern approaches

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ABSTRACT

Traditionally the green algae were classified in orders or classes according to the morphological species concept. For example, monadoid species (flagellates) were summarized in the order Volvocales, coccoids in the Chlorococcales, filaments in the Ulotrichales or Chaetophorales, and siphonocladous algae in the Cladophorales or Siphonocladales. Later, a new classification was proposed based on ultrastructural investigations of the basal bodies in the flagellar apparatus and cell division. The species with basal bodies in clockwise (CW) or directly opposite (DO) orientation were classified in the class Chlorophyceae, the counterclockwise (CCW)-orientated species in the Ulvophyceae and Trebouxiophyceae (= Pleurastrophyceae). Phylogenetic analyses of nuclear-encoded SSU and ITS rDNA sequences have basically confirmed the classification based on ultrastructural characters. However, most genera and orders are polyphyletic and the relationships between

many of the phylogenetic lineages remain unclear. Traditionally taxonomic approaches often depend on single or even negetative "characters" (e.g. absence of zoospore formation or pyrenoids). The authors feel that in some cases these may be given excessive "weight" and advocate the usage of polyphasic approaches (e.g. secondary structures of SSU and ITS rDNA sequences, results of crossing experiments, sporangium autolysin data, and studies of life cycles, multigene approaches, amplified fragment length polymorphism [AFLP]) for the classification of green algae. New generic and species concepts (*Z*- and CBC-clade concepts, biological species concept, phylogenetic concepts) can be designed for many orders and most of the classes in the Viridiplantae on the basis of this approach.

INTRODUCTION

The green algae are photosynthetic eukaryotes characterized by the presence of chloroplasts with two envelope membranes, stacked thylakoids and chlorophyll *a* and *b*. (Few genera like *Prototheca*, *Polytoma*, *Polytomella*, and *Hyalogonium* are colourless, but the cells contain leucoplasts, which secondarily lost their pigments; Pringsheim, 1963.) All green algae produce starch as the main reserve polysaccharide, which is deposited inside the plastids. The plastids arrived in a single primary endocytobiosis (endosymbiosis) event, where a cyanobacterium was taken up by a colourless eukaryote host (see reviews: Delwiche and Palmer, 1997, Delwiche, 1999, Keeling, 2004; see also chapters 2 and 3 this volume). The green algae are one of the most diverse groups of eukaryotes, showing morphological forms ranging from flagellated unicells, coccoids, branched or unbranched filaments, to multinucleated macrophytes and taxa with parenchymatic tissues (Figure 7.1). They are distributed worldwide and can be found in almost every habitat from Arctic and Antarctic regions to oceans and freshwater lakes as well as in soil from temperate and arid areas. Green algae are also found in different symbioses including lichens, protozoa, and foraminifers, or as parasites on tropical plants. There are estimated to be at least 600 genera with 10,000 species within the green algae (Norton et al., 1996). Estimations of age vary from 600 My ago (Tappan, 1980, van den Hoek et al., 1988) to 1,500 Ma (Yoon et al., 2004).

Based on the structure and pigment composition of their plastids as mentioned above, green algae and land plants are closely related. This hypothesis was put forward a long time before

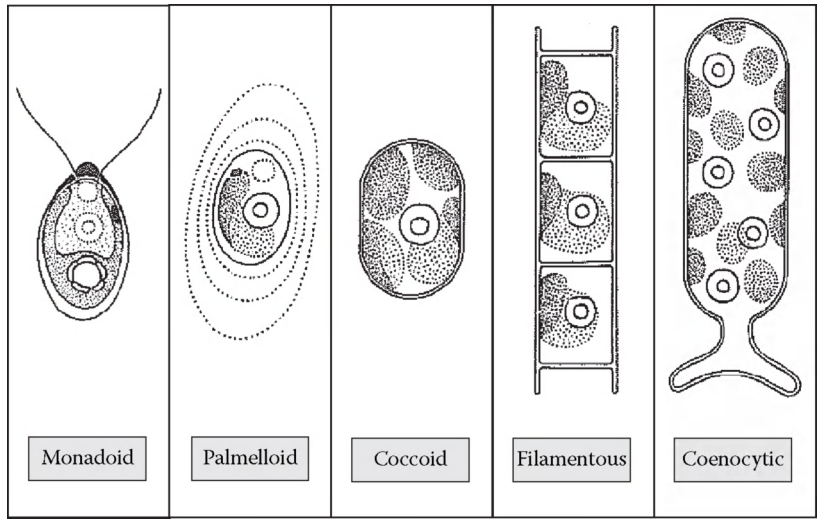

FIGURE 7.1 Different morphological organization in green algae (modified after Ettl, H. and Gärtner, G., *Syllabus der Boden-, Luft- und Flechtenalgen*. Gustav Fischer, Stuttgart, 1995). Parenchymatous and siphonocladous organization are not illustrated.

molecular and ultrastructural data were available. The present contribution will focus on green algae in their narrow sense (Chlorophyta *sensu* Bremer, 1985; see also Bremer et al., 1987). The systematics of the Streptophyta (*sensu* Bremer or Charophyta *sensu* Cavalier-Smith, 1993) is discussed in detail in chapter 8 in this volume.

How are green algae classified?

The classification of green algae is a topic of many publications, especially in the context of the origin of land plants, and is still controversial. We will not repeat all these discussions here (see citations below). More than 20 classes have been described during the 250 years since the introduction of classification by Linnaeus (Silva, 1980), some of them partly contradict each other. Depending upon which characters (morphological, ultrastructural, molecular) were used for classification, the same species can belong to different classes. All classes, if correctly described, are valid according to the International Code for Botanical Nomenclature (Greuter et al., 2000). The priority rule is only valid at the generic and species level. For example, *Ulothrix zonata* Kützing and *Uronema belkae* (Mattox et Bold) Lokhorst, both belong to the Ulotrichophyceae according to their morphology (unbranched filaments), but to two different classes (*Ulothrix zonata*—Ulvophyceae; *Uronema belkae*—Chlorophyceae) according to the ultrastructural orientation of the basal body in the flagellar apparatus in their zoospores. To date a wide range of criteria and approaches have been used for classification at higher levels, which may be broadly considered under the following three different concepts discussed below.

The morphological concept

Traditionally, the green algae were classified according to the morphological species concept based on the organization level of the vegetative state as shown in Figure 7.1. The rationale of this classification was that unicellular flagellates are primitive within the green algae, and that they evolved initially into coccoid and sarcinoid chlorophytes, and later into colonial, filamentous, coenocytic, and siphonous forms. Blackman (1900), and Blackman and Tansley (1902) proposed the first subdivision into classes and orders, which was refined over almost a century by several other authors (Pascher, 1931, Fritsch, 1935, Fott, 1971, see also Round, 1963, 1984, Bold and Wynne, 1985). In the 1960s and 1970s, a modified classification of the green algae was undertaken using experimental studies of life cycles and architecture ("Bauplan") of flagellated cells (for details see Christensen, 1962; Round, 1971; Kornmann, 1973; van den Hoek and Jahns, 1978; Ettl, 1981, 1983; Ettl and Komárek, 1982). These authors proposed seven classes: Prasinophyceae, Chlamydophyceae, Chlorophyceae, Codiolophyceae, Oedogoniophyceae, Bryopsidophyceae, and Zygnematophyceae.

The ultrastructural concept

Mattox and Stewart (1984) proposed a new classification based on the ultrastructure of the basal body in flagellated cells (Figure 7.2) and cytokinesis during the mitosis (Figure 7.3). The underlying principle of their classification scheme was that the first radiation of the green algae took place at the flagellate level, resulting in a multitude of ancient lineages of flagellates, some of which then went on to give rise to non-flagellate coccoid, sarcinoid, filamentous, siphonocladous, and siphonous representatives. Five classes were proposed: The Micromonadophyceae (uni-quadriflagellates) include all the prasinophytes except the Tetraselmidales, which they transferred to the Pleurastrophyceae. This class was admittedly paraphyletic, exhibiting primitive characteristics for many of the features used to define the other classes of green algae. Likewise, the class Prasinophyceae is defined by the presence of a primitive trait (i.e., the possession of organic body scales and flagellar scales) and by the absence of more advanced traits. These primitive, ancestral traits cannot be used to characterize particular groups within the Chlorophyta, since they are common to green algae as a whole. The Pleurastrophyceae and Ulvophyceae are characterized by a counterclockwise

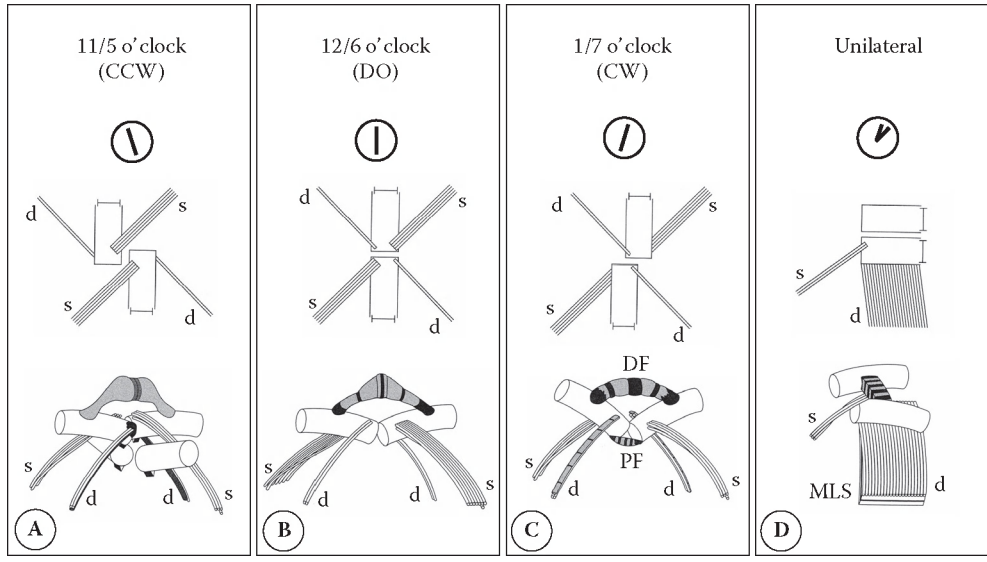

FIGURE 7.2 Different types of flagellar apparatus found among the green algae, viewed from the top (upper figure) and from the side (lower figure) (modified after Graham, L.E. and Wilcox, L.W., *Algae*. Prentice Hall, Upper Saddle River, NJ, 2000). The apparatuses generally include two or four basal bodies (shown here as rectangles or cylinders), microtubular roots (s or d), and distal (DF) or proximal (PF) connecting fibers. (A) Flagellar apparatus with cruciate roots and basal bodies displaced in counterclockwise (CCW) direction (Trebouxiophyceae and Ulvophyceae). (B) Flagellar apparatus with cruciated roots showing directly opposed (DO) displacement flagellar basal bodies (Chlorophyceae). (C) Flagellar apparatus with clockwise (CW) displaced flagellar basal bodies (Chlorophyceae). (D) Flagellar apparatus of the Streptophyta with asymmetrical (unilateral) distribution of the flagellar roots, showing the characteristic multilayered structure (MLS).

orientation of the basal body (CCW; Figure 7.2A); the Chlorophyceae are characterized by a directly opposite (DO; Figure 7.2B) or clockwise (CW; Figure 7.2C) orientation. The architecture of the flagellar apparatus in flagellated cells provides a very important taxonomic and phylogenetic character and is reviewed in detail by Melkonian (1982) and Melkonian and Surek (1995), and for the Ulvophyceae by Sluiman (1989). The Zygnematophyceae were transferred as an order to the Charophyceae based on biochemical features and development during the cell division (see Figure 7.3G through Figure 7.3H; for more details see van den Hoek et al., 1995). In addition to the phragmoplast, the Charophyceae *sensu* Mattox and Stewart are characterized by the unilateral root of their flagellar apparatus (Figure 7.2D). Most of the other classes earlier proposed by Ettl, Kornmann, and others (citations see above) were rejected and incorporated into the five classes *sensu* Mattox and Stewart.

Van den Hoek et al. (1988), who argued that the ultrastructural characters of the flagellated cell were insufficient, on their own, to characterize classes within the Chlorophyta, refined this five-class system by also taking into account the structure of the vegetative cells, mitosis and cell division, the composition of the cell walls, and the life history. These authors established seven classes (including the streptophycean algae), which combined the morphological and ultrastructural concepts: Prasinophyceae, Chlamydophyceae, Ulvophyceae, Chlorophyceae, Zygnematophyceae, Trentepohliophyceae, and Charophyceae.

The "Ulvophyceae" *sensu lato* were later subdivided into five new classes (Ulvophyceae *sensu stricto*, Cladophorophyceae, Bryopsidophyceae, Dasycladophyceae, and Trentepohliophyceae)

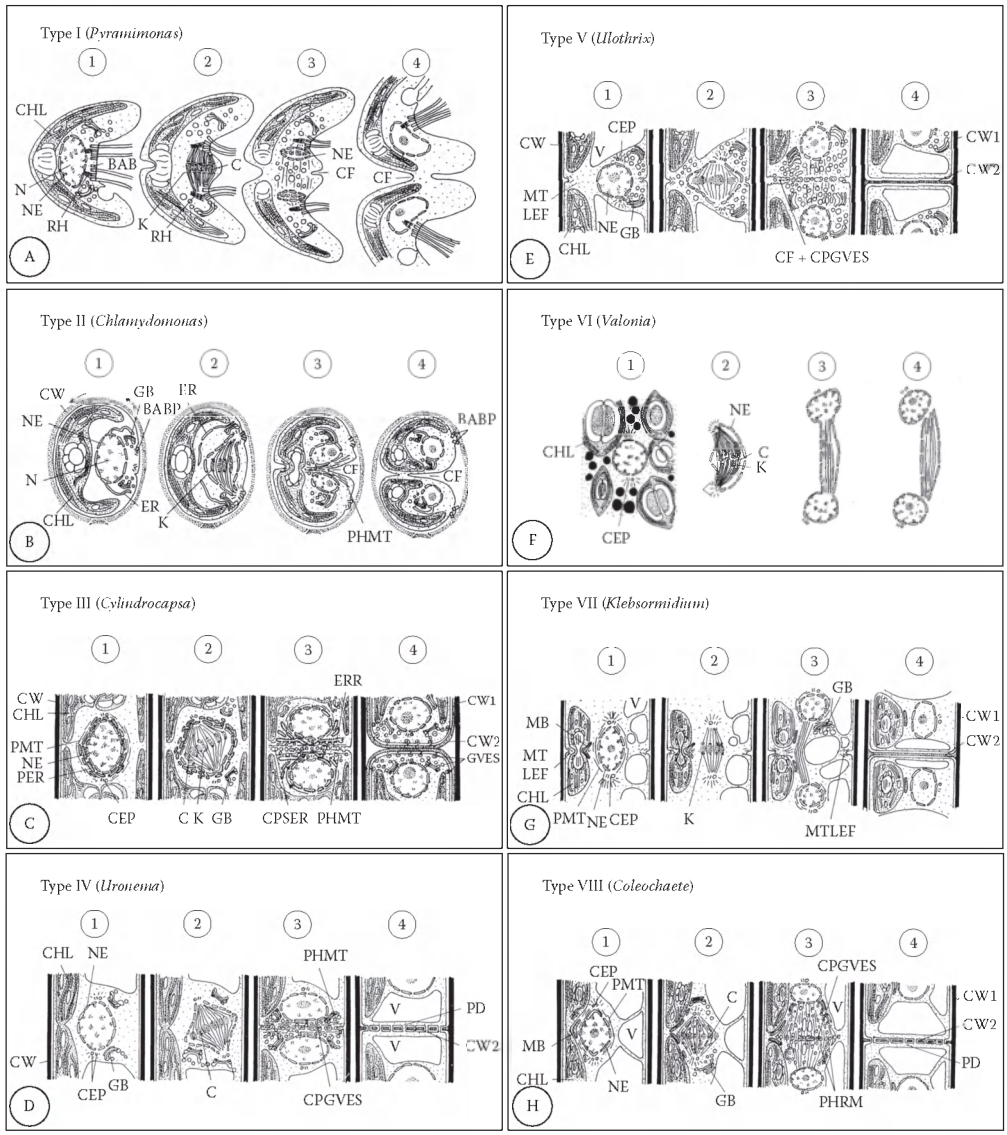

FIGURE 7.3 Different mitosis-cytokinesis types occuring among the green algae (modified after van den Hoek et al., 1988). 1. Early prophase. 2. Metaphase. 3. Late telophase. 4. Early interphase. BAB = basal body; BABP = pair of basal bodies; C = chromosome; CEP = pair of centrioles; CF = cleavage furrow; CHL = chloroplast; CPGVES = cell plate of Golgi-derived vesicles; CPSER = cell plate of smooth ER-vesicles; CW = cell wall; CW1 = old cell wall; CW2 = young cell wall; ER = endoplasmic reticulum; EER = rough endoplasmic reticulum; GB = Golgi body; GVES = Golgi-derived vesicles; K = kinetochore; MB = microbody; MTLEF = microtubules along leading edge of cleavage furrow of plasma membrane; N = nucleus; NE = nuclear envelope; PD = plasmodesma; PER = perinuclear endoplasmic reticulum; PHMT = phycoplast microtubule; PHRT = phragmoplast microtubule; PMT = perinuclear microtubules; RH = rhizoplast; V = vacuole. (A) Open mitosis with a persistent telophase spindle; cytokinesis by cleavage furrow (*Pyramimonas*). (B) Closed mitosis with a non-persistent telophase spindle; cytokinesis by a cleavage furrow in a phycoplast (*Chlamydomonas*). (C) Closed mitosis with a non-persistent spindle; cytokinesis by a cell plate of smooth ER-vesicles in a phycoplast (*Cylindrocapsa*). (D) Closed mitosis with a non-persistent telophase spindle; cytokinesis by a cell plate of Golgi-derived vesicles in a phycoplast (*Uronema*). (E) Closed mitosis with a persistent telophase spindle; cytokinesis by a cleavage furrow to which Golgi-derived vesicles are added (*Ulothrix*). (F) Closed mitosis with a prominent persistent telophase spindle, causing a typical dumbbell shape; cytokinesis does not immediately follow mitosis (*Valonia*). (G) Open mitosis with a prominent persistent telophase spindle; cytokinesis by cleavage furrow (*Klebsormidium*). (H) Open mitosis with a prominent persistent telophase spindle; cytokinesis by a cell plate of Golgi-derived vesicles in a phragmoplast (*Coleochaete*).

based on apparent differences in thallus architecture, cellular organisation, chloroplast morphology, cell wall composition, and life histories (van den Hoek et al., 1993, 1995). These classes were not validly described according to the rules of ICBN (no Latin diagnosis; not type genus designated; see Table 7.1).

TABLE 7.1
Classification of Green Algae According to the Different Concepts Using Morphological, Ultrastructural, and Molecular Approaches*

Class Author	Monophyletic clade supported in Figure 7.4 and Figure 7.5	Validly described according to ICBN	Remarks (see additional comments in Silva, 1980)
MORPHOLOGICAL CONCEPT			This concept used the morphology of the vegetative state of the organisms for classification.
Prasinophyceae Moestrup et Throndsen	−	+	Originally circumscribed and designated as a class by Christensen (1962) and was validated by Moestrup and Throndsen (1988); this class contains all flagellates with organic scales on the surface of cells, including the flagella; phylogenetically paraphyletic, containing several classes.
Chlamydophyceae Ettl	(+)	+	This class contains all monadoid and coccoid green algae with *Chlamydomonas*-like cells or zoospores, rejected by Mattox and Stewart (1984) and Deason (1984), because the Dunaliellales were not included and the subdivision in four orders is artificial, which was already mentioned by Ettl (1981); corresponds with CW-group (see Figure 7.5), needs emendations at the ordinal level.
Codiolophyceae Kornmann	+	−	This class contains all algae that form a "*Codiolum*"-stage in their life cycle; rejected by Mattox and Stewart (1984), because Ulvales are not included; invalidly described according to ICBN (no Latin diagnosis); if the Ulvales are included, monophyletic group.
Chlorophyceae Wille ex Warming	−	+	This class originally includes all green algae. Later, subsections were separated and described as other classes. Mattox and Stewart (1984) defined this class and included in it only species with CW or DO orientation (see Figure 7.2) of the basal body in their flagellated cells. *Chlorococcum* was chosen as the type genus by Christensen (1994); no solid molecular support for recognition of the monophyletic nature of the class.
Oedogoniophyceae Round	+	−	This class contains the three filamentous genera, *Oedogonium*, *Oedocladium,* and *Bulbochaete* because of their special life cycle; included as order in the Chlorophyceae by Mattox and Stewart (1984), invalidly described (no Latin diagnosis), monophyletic group.
Bryopsidophyceae Bessey	n.d.	+	In this class the two orders Bryopsidales and Halimedales, are summarized which were included in the Ulvophyceae *sensu* Mattox and Stewart (1984); no SSU rDNA sequences available.
Zygnematophyceae Round	+	−	This class summarized all conjugating algae, which were previously described as Conjugatophyceae by Engler (1892). Because *Conjugata* (= *Spirogyra*) and *Zygnema* are not synonyms, Zygnematophyceae is not a legitimate substitute for the Conjugatophyceae (see Silva, 1980); invalidly described (no Latin diagnosis), monophyletic group.

TABLE 7.1 (CONTINUED)
Classification of Green Algae According to the Different Concepts Using Morphological, Ultrastructural, and Molecular Approaches*

Class Author	Monophyletic clade supported in Figure 7.4 and Figure 7.5	Validly described according to ICBN	Remarks (see additional comments in Silva, 1980)
ULTRASTRUCTURAL CONCEPT (*sensu* Mattox and Stewart 1984)			This concept used only the basal body orientation of the flagellar apparatus and cytokinesis (mitosis) for classification.
Micromonadophyceae Mattox et Stewart	–	–	This class contains all prasinophytes as described above except the order Tetraselmidiales, which Mattox and Stewart (1984) transferred to the Pleurastrophyceae; invalidly described (no type genus designated), paraphyletic group.
Pleurastrophyceae Mattox et Stewart	–	–	This class contains algae with CCW basal body orientation (see Figure 7.2). Mattox and Stewart (1984) also included *Tetraselmis* in this class; invalidly described (no type genus designated), the nominal type genus *Pleurastrum* and their type species *P. insigne* belong to the *Stephanosphaera*-clade of the CW group (Friedl, 1996; see Figure 7.5), no solid molecular support for recognition of the monophyletic nature of the class.
Ulvophyceae Mattox et Stewart	–	–	This class also contains algae with CCW basal body orientation (see Figure 7.2). Besides the orders Ulvales and Ulotrichales, Mattox and Stewart (1984) also included in this class the orders Bryopsidales, Dasycladales, Trentepohliales, and Cladophorales/Siphonocladales; invalidly described (no type genus designated), the nominal type genus *Ulva* and their type species *U. lactuca* belong to the *Ulva*-clade (see Figure 7.4B), no solid molecular support for recognition of the monophyletic nature of the class.
Chlorophyceae Wille ex Warming	–	+	See above.
Charophyceae Rabenhorst	+	+	This class contains the order Charales, which is characterized by special differentiation in nodial and internodial cells, a complex life cycle, and a unilateral basal body orientation (see Figure 7.2) and phragmoplast (Figure 7.3). Originally described by Rabenhorst (1863), a defined characterization is given by Mattox and Stewart (1984) with Latin diagnosis, but without designation of the type genus; emendations necessary, monophyletic group.
ULTRASTRUCTURAL CONCEPT (*sensu* van den Hoek et al., 1988, 1995)			In addition to the concept presented by Mattox and Stewart (1984), the morphology, life cycle, and biochemical characters were used for this concept.
Prasinophyceae Moestrup et Throndsen	–	+	See above.
Chlamydophyceae Ettl	(+)	+	See above.
Ulvophyceae *s.str.* van den Hoek, Mann et Jahns	+	+	This class contains all species belonging to the Codiolophyceae (see above) *sensu* Kornmann (1973) including the Ulvales; invalidly described (no Latin diagnosis and no type genus designated), monophyletic group

(*Continued*)

TABLE 7.1 (CONTINUED)
Classification of Green Algae According to the Different Concepts Using Morphological, Ultrastructural, and Molecular Approaches*

Class Author	Monophyletic clade supported in Figure 7.4 and Figure 7.5	Validly described according to ICBN	Remarks (see additional comments in Silva, 1980)
Bryopsidophyceae Bessey	n.d.	+	See above.
Cladophorophyceae van den Hoek, Mann et Jahns	+	–	In this class are summarized the two orders Cladophorales and Siphonocladales, which were included to the Ulvophyceae *sensu* Mattox and Stewart (1984); invalidly described (no Latin diagnosis); monophyletic group.
Dasycladophyceae van den Hoek, Mann et Jahns	+	–	This class contains the order Dasycladales, which Mattox and Stewart (1984) included to the Ulvophyceae; invalidly described (no Latin diagnosis); monophyletic group.
Chlorophyceae Wille ex Warming	–	+	See above.
Zygnematophyceae Round	+	–	See above.
Trentepohliophyceae van den Hoek, Stam et Olsen	+	–	This class contains order Trentepohliales with the genera *Trentepohlia*, *Cephaleuros*, *Phycopeltis,* and *Stomatochroon*, which Mattox and Stewart (1984) included to the Ulvophyceae; invalidly described (no Latin diagnosis); monophyletic group.
Charophyceae Rabenhorst	+	+	See above.
MOLECULAR CONCEPT (*sensu* Lewis and McCourt 2004)			This classification is based on the molecular phylogeny of SSU rDNA or *rbc*L sequences.
Chlorophyta	+	+	This division contains all algae, their cells divide via phycoplast during cytokinesis (see Figure 7.3); solid molecular support for recognition of the monophyletic nature of the division.
Chlorophyceae Wille ex Warming	–	+	See above.
Trebouxiophyceae Friedl	–	+	This contains all taxa belonging to the class Pleurastrophyceae *sensu* Mattox and Stewart (1984) excluding the Tetraselmidales (see above) and a group of autosporic coccoid green algae; a new name was necessary, because the genus *Pleurastrum* belongs to the Chlorophyceae (Friedl, 1996); no solid molecular support for recognition of the monophyletic nature of the class.
Ulvophyceae Mattox et Stewart	–	–	See above.
Prasinophyceae Moestrup et Throndsen	–	+	See above.
Streptophyta = Charophyta	+	+	This division contains all algae, bryophytes, ferns, and higher plants, their cells divide via phragmoplast during cytokinesis (see Figure 7.3); solid molecular support for recognition of the monophyletic nature of the division.

TABLE 7.1 (CONTINUED)
Classification of Green Algae According to the Different Concepts Using Morphological, Ultrastructural, and Molecular Approaches*

Class Author	Monophyletic clade supported in Figure 7.4 and Figure 7.5	Validly described according to ICBN	Remarks (see additional comments in Silva, 1980)
Mesostigmatophyceae Marin et Melkonian	−	+	This class contains the two genera *Mesostigma* and *Chaetosphaeridium*, the latter genus belongs to the *Coleochaete*-clade (see Figure 7.4A, see also chapter 8, this volume); emendation necessary (exclusion of *Chaetosphaeridium*), then monophyletic group.
Chlorokybophyceae Bremer	+	−	This class contains few strains of a single sarcinoid species *Chlorokybus atmophyticus*; invalidly described (no Latin diagnosis); monophyletic group.
Klebsormidiophyceae van den Hoek, Mann et Jahns	−	−	Originally described by Jeffrey (1982) containing all streptophycean algae (exclusive *Coleochaete*). Van den Hoek et al. (1995) included the genera *Klebsormidium*, *Chlorokybus*, *Chaetosphaeridium*, *Coleochaete*, *Stichococcus*, and *Raphidonema* in this class, the last two genera belong to the Trebouxiophyceae, the others were transferred to other classes; invalidly described (no Latin diagnosis); monophyletic group, if confined to *Klebsormidium* and relatives (see Figure 7.4).
Zygnematophyceae Round	+	−	See above.
Coleochaetophyceae Bessey ex Woods	+	+	This class contains species of the genus *Coleochaete* and *Chaetosphaeridium*; Jeffrey (1982) described this class in the same way (invalidly without Latin diagnosis); monophyletic group.
Charophyceae Rabenhorst	+	+	See above.

Note: += supported; −= not supported if class is revised; nd = no ssu sequencing data available.
*The table contains only classes that are widely accepted by the science community. For further described classes, see Silva (1980).

On the basis of the type of cell division (Figure 7.3), the green algae (inclusive of the higher plants) summarised as Viridiplantae (*sensu* Cavalier-Smith, 1981; Sluiman, 1985), or Chlorobionta (*sensu* Bremer, 1985; Bremer et al., 1987) were subdivided into two divisions—the Streptophyta (including Zygnematophyceae, Charophyceae, and higher plants), which form a phragmoplast during the cell division, and the Chlorophyta, which contain the other five classes, which mostly form a phycoplast (Pickett-Heaps, 1975).

The Molecular Concept (Phylogenetic Concept)

In the 1990s, the application of phylogenetic analyses of molecular markers was introduced into the systematics and taxonomy of algae. Typical genetic markers used are the nuclear ribosomal operon (SSU, 5.8S, and LSU including ITS-1 and ITS-2), actin, several chloroplast genes (*rbc*L, *atp*B, and others), and mitochondrial genes (*nad*5). Phylogenetic analyses of SSU rDNA have provided support for the original suggestion, based on ultrastructural data, that there are two main lineages among the green plants (Friedl, 1997): the first one comprising charophycean algae and

the land plants (the Streptophyta *sensu* Bremer, 1985), the other lineage consisting of the remaining green algae (Chlorophyta *sensu* Bremer, 1985). According to these analyses, the following lineages were revealed within the green algae (including the main publications): Prasinophyceae (e.g. Steinkötter et al., 1994; Nakayama et al., 1998; Guillou et al., 2004), Chlorophyceae (e.g. Hepperle et al., 1998; Pröschold et al., 2001; Krienitz et al., 2003; Friedl, 1997), Trebouxiophyceae (e.g. Friedl, 1995; 1997; Krienitz et al., 2004), Ulvophyceae (e.g. O'Kelly et al., 2004a, 2004b, 2004c), plus the streptophyte algae (e.g. Huss and Kranz, 1997; Marin and Melkonian, 1999; Karol et al., 2001).

At present these classes have been largely accepted by the science community; however, there is still discussion, because some lineages showed a "Long-Branch Attraction" (LBA) phenomenon *sensu* Philippe (2000) and the relationship among these lineages remains unclear. Based on the currently combined evidence (morphology, ultrastructure, and molecular phylogeny), the recognition of more classes can be revealed, and this is discussed below using the class Ulvophyceae as an example.

Mattox and Stewart (1984) and O'Kelly and Floyd (1984) defined the Ulvophyceae primarily based on ultrastructural features of the flagellar apparatus (i.e., a CCW orientation of the basal body). This class was furthermore regarded as more "advanced" in view of the fact that their vegetative thallus is non-flagellated and presumably derived from scaly green flagellates. Five orders were recognized within the Ulvophyceae (Ulotrichales, Ulvales, Siphonocladales/Cladophorales, Dasycladales, and Caulerpales) based on modifications of the flagellar apparatus, differences in zoosporangial and gametangial structures, and life histories. This classification scheme has been largely adopted until today (Lewis and McCourt, 2004); however, molecular data (SSU rDNA and *rbc*L) have, so far, not been able to provide solid support for recognition of the monophyletic nature of the class Ulvophyceae *sensu* Mattox and Stewart (Zechman et al., 1990; Watanabe et al., 2001; López-Bautista and Chapman, 2003; see also Figure 7.4). Instead, molecular phylogenies reveal five separate, well-supported clades for which the mutual interrelationships remain ambiguous. These phylogenetic results would favour the recognition of five separate classes (Ulvophyceae *sensu stricto*, Cladophorophyceae, Bryopsidophyceae, Dasycladophyceae, and Trentepohliophyceae), as has been proposed by van den Hoek et al. (1995) based on apparent differences in thallus architecture, cellular organisation, chloroplast morphology, cell wall composition, and life histories. We refer to the following papers for molecular phylogenetic studies within each of these five lineages: Ulvophyceae (Hayden and Waaland, 2002; Friedl and O'Kelly, 2002; O'Kelly et al., 2004a, 2004b, 2004c), Cladophorophyceae (Bakker et al., 1994; Hanyuda et al., 2002; Leliaert et al., 2003), Bryopsidophyceae (Woolcott et al., 2000; Lam and Zechman, 2006), Dasycladophyceae (Olsen et al., 1994; Zechman, 2003), and Trentepohliophyceae (López-Bautista and Chapman, 2003).

Lewis and McCourt (2004) have dealt with these classification problems by only presenting a "working classification of green algae and land plants." To summarize the current state of classification, we present a phylogenetic tree of SSU rDNA sequences including representatives of all lineages available in GenBank (Figure 7.4). The clades in Figure 7.4, which form monophyletic lineages and are well-supported in bootstrap analyses, are named after a representative species or genus without ranking at higher levels. The different classification systems using morphological, ultrastructural, and molecular concepts are summarized with additional information in Table 7.1.

In the next section, we want to focus on classification at the generic and species level, because many questions need to be clarified before a new or revised classification at the higher level can be proposed. To give an example of a problematic situation, the order Ulotrichales (traditionally contains only unbranched filaments) is classified within the class Ulvophyceae based on the ultrastructural results and the molecular position of a single taxon of the genus *Ulothrix*, *U. zonata* (O'Kelly and Floyd, 1984; see also Lewis and McCourt, 2004). However, the situation is further "confused" as the type species of *Ulothrix*, *U. tenuissima* Kützing, has not been investigated employing ultrastructural and molecular methods. According to the ICBN, the name of an order must be combined and connected to the type genus and its type species.

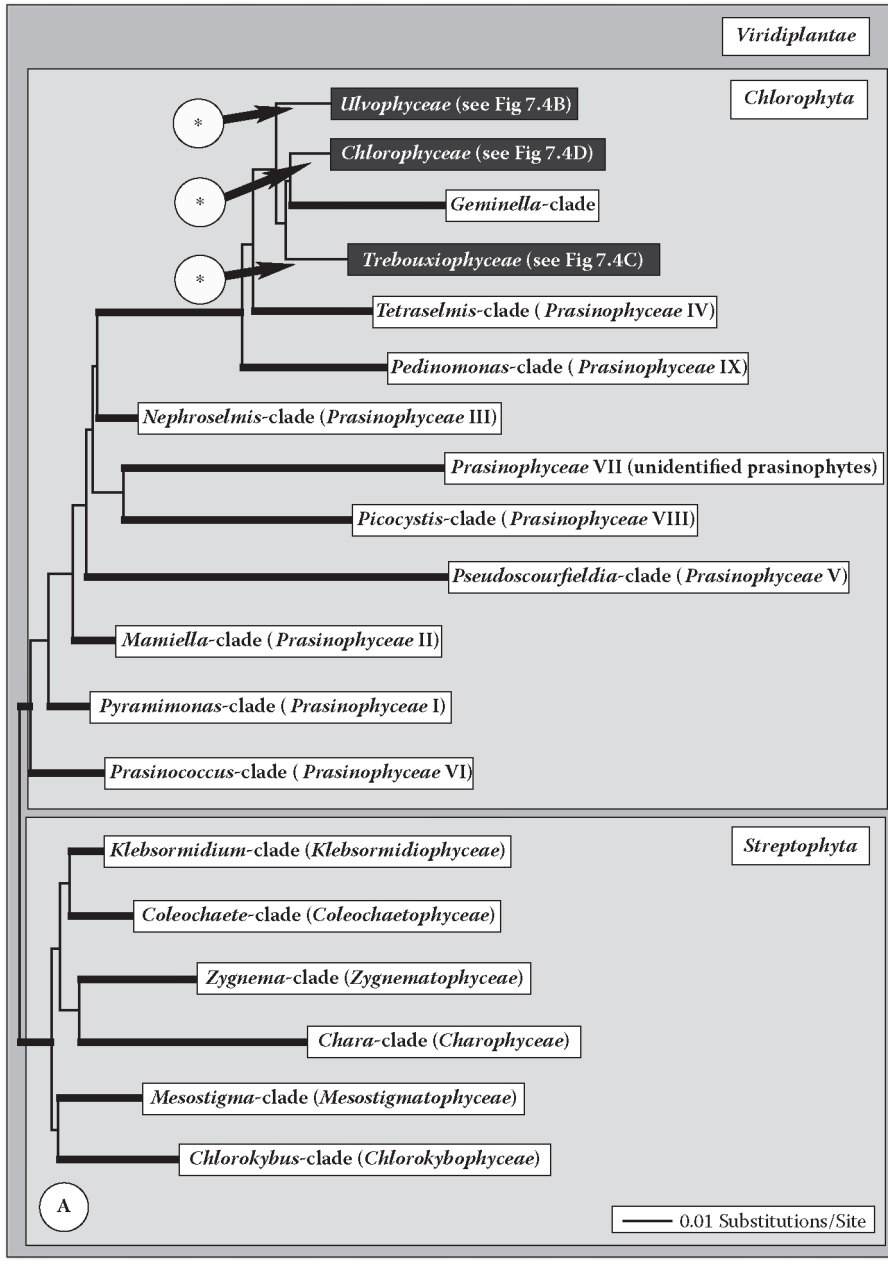

FIGURE 7.4 Molecular phylogeny of the Viridiplantae based on SSU rDNA sequence comparisons. The phylogenetic tree shown was inferred by the neighbour-joining method based on distances of 1668 aligned positions of 469 taxa using PAUP 4.0b10 (Swofford, 2002). Subsections of this dataset (parts A through D) were analyzed using the best model (TrN+I+G; Tamura and Nei, 1993) calculated by Modeltest 3.7 (Posada and Buckley, 2004; Posada and Crandall, 1998). Bootstrap percentage values (>70%) are marked by bold branches given for neighbour-joining (using TrN+I+G model; 1000 replicates). The monophyletic clades are provisionally named after a representative taxon. (A) Phylogenetic tree of the Chlorophyta and Streptophyta (67 taxa). The three classes Ulvophyceae, Chlorophyceae, and Trebouxiophyceae are represented by few taxa in this analysis (best model: TrN+I+G; I = 0.49, G = 0.58). The clades containing species of the three subsections (common branch marked by an asterisk) were not supported in bootstrap analyses; however, they were separately analyzed in parts B through D and marked in black.

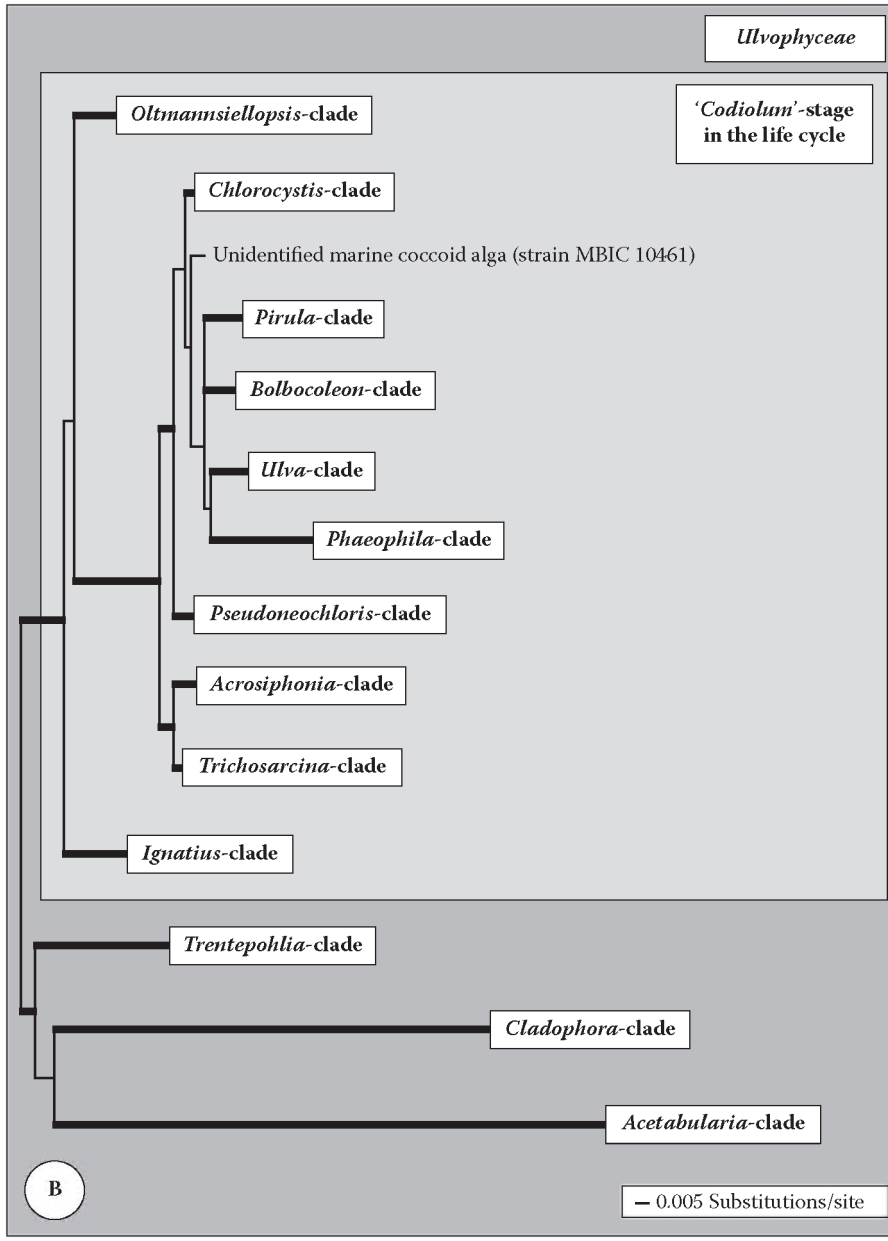

FIGURE 7.4 (CONTINUED) (B) Phylogenetic tree of the Ulvophyceae (142 taxa; best model: TrN+I+G; I = 0.35, G = 0.58).

CLASSIC VERSUS MODERN APPROACHES: PROBLEMS WITH IDENTIFICATION OF SPECIES AND GENERA

At the generic and species levels, algae (especially microalgae) are traditionally classified according to morphological and cytological characters of vegetative stages in their life cycle. Phylogenetic analyses of ribosomal genes (SSU and ITS rDNA sequences) have demonstrated that this morphological concept is artificial for most of the algal genera and needs to be revised. For example, within the Volvocales (monadoid unicells and colonies) 60 genera with 2000 species have been described on the basis of phenotypic characters. The largest genus *Chlamydomonas* contains 800 species,

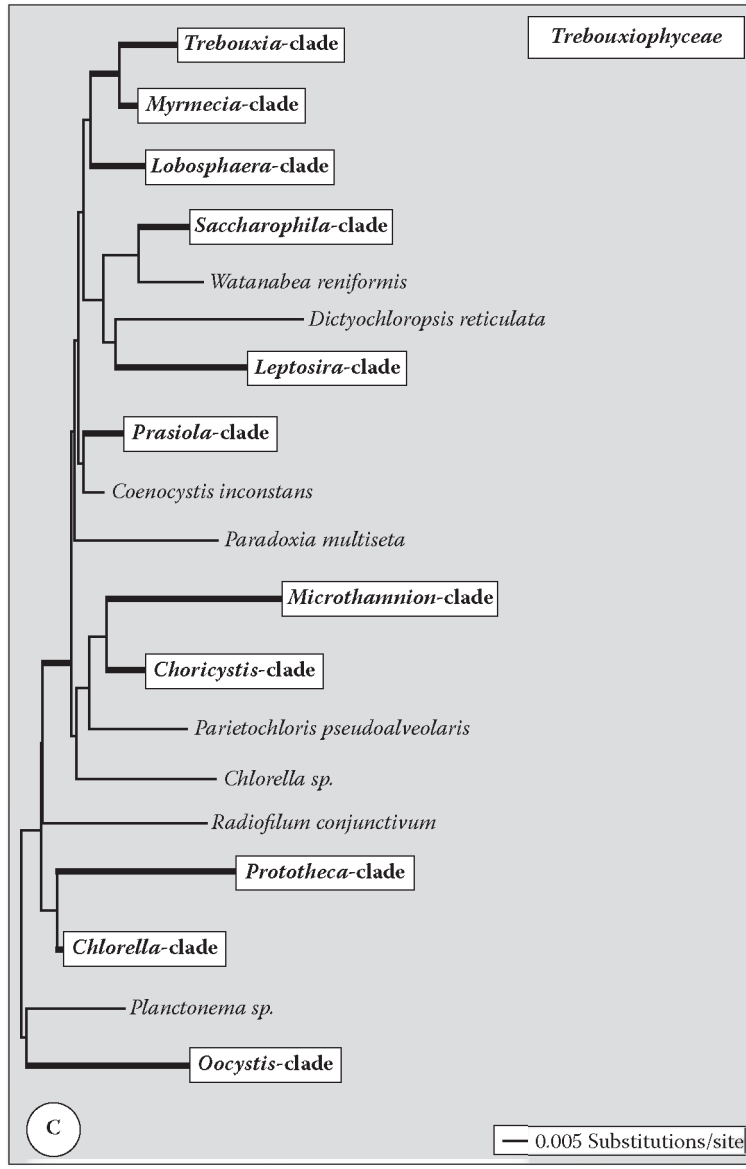

FIGURE 7.4 (CONTINUED) (C) Phylogenetic tree of the Trebouxiophyceae (77 taxa; best model: TrN+I+G; I = 0.52, G = 0.61).

which are characterized by different cell sizes and shapes, different chloroplast shapes, the number and position of pyrenoids within the chloroplast, and the position of eyespot and nucleus within the cell. However, according to phylogenetic analyses, species of the unicellular genus *Chlamydomonas* form eight monophyletic lineages within the Chlorophyceae. Strains belonging to other genera are in the same lineage as some of these "*Chlamydomonas*" (Pröschold et al., 2001; Figure 7.5).

Coleman (2000) introduced new generic and species concepts (the Z-clade and CBC-clade concepts) based on compensatory base changes (CBC) in ITS sequences ("genetic signatures" or even "DNA barcode") compared with the mating ability of species. In contrast to the biological species concept (*sensu* Mayr, 1948), strains of the same Z(ygote)-clade can at least form zygotes, though they may or may not germinate and produce fertile F1 generations. A Z-clade contains one,

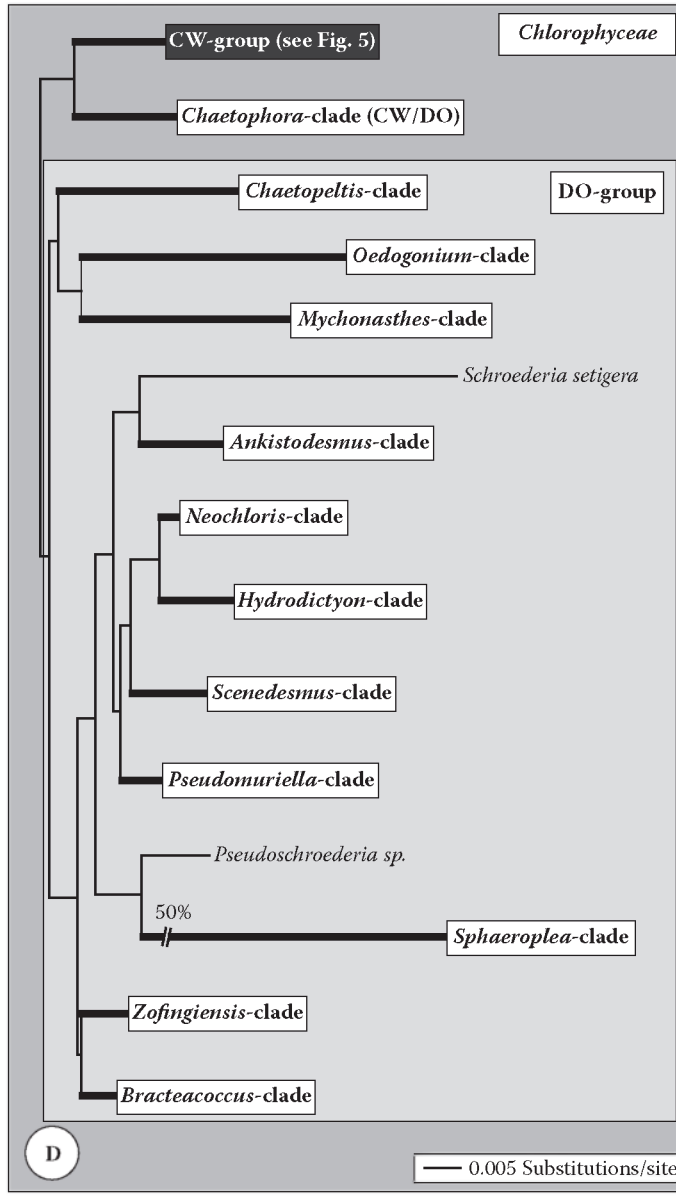

FIGURE 7.4 (CONTINUED) (D) Phylogenetic tree of DO-group of the Chlorophyceae (80 taxa). The CW-group of the Chlorophyceae is represented by few taxa in this analysis (best model: TrN+I+G; I = 0.57, G = 0.67).

or a very few, biological species, each with genetically very similar members sharing identical ITS rDNA sequences (certainly no CBCs). Therefore, ITS sequences have proven to be very good tools for evolutionary comparisons, especially at the biological species level (Coleman, 2003). For example in *Chlamydomonas allensworthii* Starr, Marner et Jaenicke, two CBC clades (which correlate with their pheromone production) and five Z-clades could be recognized (Coleman et al., 2001; Figure 7.6). This demonstrated that in the "morpho-species" *Chlamydomonas allensworthii* at least five biological species are present. These species are not correlated to their geographical origins. Furthermore, morphological differences are not observed in the strains investigated and only two different pheromones are found: lurlenol and lurlenic acid (Jaenicke and Starr, 1996),

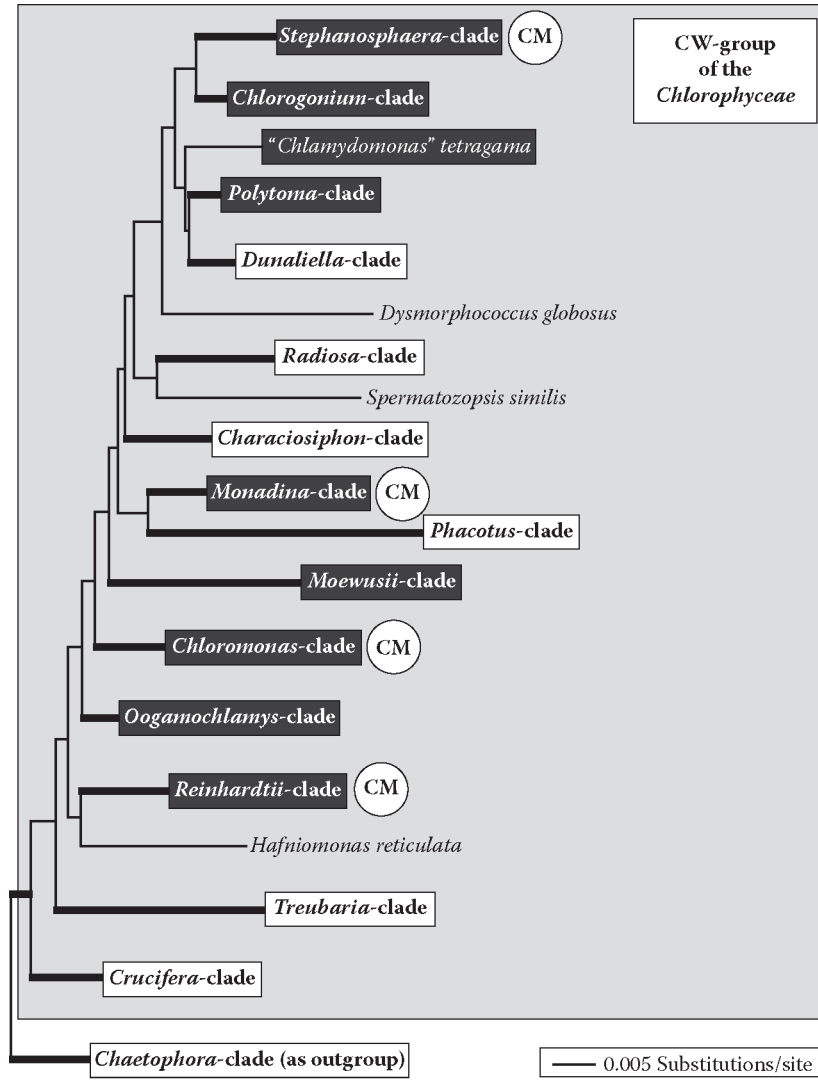

FIGURE 7.5 Molecular phylogeny of the CW-group (131 taxa) based on SSU rDNA sequence comparisons using the *Chaetophora*-clade *sensu* Pröschold et al. (2001) as outgroup. The phylogenetic tree shown was inferred by the neighbour-joining method based on distances of 1668 aligned positions calculated by the model of Tamura and Nei (Tamura and Nei, 1993; TrN+I+G; I = 0.51, G = 0.57), which was calculated as best model by Modeltest 3.7 (Posada and Buckley, 2004; Posada and Crandall, 1998). Bootstrap percentage values (>70%) are marked by bold branches given for neighbour-joining (using TrN+I+G model; 1000 replicates). The monophyletic clades are provisionally named after a representative taxon. The clades containing species of *Chlamydomonas* and *Chloromonas* (CM) are marked in black.

which also correlate with the phylogenetic analyses presented in Figure 7.6. For analysis of compensatory base changes, the secondary structures of SSU and ITS rDNA sequences have to be determined by folding, which requires accuracy of sequencing. However, this is not always available, particularly in the sequences published in GenBank prior to 1997–1998 and the advent of automatic sequencing. Therefore, for this type of analysis we recommend resequencing where more than two ambiguities or mismatches are found.

As demonstrated above (see also examples presented in Lewis and McCourt, 2004), most of the genera in the Chlorophyceae (and in the Chlorophyta in general) are polyphyletic and a revision

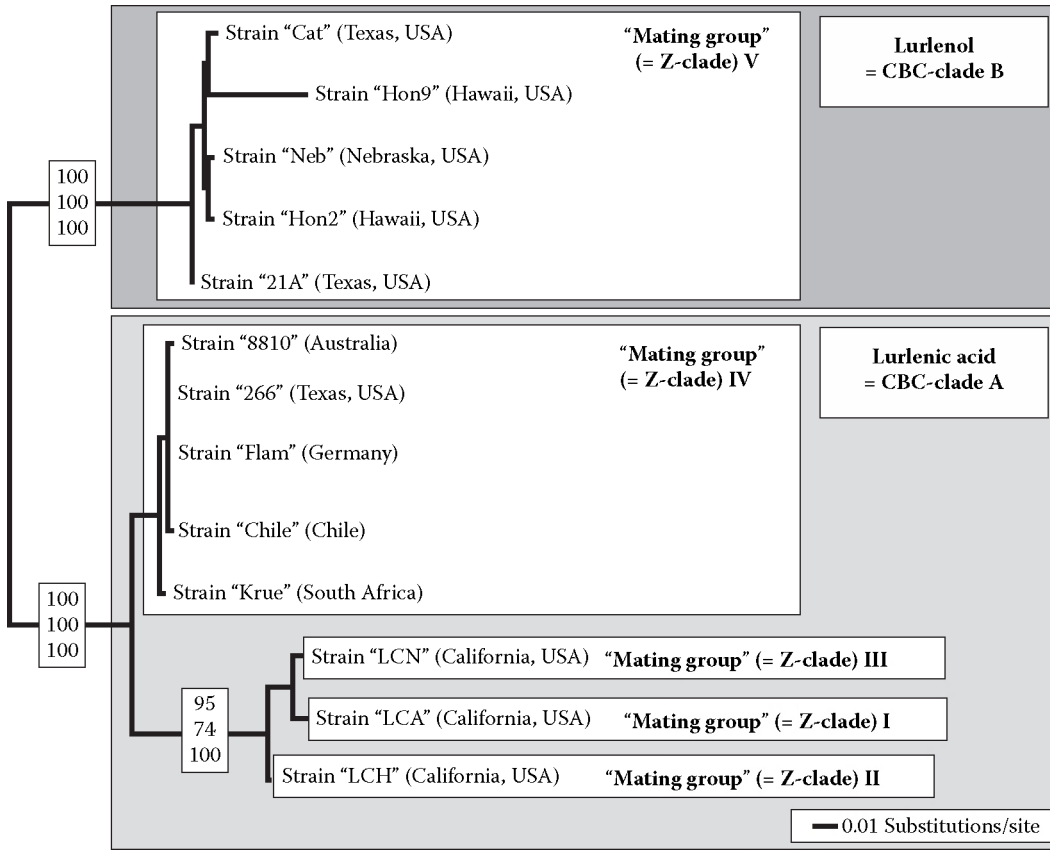

FIGURE 7.6 Molecular phylogeny of *Chlamydomonas allensworthii* based on ITS rDNA sequence comparisons. The phylogenetic tree shown was inferred by maximum likelihood method using K80+G model (Ti/Tv = 1.65; G = 0.007; Kimura, 1980) calculated as best model by Modeltest 3.7 (Posada and Buckley, 2004; Posada and Crandall, 1998). Bootstrap percentage values (>50%) are given for maximum likelihood (using K80+G; bold italic), neighbour-joining (using K80+G; bold), and unweighted maximum parsimony (not bold). Two CBC clades recognized are marked gray and correspond to the pheromone production (lurlenol and lurlenic acid). The Z-clades are highlighted.

needs to be performed that follows the regulations of the ICBN. According to the ICBN, each genus validly described has a type species, which has a type specimen deposited in a public herbarium (for macroalgae), or a type figure in a publication (for microalgae). Following these rules, a genus name can only remain for a group (clade, monophyletic lineage), which contains the type species. However, for many type species of genera (especially for microalgae), no "type material" (mostly only figures) is available as reference, thus no DNA could be extracted for phylogenetic comparisons. To solve this problem, an epitype (a new herbarium specimen for macroalgae or a cryopreserved culture for microalgae) or a neotype, when the type specimen is lost or destroyed, can be designated and deposited in a public herbarium or culture collection. For example in the genus *Chlamydomonas*, Pröschold and Silva (2007) have proposed *C. reinhardtii* P.A. Dangeard as conserved type of the genus and designated the culture strain SAG 11-32b (= CCAP 11/32A, = UTEX 90) cryopreserved as epitype, to emend an ancient crude drawing. In microalgae in particular, this procedure needs to be performed for all genera in order to link traditional and modern approaches, which are dependent on reference material that is available for the science community. This material (cultures, extracted DNA) can be provided by culture

collections; however, the reference material is needed in a genetically stable condition and in a metabolic inactive state (only this is accepted by ICBN).

For microalgae, cryopreservation is the long-term solution, provided in major culture collections (Day and Brand, 2005). The long-term stability of reference material is essential for all research fields in biology; later comparisons can utilize different molecular techniques, e.g. amplified fragment length polymorphism (AFLP) as demonstrated for *Chlorella vulgaris* Beijerinck (see details in Müller et al., 2005).

TAXONOMIC REVISION OF GENERA AND SPECIES USING POLYPHASIC APPROACHES

Traditional taxonomic approaches often depend on single or even negative "characters" (e.g. absence of zoospore formation). For example, in unicellular flagellates, the genus *Chloromonas* is separated, by the absence of pyrenoids in the chloroplast, from the genus *Chlamydomonas*. Phylogenetic analyses have demonstrated that some strains of *Chloromonas* and *Chlamydomonas* can belong to the same clade and, in the case of *Chloromonas reticulata* (Goroschankin) Wille, to the same species (containing strains with or without pyrenoids; see details in Pröschold et al., 2001). Nozaki et al. (1998) have demonstrated for *Chlorogonium*, another unicellular flagellate, that the presence or absence of pyrenoids is dependent on culture conditions (autotrophic: in light in mineral medium, or heterotrophic: in dark in medium with organic compounds like yeast extract), where the algae grow. An additional example includes the species *Chlorella vulgaris* and *Micractinium pusillum* Fresenius, which are closely related according to phylogenetic analyses (Krienitz et al., 2004; Luo et al., 2005, 2006). Cultured under axenic conditions in defined culture medium, both showed smooth-walled unicells by light microscopy. However, if a grazer is added to the algal culture, in this case the rotifer *Brachionus calciflorus* Pallas, strains of *Micractinium pusillum* form colonies and cell wall spines, which are typical for this genus (Luo et al., 2005, 2006). In contrast, no phenotypic differences are observed in cultures of *Chlorella vulgaris* under the influence of a grazer.

For the green seaweeds *Ulva*, *Enteromorpha*, and *Monostroma*, a similar morphological polymorphism is known. If *Ulva lactuca* Linnaeus is grown under axenic condition, the thalli lose the ability to produce the natural foliose morphology. Adding marine bacteria isolated from *Ulva* thalli to the axenic cultures causes the algae to grow an *Enteromorpha*-like thallus (Bonneau, 1977; Provasoli and Pintner, 1980). The influence of marine bacteria on morphogenesis has also been reported for *Ulva pertusa* Kjellman (Nakanishi et al., 1996), *Enteromorpha linza* (L.) J. Agardh, and *E. intestinalis* (L.) Nees (Fries, 1975). The phylogenetic analyses of ITS rDNA sequences have confirmed that *Ulva* and *Enteromorpha* are not distinct genera, and therefore, *Enteromorpha* was included into the genus *Ulva* (Tan et al., 1999, Hayden et al., 2003). The associated bacteria isolated from *Monostroma oxyspermum* Kützing also have a morphogenetic influence on axenic cultures of this alga as well as on *Ulva pertusa* and *U. intestinalis* (= *Enteromorpha intestinalis*). The bacteria were characterized by phylogenetic analyses as *Cytophaga-Flavobacterium-Bacteroides* (CFB) complex (Matsuo et al., 2003), and a morphogenetic inducer (Thallusin) could be isolated from these bacteria, which revealed its importance for the natural growth of the seaweeds tested (Matsuo et al., 2005). The extreme phenotypic plasticity of siphonocladalean macroalgae (e.g. *Anadyomene*, *Ernodesmis*, *Microdictyon*, *Siphonocladus*, and *Struvea*) can also be demonstrated by growing isolates under different culture conditions. Certain morphological characters, including some that are traditionally used for generic delineation, are found to be variable and dependent on environmental conditions like temperature and light regimes (e.g. the formation of blades in *Anadyomene* and *Microdictyon*). In view of this morphological variability in many green algae, we would like to stress the importance of field studies and comparative studies between material collected in the field and material grown in culture. In several green algae, detailed field studies, including the investigation of ecology and phenology, have been of significant assistence to the clarification of the taxonomic identity of several taxa (see for example López-Bautista et al., 2006, for the order Trentepohliales).

The major work of a taxonomic revision of genera and species regards consideration of morphological plasticity under different culture and environmental conditions. It needs also a wider context. We propose here the usage of a polyphasic approach (plasticity of phenotype and different life stages under different conditions, biochemical and physiological approaches, phylogenetic concepts, comparison of species concepts, multi-gene approach), which we demonstrate for the following examples presented below.

Polyphasic approaches used for characterization of the genera *Oogamochlamys* and *Lobochlamys*

A group of "former" *Chlamydomonas* species were transferred to these two newly described genera by Pröschold et al. (2001), on the basis of phylogenetic analyses of SSU rDNA sequences. Both genera belong to the *Oogamochlamys*-clade (well-supported monophyletic lineage shown in Figure 7.5), and can be clearly identified by using multiple approaches, which are not causally connected. The genus *Oogamochlamys* is characterized by chloroplast morphology (parietal, massive plastids with ridges on the surface), multiple pyrenoids irregularly distributed, and homothallic protandric oogamy; the genus *Lobochlamys* has a cup-shaped chloroplast with incisions, cell wall with mucilage layer around the flagellated cells, and homo- or heterothallic isogamous sexual reproduction (Pröschold et al., 2001). The two genera, each have several unique compensatory base changes (CBC) in the secondary structure of SSU rRNA (e.g. in Helix E23_1 and E23_2 demonstrated here in Figure 7.7). Using the Z-clade concept *sensu* Coleman (2000), three species can be identified in *Oogamochlamys*: *O. gigantea* (Dill) Pröschold, Marin, Schlösser et Melkonian; *O. zimbabwiensis* (Heimke et Starr) Pröschold, Marin, Schlösser et Melkonian; and *O. ettlii* (Dill) Pröschold, Marin, Schlösser et Melkonian—furthermore, these can be characterized by morphology (Figure 7.8). The species of *Lobochlamys*, *L. segnis* (Ettl) Pröschold, Marin, Schlösser et Melkonian and *L. culleus* (Ettl) Pröschold, Marin, Schlösser et Melkonian, are characterized by different sporangia wall lytic enzymes (Figure 7.9).

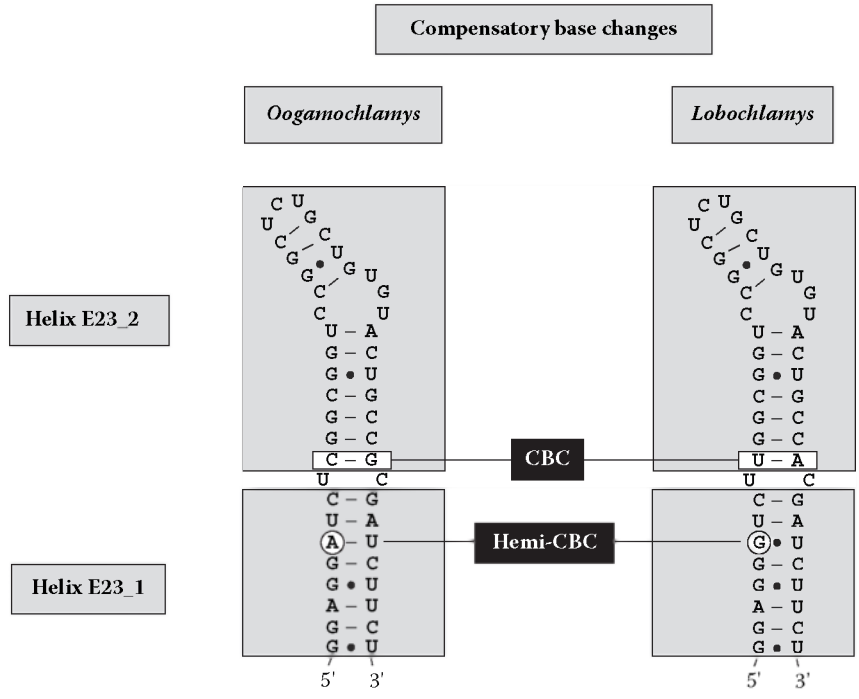

FIGURE 7.7 Comparison of Helices E23_1 and E23_2 of the SSU rRNA secondary structure between *Oogamochlamys* and *Lobochlamys*. Compensatory bases changes (Hemi-CBC and CBC) are highlighted.

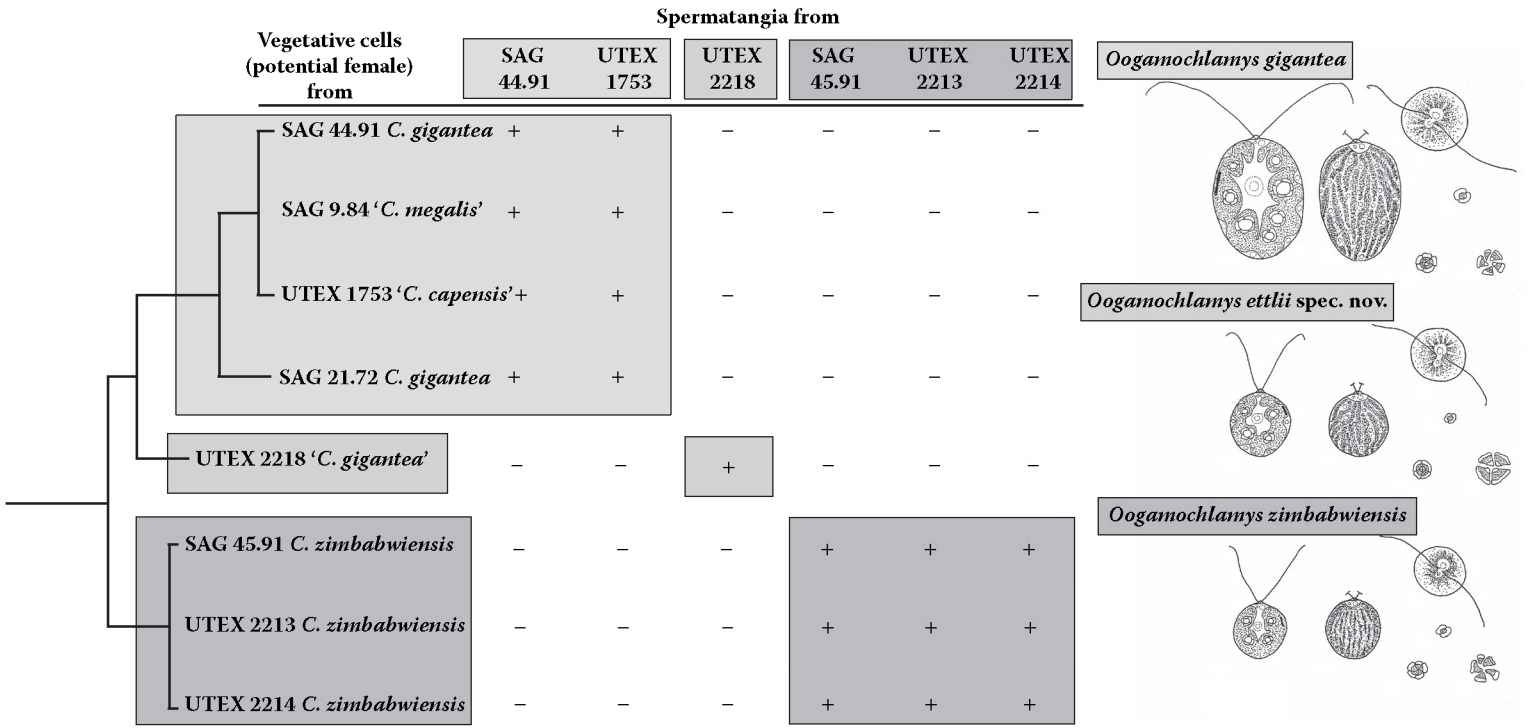

FIGURE 7.8 Molecular phylogeny of *Oogamochlamys* based on SSU rDNA sequence comparisons. The phylogenetic tree shown was inferred by the maximum-likelihood method using the TrNef+I+G (equal base frequencies, I = 0.72, G = 0.63) calculated as best model. The results of crossing experiments are marked in boxes for *O. gigantea*, *O. ettlii*, and *O. zimbabwiensis* (+ = zygote formation; – = no zygote formation). The strains named in the tree are the original designation in the culture collection. The morphological re-investigation of these strains is shown on the right.

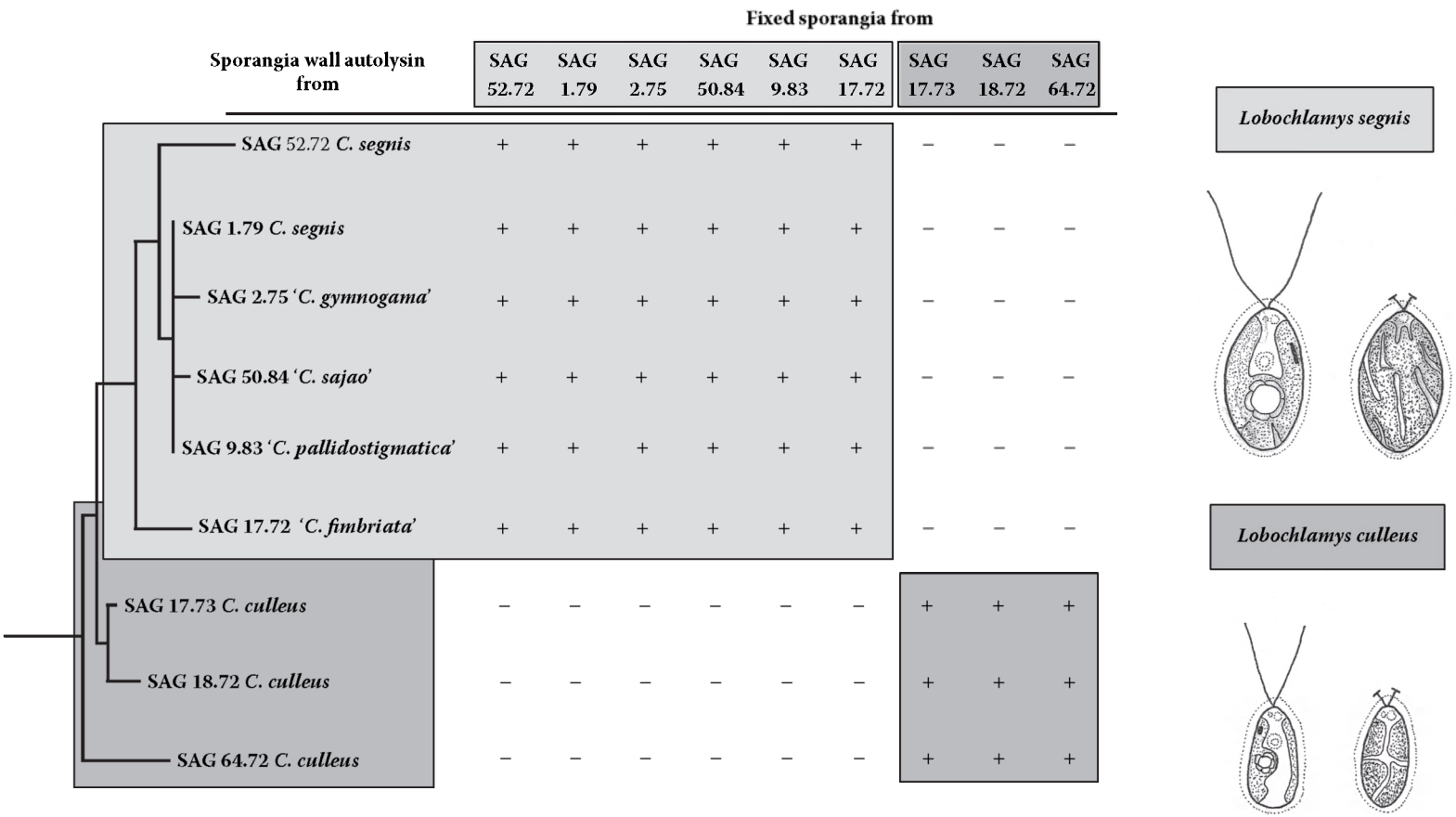

FIGURE 7.9 Molecular phylogeny of *Lobochlamys* based on SSU rDNA sequence comparisons. The phylogenetic tree shown was inferred by the maximum-likelihood method using the TrNef+I+G (equal base frequencies, I = 0.72, G = 0.63) calculated as best model. The results of biological tests using the sporangium autolysin on fixed sporangia are marked in light gray for *L. segnis* and in dark gray for *L. culleus* (+ = release of zoospores; − = no release of zoospores) according to the VLE groups 9 (*L. segnis*) and 10 (*L. culleus*; Schlösser, 1976, 1984). The strains named in the tree are the original designation in the culture collection. The morphological re-investigation of these strains shown on the right.

Schlösser (1976, 1984) determined that these enzymes (called sporangium autolysin, or vegetative lytic enzymes [VLEs]) are produced by zoospores for release from the sporangium cell wall, are stage and species specific, and can be used for classification ("autolysin concept"). Fifteen VLE groups were distinguished among 65 strains of different *Chlamydomonas* species examined (Schlösser, 1976, 1984), from which VLE group 9 correspond to *L. segnis* and 10 to *L. culleus*.

Delimiting phylogenetic species by a multi-gene approach in *Micromonas* and *Halimeda*

Different empirical methods for delimiting species have been described recently (Sites and Marshall, 2003). In one of these methodologies, termed the "exclusivity criterion," phylogenetic species are delimited, using genealogical concordance of multiple independent loci (Dettman et al., 2003). With this method, species are delimited based on two requirements: species are exclusive groups (those in which all members are more closely related to each other than to any organism outside of the group) and species reside at the boundary between reticulate and divergent genealogy, where unlinked genes should have concordant genealogical histories (Sites and Marshall, 2003). Within single interbreeding species (or in case of hybridisation between lineages), the mixing effects of recombination between genes would cause unlinked loci to have different genealogies, but between genetically isolated species, the extinction of ancestral alleles by genetic drift would lead to the congruence of genealogies. Hence, the transition between deep genealogical concordance and shallow genealogical discordance can be used to recognize phylogenetic species (Taylor et al., 2000). A recent paper by Slapeta et al. (2006) serves as one of the few examples in which multi-gene phylogenies are employed to delimit species in the green algae. In this study, phylogenetic analyses of four independent nuclear, plastid, and mitochondrial loci (rDNA, ß-tubulin, *rbc*L, *cox* 1) has led to the recognition of numerous cryptic species in the marine prasinophyte, *Micromonas pusilla* (Butcher) Manton et Parke. Multiple semi-cryptic species were also revealed in the tropical seaweed *Halimeda* based on nuclear ribosomal and plastid DNA sequences (Verbruggen et al., 2005a, 2005b). In this study the hypothesis was formulated that reticulate evolution could have been involved in speciation within *Halimeda*, based on discordances between phylogenetic trees inferred from nuclear and plastid DNA sequences. Both studies confirm the fact that the morphospecies concept is untenable because it overlooks a large genetic and species diversity, both in green macro- and microalgae, even in the smallest eukaryotes.

These examples have clearly demonstrated that the usage of polyphasic approaches could be the answer to systematic and taxonomic questions, at least at the generic and species levels. Not all of the different methods and concepts described here for taxonomic revisions are suitable for all groups of green algae, and others need to be developed (ecological, physiological, and biochemical data). In addition, descriptions of habitat and origin especially for seaweeds should also be included. However, many of these methods examined only for a particular group can be used for other groups as well. For example, the specificity of autolysins presented here for *Chlamydomonas* and its relatives is also found in filamenteous green algae. Schlösser (1987) distinguished fragmentation autolysins in *Uronema confervicola* Lagerheim, *Radiofilum transversale* (de Brébisson) Christensen, and in an unidentified species of *Geminella*. The autolysin of *Uronema confervicola* react in bioassays on other strains of *Uronema* and strains of other genera including *Chaetophora*, *Draparnaldia*, *Fritschiella*, and *Stigeoclonium* (all branched filaments), which all belong to the *Chaetophora*-clade according to the phylogenetic analyses presented in Figure 7.4. However, no fragmentation could be observed when *Uronema* autolysin was applied to strains of the genera *Ulothrix*, *Geminella*, *Klebsormidium*, *Trentepohlia*, and others. This is particularly interesting as phylogenetic analyses positioned these taxa in other clades (see Figure 7.4).

Differentiation of genera and species based on single characters (e.g. morphology of the vegetative cell alone) is often not possible and inevitably leads to ambiguous classifications. This was previously

recognized by several authors prior to the availability of ultrastructural and molecular phylogenetic data for characterization. For example, Kornmann and Sahling (1983) studied the life cycle of *Chlorocystis cohnii* (Wright) Reinhard (Figure 7.10) and *Halochlorococcum marinum* P. Dangeard, two marine coccoid green algae, and found a *Codiolum* stage as a zygote in sexual reproduction, which is characteristic for filamenteous and parenchymatous marine green algae (e.g. *Acrosiphonia*, *Urospora*, or *Ulva*). They concluded that both taxa are closely related to these algae and described a new order Chlorocystidales in the Codiolophyceae (= Ulvophyceae *sensu stricto*). However, Komárek and Fott (1983) integrated both species as marine representatives of the freshwater genera *Chlorochytrium* and *Spongiochloris* based on the morphology of their vegetative cells. *Chlorocystis* and *Chlorochytrium* have a cup-shaped plastid, *Halochloroccum* and *Spongiochloris* have a reticulated chloroplast, and otherwise the cell morphology of all four is very similar. Phylogenetic analyses confirmed the placement of *Chlorocystis* and *Halochlorococcum* in the Ulvophyceae as proposed by Kornmann and Sahling (Pröschold et al., unpublished data). In contrast, *Chlorochytrium* and *Spongiochloris* on the basis of ultrastructural and molecular data belong to the Chlorophyceae (Watanabe and Floyd, 1994, Lewis, 1997; *Stephanosphaera*-clade in Figure 7.5).

CONCLUSIONS

Biodiversity of green algae based on taxonomic revision using polyphasic approaches

As demonstrated here, polyphasic approaches can distinguish and delimit species and genera, which lead on one hand to a reduction of described species (e.g. Figure 7.9) and on the other hand to more biological species, which are morphologically identical (e.g. Figure 7.6). For example, the 800 described species of *Chlamydomonas* can be reduced to approximately 100 to 150 species using these approaches (Pröschold, unpublished data). However, Fawley et al. (2004) have shown that the biodiversity of green microalgae is much higher than expected. They isolated 273 strains with 93 distinct SSU rDNA sequences from four different sites in North Dakota and Minnesota (USA). Only four of these matched with any sequences published in GenBank. It is widely accepted that microbial diversity differs fundamentally from biodiversity of larger animals and plants. Furthermore, it has been suggested that free-living microbes have a cosmopolitan distribution (Fenchel and Finlay, 2003) and that most protistan organisms, smaller than 1 mm in size, have worldwide distribution wherever their required habitats are realised. This is a consequence of ubiquitous dispersal driven by huge population sizes, and consequently low probability of local extinction. Organisms larger than 10 mm are much less abundant, and rarely cosmopolitan (Finlay, 2002; Finlay and Fenchel, 2004). However, these hypotheses are only based on the phenotypic approach (morpho-species) and are dependent on clear identification of the microorganisms. For microalgae the hypotheses are still under discussion (Finlay and Fenchel, 2002; Coleman, 2002) and are not yet proven. Polyphasic approaches as suggested here have indicated that the biodiversity of microalgae is much higher than the described diversity according to the morpho-species concept. For example, the morpho-species *Gonium pectorale*, a member of the colonial Volvocales, contains at least five independent interbreeding biological species (Fabry et al., 1999) with little correlation to their origin. It seems that sex and the type of interbreeding (heterothally: self-sterile, need two gametes originating from different clones, or homothally: self-fertile, gametes originating from a single clone) need to be considered in the context of biogeographical distribution. Similarly in *Cladophora* (one of the largest green macro-algal genera), the number of recognized morpho-species has been reduced from over 1000 to about 100 based on comparative morphological studies performed on field, culture, and herbarium material (e.g. van den Hoek, 1963, 1982). More recently, molecular studies have revealed that some of these morphological species (e.g. *C. vagabunda* (L.) van den Hoek, which occupies one huge continuous geographic area) represent different divergent lineages that can be related to thermal ecotypes (Bakker et al., 1995; Breeman et al., 2002).

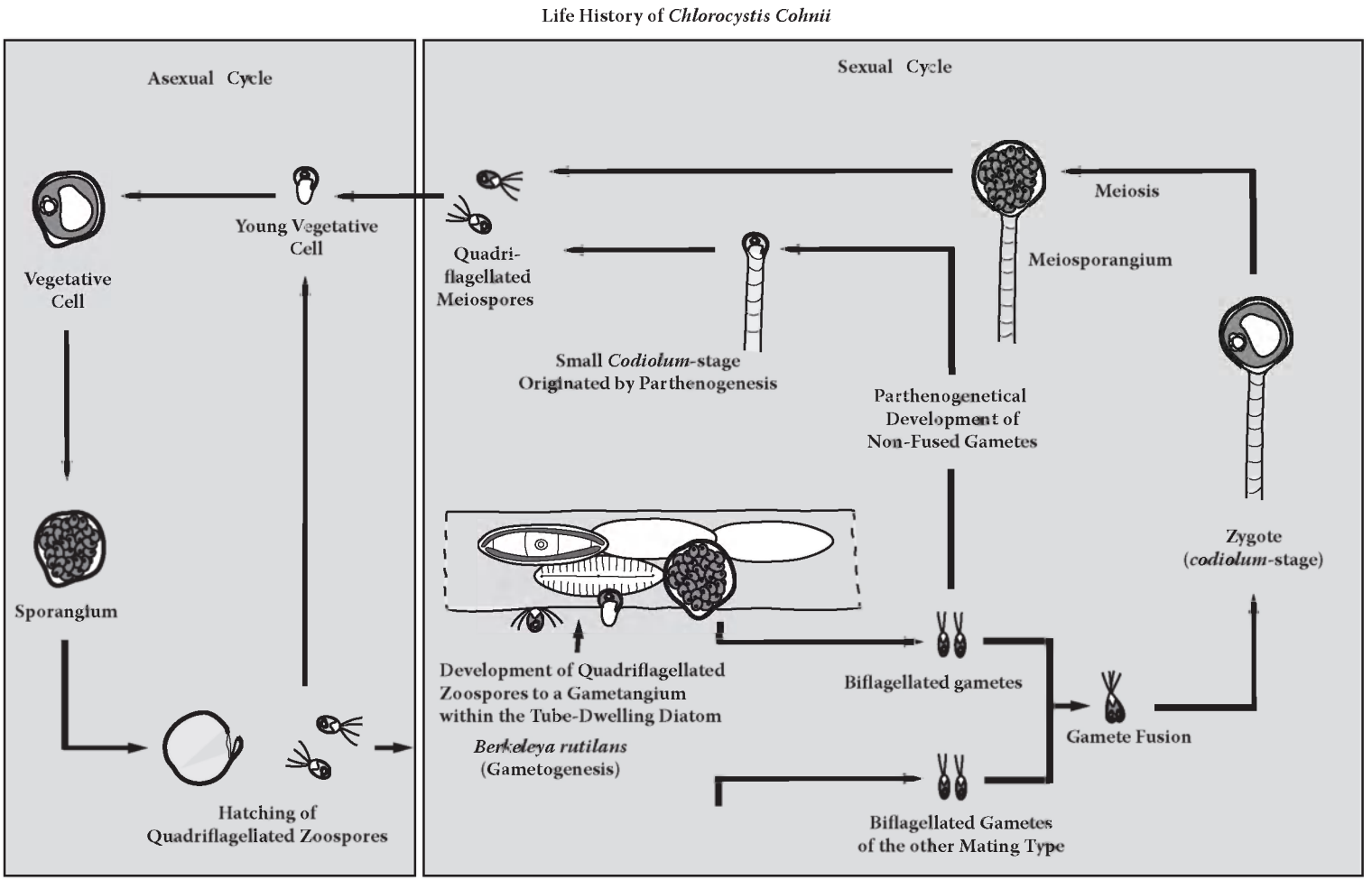

FIGURE 7.10 Life cycle of the marine coccoid green alga *Chlorocystis cohnii* (Ulvophyceae). (Redrawn after Kornmann, P. and Sahling, P.-H., *Helgoländer Meeresuntersuchungen*, 36: 1–65, 1983. With permission.)

Depending on the interpretation of these molecular and temperature tolerance data, *C. vagabunda* can be regarded as a single species, or alternatively a cryptic species complex (multiple species). Similar results were found by van der Strate et al. (2002), who demonstrated, based on ITS sequence divergence, differential microsatellite amplification and thermal ecotypes, that the Atlantic taxon *Cladophoropsis membranacea* (Hofman Bang ex C. Agardh) Børgesen consists of at least three cryptic species that have overlapping biogeographies.

Reconstructing the green algal tree by multi-gene and whole-genome analysis

Although the challenges associated with reconstruction of deep relationships are fundamentally different from those of shallow ones, multi-gene approaches are fast becoming the norm in both types of studies. In shallow reconstruction problems, where the complex effects of reticulation and lineage sorting are persistently present, gene genealogies of multiple independent, fast-evolving loci are analyzed to answer questions related to species concepts, speciation events, hybridisation, and gene flow in or between species (see, for example, Slapeta et al., 2006). In deep phylogenies, lineages are widely separated in time, resulting in few problems with reticulation and lineage sorting. Yet, deep phylogenetic reconstructions suffer from a number of other persistent problems. One of these is the well-known problem of long-branch attraction that can be caused by a variety of factors, including the fact that the set of lineages is relatively incomplete due to extinction events. A related problem to long-branch attraction is substitution saturation, which particularly hampers phylogenetic reconstruction of deep branches.

Phylogenetic relationships within the green algae have been based primarily on sequence analyses of the nuclear-encoded SSU rDNA. Based on this single marker, phylogenetic relationships among the major groups of green algae have been found particularly difficult to resolve, because the internal branches, grouping the different orders and classes, are generally very short relative to the subsequent evolutionary history of the group (with the exception of a few internal branches that have undergone considerable rate acceleration of the SSU rDNA, such as the ones leading to the Bryopsidales, Cladophorales, and Dasycladales). These short internal, often weakly supported branches can be attributed to a number of factors including conflict between characters due to homoplasy within a sequence, insufficient sequence length, rate variation across characters or taxa, the presence of taxa with unstable positions that may reduce support levels in the tree as a whole (Sanderson and Shaffer, 2002), or an historical signal of a rapid evolutionary radiation (Wortley et al., 2005). The factors most open to investigation in the challenge of the green algal tree reconstruction are sequence length and taxon sampling. The relative contribution of taxon number and gene number to accuracy in phylogenetic inference is still a major issue in phylogenetics and has been widely discussed (Graybeal, 1998). The general consensus today is that increasing taxon number correlates with a slight decrease in phylogenetic accuracy, while increasing sequence length or gene number has a significant positive effect on phylogenetic accuracy (Rokas and Caroll, 2005). Indeed a number of recent studies have shown that large-scale molecular sequencing projects and subsequent concatenation of multiple markers should be able to resolve the deep and problematic phylogeny of ancient eukaryotic groups, including the green algae (see for example Simpson et al., 2006).

The challenge of constructing the green algal tree by employing multi-gene or whole-genome molecular analysis techniques is still in its infancy. At present these phylogenies have been based primarily on concatenated gene sequences from organellar genomes from a relative small number of taxa (see Pombert et al., 2004, 2005, 2006, including references). Organellar genomes are particularly useful for phylogenomic reconstruction because of their relatively high gene content, condensed in comparison to nuclear genomes. Also, organellar genes are typically single-copy, in contrast to many nuclear genes that are multi-copy in nature, often having a confounding effect on phylogenetic reconstruction.

Pombert et al. (2005, 2006) analyzed the concatenated amino acid and nucleotide sequences derived from 58 protein-coding genes that are shared among the chloroplast DNAs of *Pseudendoclonium*,

Oltmannsiellopsis, *Chaetosphaeridium*, *Chlamydomonas*, *Chlorella*, *Mesostigma*, *Marchantia*, *Nephroselmis*, and *Nicotina*. As expected, their results confirmed the long-standing view in which the chlorophytes and streptophytes form two distinct lineages. In addition this whole-genome analysis supported the hypothesis that the Ulvophyceae is "sister" to the Trebouxiophyceae but could not eliminate the hypothesis that the Ulvophyceae is "sister" to the Chlorophyceae. The latter hypothesis was also supported by phylogenetic analysis of gene order data and by independent structural evidence based on shared gene losses and rearrangement break points within ancestrally conserved gene clusters. Phylogenetic analyses of seven concatenated mtDNA-encoded protein sequences also revealed a close relationship between the ulvophyte *Pseudendoclonium* and chlorophycean taxa, with the trebouxiophyte *Prototheca* occupying a basal position (Pombert et al., 2004). The difficulty in unequivocally resolving the basal divergences within the green algae, even with large numbers of concatenated genes, might be attributed to a real rapid, early radiation of the green algae, rather than to data availability. An additional potential cause is that various protein-encoded genes might have lost their phylogenetic information due to substitution saturation, plaguing the phylogenetic analysis involving deep branches in the green algal tree. The chloroplast and mitochondrial genome sequences of additional green algae will be required to provide unambiguous support for the ancient divergences within the green algae (Pombert et al., 2005). Currently the "Organelle Genome Megasequencing Program" (http://megasun.bch.umontreal.ca/ogmp/) and a research project funded through the "Assembling of the Tree of Life program" (http://ucjeps.berkeley.edu/TreeofLife/) are involved in sequencing and analysing a large number of organellar genomes, including those of various members of charophytes, trebouxiophytes, ulvophytes, and chlorophycean green algae (Monique Turmel, Université de Laval, Canada, personal communication).

OUTLOOK

How should we approach taxonomic revision using polyphasic approaches?

The key to answering this question is to have reference material for comparisons. In many cases this material can be provided by major culture collections, where strains should be deposited. This sounds like a relatively trivial point, but in many publications in the scientific literature no strain numbers are given and only species names have been designated. As indicated in this review, most of the genera and species are polyphyletic and without strain designations, comparisons are at least difficult and often not possible. This was shown to be the case of the "model" organism, *Chlamydomonas reinhardtii*, where the two mating types and strains of F1 generations from a single zygote isolated by G.M. Smith in 1945 were sent from one research laboratory to others over 60 years (for details, see genealogy in Pröschold et al., 2005).

Starting with reference material from a culture collection, the SSU and ITS rDNA and possibly other "marker" genes should be sequenced and phylogenetically analysed using different molecular methods (maximum likelihood, maximum parsimony, distance, and Bayesian). In case of SSU and ITS rDNA, the secondary structures should be elucidated for detection of compensatory base changes and synapomorphies. Therefore, for this type of analysis we recommend resequencing where more than two ambiguities or mismatches are found. Using these data as a starting point, all strains of a clade should then be studied using the polyphasic approaches as described above. For these approaches, experience and expertise are necessary, which can be provided only by utilizing the knowledge of a "taxonomic college" (scientific experts of a certain groups of algae and protozoa). Finally, with the input of these experts the strains of particular groups of green algae can be revised and then be cryopreserved, if possible, for long-term stability. In addition, the extracted DNA of these strains could be preserved. This process has been initiated at the Culture Collection of Algae and Protozoa (CCAP, Dunbeg by Oban, Scotland), and it is hoped this approach will in due course involve a wide spectrum of phycological taxonomists and other culture collections.

We are confident that the reconstruction of the ancient divergences within the green algae will be greatly facilitated by the ongoing sequencing efforts of a large number of genes (including complete plastid and mitochondrial genomes, and a variety of novel nuclear markers) of a wide range of green algae. This will undoubtedly get us closer to understanding the "true" phylogeny of the green algae. In the short term we believe that the SSU rDNA phylogeny presented here could be easily complemented with complete LSU rDNA sequences (although this gene has been found difficult to amplify in some green algal groups, for example, the Bryopsidales). This gene has proved to be more variable and phylogenetically more informative than the SSU rDNA in several eukaryotic groups including green algae (see, for example, Buchheim et al., 2001; Shoup and Lewis, 2003; Leliaert et al., 2003). LSU rDNA has furthermore the advantage over SSU rDNA in that it contains three hypervariable regions (see Wuyts et al., 2001), which make it suitable to be phylogenetic informative at the species level.

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