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MORPHOLOGY AND PHYLOGENETIC POSITION OF THE FRESHWATER GREEN MICROALGAE *CHLOROCHYTRIUM* (CHLOROPHYCEAE) AND *SCOTINOSPHAERA* (SCOTINOSPHAERALES, ORD. NOV., ULVOPHYCEAE)[1]

Pavel Škaloud,[2] *Tomáš Kalina, Katarína Nemjová*

Charles University in Prague, Faculty of Science, Department of Botany, Benátská 2, 128 01, Prague 2, Czech Republic

Olivier De Clerck, and Frederik Leliaert

Phycology Research Group, Biology Department, Ghent University, Krijgslaan 281 S8, 9000, Ghent, Belgium

The green algal family Chlorochytriaceae comprises relatively large coccoid algae with secondarily thickened cell walls. Despite its morphological distinctness, the family remained molecularly uncharacterized. In this study, we investigated the morphology and phylogenetic position of 16 strains determined as members of two Chlorochytriaceae genera, *Chlorochytrium* and *Scotinosphaera*. The phylogenetic reconstructions were based on the analyses of two data sets, including a broad, concatenated alignment of small subunit rDNA and *rbc*L sequences, and a 10-gene alignment of 32 selected taxa. All analyses revealed the distant relation of the two genera, segregated in two different classes: Chlorophyceae and Ulvophyceae. *Chlorochytrium* strains were inferred in two distinct clades of the *Stephanosphaerinia* clade within the Chlorophyceae. Whereas clade A morphologically fits the description of *Chlorochytrium*, the strains of clade B coincide with the circumscription of the genus *Neospongiococcum*. The *Scotinosphaera* strains formed a distinct and highly divergent clade within the Ulvophyceae, warranting the recognition of a new order, Scotinosphaerales. Morphologically, the order is characterized by large cells bearing local cell wall thickenings, pyrenoid matrix dissected by numerous anastomosing cytoplasmatic channels, sporogenesis comprising the accumulation of secondary carotenoids in the cell periphery and almost simultaneous cytokinesis. The close relationship of the Scotinosphaerales with other early diverging ulvophycean orders enforces the notion that nonmotile unicellular freshwater organisms have played an important role in the early diversification of the Ulvophyceae.

Key index words: Chlorochytrium; **Chlorophyceae; chloroplast;** *Kentrosphaera*; **Phylogeny;** *Scotinosphaera*; **taxonomy; ultrastructure; Ulvophyceae**

***Abbreviations*: ACOI, Coimbra Collection of Algae; BBM, Bold's basal medium; BI, Bayesian inference; BV, bootstrap value; CAUP, Culture Collection of Algae of Charles University in Prague; cDNA, complementary DNA; DAPI, 4′,6-diamidino-2-phenylindole; EMBL, European Molecular Biology Laboratory; ML, maximum likelihood; PP, posterior probability; rbcL, ribulose-bisphosphate carboxylase**

Diversity of eukaryotic microorganisms is generally poorly known and likely underestimated, especially when compared to animals and land plants. In the past two decades, the use of molecular tools has revolutionized microbial diversity research, including the discovery of numerous deeply branching phylogenetic lineages (Edgcomb et al. 2002, Kawachi et al. 2002, Moriya et al. 2002, Kawai et al. 2003, Stoeck et al. 2006, Kai et al. 2008, López-García and Moreira 2008, Zhao et al. 2012).

Green algae are no exception. Despite their long taxonomic history, new lineages are frequently being identified and described as higher taxa (Rindi et al. 2006, Zhang et al. 2008, Leliaert et al. 2009, Neustupa et al. 2009, 2011, Eliáš et al. 2010, Aboal and Werner 2011, Carlile et al. 2011, Němcová et al. 2011, Somogyi et al. 2011). Many of these new higher taxa are in fact based on molecular analysis of described species. For example, molecular and ultrastructural data have shown that the Prasinophyceae, traditionally comprising a diverse array of flagellates with organic body scales, comprise a paraphyletic assemblage of early diverging lineages, which are now being defined as new orders or classes (Marin and Melkonian 2010, Leliaert et al. 2012). Similarly, the identification of an unrecognized deeply branching clade of green algae, the Palmophyllales (Zechman et al. 2010) was based on a genus that had been known for over a century (Kützing 1847). Other examples include the endophytic marine green alga *Blastophysa rhizopus* Reinke, the marine quadriflagellate *Oltmannsiellopsis viridis* (Hargraves & Steele) Chihara & Inouye, the subaerial, coccoid *Ignatius tetrasporus* Bold & MacEntee, and the epizoic, filamentous *Trichophilus welckeri* Weber-van Bosse, which have been recovered as distinct lineages of Ulvophyceae (Iima and Tatewaki

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[2]Author for correspondence: e-mail skaloud@natur.cuni.cz.

1987, Nakayama et al. 1996, Friedl and O'Kelly 2002, Watanabe and Nakayama 2007, Cocquyt et al. 2010, Suutari et al. 2010). In other cases, molecular data have resulted in unexpected taxonomic transfers. For example, the invertebrate pathogen *Helicosporidium* Keilin, considered to be either a fungus or a protozoan of uncertain affinity, was phylogenetically inferred among the green algal class Trebouxiophyceae (Tartar et al. 2002). Similarly, the uniflagellate genus *Pedinomonas* Korshikov, traditionally affiliated with either Prasinophyceae (Moestrup 1991) or Ulvophyceae (Melkonian 1990), has been recovered as a distinct clade, sister to the core chlorophytes (Marin 2012).

Although the molecular diversity of green algae has been relatively well studied (reviewed in Lewis and McCourt 2004, Leliaert et al. 2012), DNA sequence data are still lacking for many genera and families. In particular, molecular investigation of morphologically distinct genera could improve our understanding of green algal evolution (Zechman et al. 2010). One such group of morphologically remarkable and molecularly uncharacterized green algae is the Chlorochytriaceae as circumscribed by Komárek and Fott (1983). The family comprises relatively large (up to 400 μm), spherical to irregularly shaped coccoid algae with secondarily thickened, stratified cell walls. Of seven genera recognized by Komárek and Fott (1983), *Chlorochytrium* Cohn and *Scotinosphaera* Klebs have been deposited in public culture collections, enabling a detailed morphological and ultrastructural investigation, and molecular characterization.

Chlorochytrium grows endophytically in intercellular spaces of various freshwater, aquatic plants (Cohn 1872, Klebs 1881a, West 1916, Lewin 1984) or marine macro-algae (West et al. 1988). The spheroid, ovoid, or slightly irregular cells contain a single parietal chloroplast with one to several pyrenoids. The life history of *Chlorochytrium* involves biflagellate isogametes that leave sporangial wall in common gelatinous vesicle and fuse into quadriflagellate planozygotes that settle on host after short motile period. The settled planozygotes develop into vegetative cells, which enter the intercellular spaces of the host plant by the formation of tubular protrusions. Both gametes and zoospores arise from successive bipartition of the protoplasm. During a complex pattern of cell division, protoplasm pieces fuse and develop into zoids (Cohn 1872, Klebs 1881a, Kurssanov and Schemakhanova 1927).

Scotinosphaera (Klebs 1881c) comprises free-living, freshwater and terrestrial organisms with spheroid to ovoid cells, having one to several local thickenings that may develop into extensive protrusions. The cells contain a single axial chloroplast with one to several central pyrenoids and numerous lobes spreading to the cell periphery. Only asexual reproduction has been observed, which takes place by numerous biflagellate zoospores, arising from almost simultaneous cytokinesis (Klebs 1881c, Punčochářová 1992).

Chlorochytrium and *Scotinosphaera* have an intricate taxonomic history, particularly because of close morphological and ecological similarities of both genera with *Kentrosphaera* (Borzi 1883), *Endosphaera* (Klebs 1881b), and *Stomatochytrium* (Cunningham 1887). West (1904, 1916) reduced *Stomatochytrium* and *Scotinosphaera* to synonyms of *Chlorochytrium*, based on morphological similarities and endophytic habit. Later, Bristol (1920) also included *Kentrosphaera* as a synonym of *Chlorochytrium*, based on similarities in chloroplast structure. However, some of the later investigators did not accept Bristol's proposal. Smith (1933) retained the genera *Chlorochytrium* and *Kentrosphaera* based on their different habitat and reproductive characteristics. He characterized *Chlorochytrium* as an endophytic genus with sexual reproduction, and *Kentrosphaera* as free-living, reproducing only by asexual zoospores. Komárek and Fott (1983) followed Smith's recognition of *Chlorochytrium* and *Kentrosphaera* as separate genera, and listed *Chlorocystis* Reinhard, *Stomatochytrium*, *Scotinosphaera* and *Endosphaera* as synonyms of the former genus. Punčochářová (1992) regarded *Scotinosphaera* as an invalid name due to the absence of either description or illustration of the structure of its vegetative cells. However, Wujek and Thompson (2005) rectified this by recognizing *Scotinosphaera* as a validly described genus having priority over *Kentrosphaera*. On the basis of detailed morphological observations, Wujek and Thompson (2005) classified five species into *Scotinosphaera*, and considered *Chlorochytrium* as a monotypic genus.

In addition to ambiguous generic circumscriptions, the taxonomic affinity of *Chlorochytrium* and *Scotinosphaera* has long been questioned. Since their description, both genera were considered closely related. The genera were originally assigned to the green algal order Chlorococcales, and placed into various families, including the Endosphaeraceae (Smith 1950), Chlorococcaceae (Bourrelly 1966), and Chlorochytriaceae (Komárek and Fott 1983). Ultrastructural investigations, however, shed doubt on the close relation between the two genera (Watanabe and Floyd 1994). Based on different arrangement of kinetosomes and flagellar roots, *Chlorochytrium* was classified in the Chlorophyceae and *Scotinosphaera* in the Trebouxiophyceae (then Pleurastrophyceae). This classification was not adopted by Wujek and Thompson (2005), who retained both genera in Chlorophyceae according to their similar morphological features.

The aim of this study was to determine the phylogenetic position of two members of the Chlorochytriaceae, *Chlorochytrium* and *Scotinosphaera*, based on DNA data and thus to confirm or refute their close relationship suggested by morphological similarities. We show that *Chlorochytrium* is a member of the *Stephanosphaerinia* clade of the Chlorophyceae, and

comprises two separate clades. *Scotinosphaera* is unrelated to *Chlorochytrium* and forms a distinct lineage of Ulvophyceae, which warrants the description of a new order, Scotinosphaerales ord. nov. Detailed morphological, ultrastructural, and life cycle descriptions are presented.

MATERIALS AND METHODS

Collection, culturing, and microscopic analyses. Strains of *Chlorochytrium* and *Scotinosphaera* were obtained from the Culture Collection of Algae, Charles University in Prague (CAUP) and the Coimbra Collection of Algae (ACOI) (Table 1). The strains were cultivated on Bold's basal agar medium (Bischoff and Bold 1963) at 23°C, under continuous illumination of 5–15 μmol photons · m^{-2} · s^{-1} provided by 18W cool fluorescent tubes (Philips TLD 18W/33). The unialgal cultures were examined with an Olympus BX51 light microscope (Olympus Corp., Tokyo, Japan) with differential contrast, and photos taken with a mounted Olympus Z5060. For observation of nuclear cycle, the cells were fixed and stained in aceto-iron-hematoxilin-chloral hydrate (Wittmann 1965). Epifluorescence microscopy on material stained in 1% calcofluor (Sigma-Aldrich, St. Louis, MO, USA) or DAPI dye (0.5 mg · mL^{-1}; Sigma-Aldrich) was used for additional morphological observations of the cleavage wall formation and nuclear cycle, respectively. Chloroplast morphology was investigated using a laser scanning confocal microscope Leica TCS SP2 (Leica Microsystems, Wetzlar, Germany) equipped with an Argon-Krypton laser. We used a 488 nm excitation line and an AOBS filter free system collecting emitted light between 498 and 700 nm. A Leica 63x/1.4 N.A. oil immersion objective fitted on a Leica DM IRE2 inverted microscope was used. A series of optical sections through chloroplasts were captured and used for a 3-dimensional reconstruction of their morphology. The autofluorescence of the chlorophyll was exploited for visualization of the chloroplast structure. The chloroplast reconstructions were produced by the ImageJ 1.34p program (Abramoff et al. 2004), using the "Volume viewer" plugin.

Electron microscopy. For transmission electron microscopy (TEM), cells cultivated on BBM medium were prefixed in 1% solution for 2 h and then fixed in 2% solution of glutaraldehyde in the BBM for 3 h. After washing with pure BBM, the cells were post fixed for 4 h at 5°C in 1% osmium tetroxide in 0.05 M phosphate buffer, and overnight at 5°C in 2% water solution of uranyl acetate. After dehydration through an ethanol-butanol series, the cells were infiltrated and embedded in Spurr's low viscosity epoxy medium (Spurr 1969). Ultrathin sections were prepared using an Ultracut E (Reichert-Jung, Wien, Austria). The sections were contrasted using 2% water solution of uranyl acetate and lead citrate solution (Reynolds 1963). Alternative contrasting was performed with methanolic uranyl acetate solution followed by the bismuth nitrate solution (Riva 1974, Tandler 1990). Ultrathin sections were examined using a TEM Tesla 613 (Tesla, Brno, Czech Republic) or JEOL 1011 TEM (JEOL Ltd., Tokyo, Japan) equipped with a digital camera.

DNA and RNA extraction, and reverse transcription. After centrifugation and mechanical disruption of cells by shaking in the presence of glass beads (0.5 mm in diameter, Sigma-Aldrich), genomic DNA was extracted using the Invisorb Spin Plant Mini Kit (Invitek). Total RNA was extracted using a Nucleospin kit RNA XS. The extracted RNA was precipitated with 50.8 μL of a solution that consisted of 50 μL 100% ethanol and 0.8 μL 5 M NaCl and cooled for −20°C at night. After centrifugation for 5 min at 16 rcf, ethanol was discarded and

TABLE 1. List of strains used in this study.

Taxon	Strain number	Strain relatives	Origin
Scotinosphaera austriaca	CAUP H5304	–	Plankton of the Neusiedler See, Austria
Scotinosphaera facciolae	ACOI 256	CAUP H5309	Stagnant water, Serra da Boa Viagem, Portugal
Scotinosphaera gibberosa var. *polymorpha*	CAUP H5301	–	Basin, greenhouse, Bratislava, Slovakia
Scotinosphaera gibberosa var. *gibberosa*	CAUP H5302	SAG 75.80, UTEX 2913	Stone in a water basin, Plovdiv, Bulgaria
Scotinosphaera lemnae	CAUP H5303a	SAG 240-1	Dead *Lemna*, pond near Glasgow, Scotland
Scotinosphaera lemnae	CAUP H5303b	UTEX 100	Dead *Lemna*, pond near Glasgow, Scotland
Scotinosphaera willei	ACOI 251	CAUP H5310	Phytoplankton, Serra da Estrela, Portugal
Scotinosphaera sp.	CAUP H5305	UTEX 145	Soil, Bloomington, IN, USA
Scotinosphaera sp.	CAUP H5306	–	Athens, Greece
Scotinosphaera sp.	CAUP H5307	–	Soil, Sasebo City, Nagasaki Prefecture, Japan
Scotinosphaera sp.	CAUP H5308	–	Soil, Boreč Hill, Czech Republic (Škaloud 2009)
Chlorochytrium lemnae	CAUP H6901	SAG 15.85, UTEX 2315	Endophyte in *Lemna trisulca*, pond near Utrecht, The Netherlands
Chlorochytrium lemnae	CAUP H6902	SAG 16.85	Endophyte in *Lemna minor*, Oxfordshire, Noke Oxon, England
Chlorochytrium lemnae	CAUP H6903	UTEX 2283	Endophyte in *Lemna*, Oxford, England
Chlorochytrium lemnae	CAUP H6904	UTEX 2284	Endophyte in *Lemna*, Oxford, England
Chlorochytrium lemnae	CAUP H6905	CCAP 212/1, SAG 212-1	Soil, India

100 μL of 70% ethanol was added. Then the same centrifugation step followed. The ethanol was discarded and the precipitated RNA was dried at 37°C for a few minutes and subsequently diluted in 12 μL of redistilled water. For amplification of the nuclear genes cDNA was constructed from total RNA using Omniscript RT kit (Qiagen, Venlo, Netherlands).

PCR amplification, cloning of PCR products, and sequencing. Three molecular markers were PCR-amplified from the genomic DNA: nuclear-encoded small subunit (SSU) rDNA and chloroplast-encoded *rbc*L and *atp*B. In addition, four nuclear genes (40S ribosomal protein S9, 60S ribosomal protein L17, oxygen-evolving enhancer OEE1 and actin gene) were amplified from cDNA. List of primers, PCR cycling conditions, and reaction mixture composition are given in Tables S1 and S2 in the Supporting Information. After checking the quality of PCR products on agarose gel, the PCR products were purified using GenElute PCR Clean-Up Kit (Sigma-Aldrich) or MinElute Gel Extraction Kit (Qiagen). The cleaned PCR products were cloned using pGEM-T Easy Vector Systems (Promega, Madison, WI, USA) and ligated plasmids were transformed into highly efficient competent *E. coli* cells (Promega). The transformed cells were plated on LB medium and cultivated at 37°C for 16 h. The LB plates were treated with 50 μL of IPTG and 50 μL X-gal. After the cultivation, white colonies were picked up and diluted in 10 μL of redistilled water and denaturated at 95°C for 10 min. One μL of this mixture was used for the subsequent PCR amplification, using the conditions described in Cocquyt et al. (2010). PCR products were sequenced using PCR primers with either 3130xl or 3730xl Applied Biosystems automatic sequencer. The sequences are available in the EMBL Nucleotide Sequence Database under accession numbers HE860249-81 (Tables S3 and S4 in the Supporting Information). The SSU rDNA sequences of the *Chlorochytrium* strains H6901, H6902, and H6903 contained a putative group I intron, S943 (Haugen et al. 2005), which was removed prior to phylogenetic analyses.

Phylogenetic analyses. Two data sets were created for phylogenetic analyses. The first one consisted of 88 SSU rDNA and 65 *rbc*L sequences yielding a concatenated alignment of 88 taxa representing a broad range of Chlorophyta (Table S3). Two Streptophyta (*Chlorokybus* and *Mesostigma*) were selected as outgroup. Based on the results of the phylogenetic analysis inferred from the concatenated SSU-*rbc*L alignment, a second, 10-gene alignment was assembled to better resolve the position of *Scotinosphaera* within the Ulvophyceae. This alignment largely corresponded to the data set of Cocquyt et al. (2010) and included eight nuclear-encoded genes [SSU rDNA, Actin, Glucose-6-phosphate isomerase (G6PI), Glyceraldehyde-3-phosphate dehydrogenase (GapA), Oxygen-evolving enhancer protein 1 (OEE1), and the Ribosomal proteins 40S S9, 60S L3, and 60S L17] and the plastid-encoded genes *rbc*L and *atp*B from 30 representatives of Chlorophyta and two prasinophytes (*Nephroselmis* and *Ostreococcus*) as outgroup (Table S4).

For the SSU-*rbc*L data set, the SSU sequences were aligned using MUSCLE (Edgar 2004), and visually inspected in BioEdit 7.0.5.3 (Hall 1999). Ambiguously aligned regions in the SSU alignment were removed using Gblocks 0.91b (Castresana 2000) with options allowing for smaller final blocks, gap positions within the final blocks, and less strict flanking positions. This reduced the SSU alignment from 1920 to 1691 positions. The *rbc*L sequences were aligned by eye based on their amino acid sequences in BioEdit, and the third codon positions were removed resulting in an alignment of 920 positions. A suitable partitioning strategy and models of sequence evolution were selected using the Bayesian Information Criterion with Partitioned Model Tester 1.01 (Verbruggen 2010), resulting in a 3-partition strategy (SSU, *rbc*L 1st and 2nd codon position) with uncoupled GTR+G8 models for each partition. The SSU-*rbc*L alignment was analyzed with Bayesian inference (BI) and maximum likelihood (ML) using MrBayes (Ronquist and Huelsenbeck 2003) and RAxML (Stamatakis et al. 2008), respectively. For both analyses unlinked GTR+G models (CAT approximation in RAxML) were applied to each partition. The BI analysis consisted of two runs of 10 million generations with sampling every 1000 generations. Convergence of the log-likelihood and model parameters was checked in Tracer v. 1.4 (Rambaut and Drummond 2007). A burn in sample of 1,500 trees was removed before constructing the majority rule consensus tree. Two additional analyses were performed to better resolve the positions of *Chlorochytrium* and *Scotinosphaera*, and to assess genetic variation within the two genera. These analyses were based on smaller but denser taxon sets and complete SSU-*rbc*L sequences (i.e., with no positions excluded; Table S3).

For the 10-gene alignment, the SSU sequences were aligned using MUSCLE and the protein-coding genes were aligned by eye based on their amino acid sequences in BioEdit. The 10 loci were concatenated, yielding an alignment of 10,190 positions, which was 58% filled at the nucleotide level. In some cases, sequences from different species were concatenated if their monophyly with respect to other taxa in our alignment could be demonstrated (Campbell and Lapointe 2009, Cocquyt et al. 2010). Alignment positions were not excluded a priori, but instead 25% of the fastest evolving sites were removed as suggested and described in Cocquyt et al. (2010), reducing the alignment to 7,642 positions. The alignment was partitioned and models were selected following Cocquyt et al. (2010), except that we did not partition the SSU into stems and loops. BI (MrBayes) and ML (RAxML) analyses were analyzed using unlinked GTR+G models for seven partitions [SSU: one partition; first, second, and third codon positions of the nuclear and plastid genes (three times two partitions)]. All alignments were submitted to TreeBase (http://www.treebase.org/treebase-web/home.html) and are available under No. S13318.

RESULTS

Molecular phylogenetic analyses. Bayesian inference and ML analyses of the SSU-*rbc*L alignment yielded similar tree topologies. The Bayesian tree with indication of ML bootstrap values is shown in Figure 1. The overall tree topology was congruent with published phylogenies of Chlorophyta (Leliaert et al. 2012), showing a paraphyletic assemblage of early branching prasinophytes and three large classes (Chlorophyceae, Ulvophyceae and Trebouxiophyceae) that make up the core chlorophytan clade. The Trebouxiophyceae was recovered as a nonmonophyletic group; monophyly of the Chlorophyceae and Ulvophyceae was moderately to poorly supported. *Chlorochytrium* and *Scotinosphaera* were distantly related and segregated in two different classes: Chlorophyceae and Ulvophyceae.

Chlorochytrium was inferred as a member of the *Stephanosphaerinia* clade within the Chlorophyceae. To accurately resolve the phylogenetic position of all investigated *Chlorochytrium* strains, a separate phylogenetic analysis of *Stephanosphaerinia* was performed (Fig. 2A). The five sequenced strains of *Chlorochytrium lemnae* were separated in two distinct clades that do not show a sister relationship.

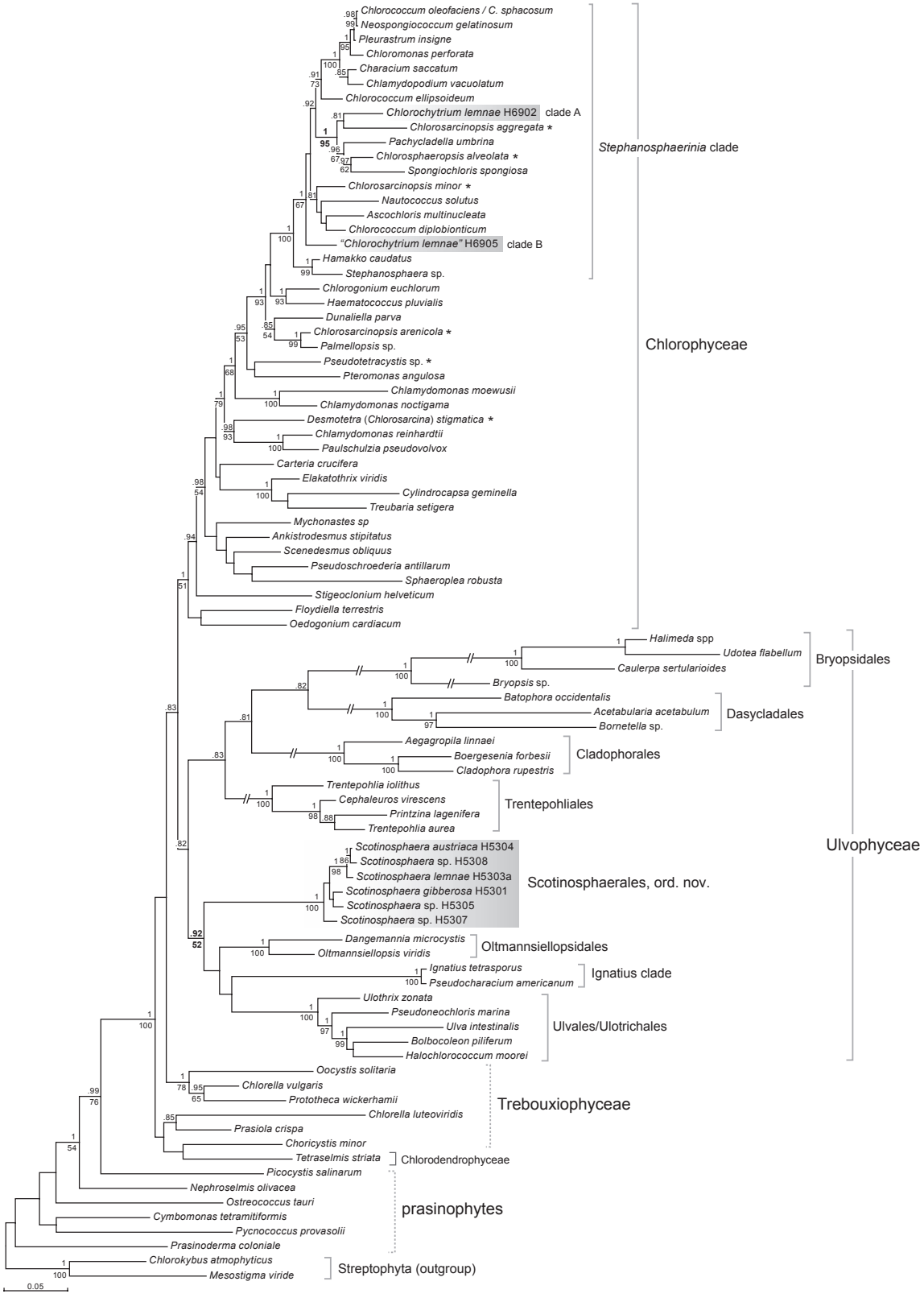

FIG. 1. Phylogeny of the Chlorophyta obtained by Bayesian inference of the concatenated SSU-*rbc*L alignment. The Bayesian majority rule tree showing all compatible bipartitions is shown with node support given as Bayesian posterior probabilities (above branches) and maximum-likelihood (ML) bootstrap values (below branches); values <0.8 and 50, respectively, are not shown. Very long branches in the Ulvophyceae have been scaled 25% (indicated by slashes). Species traditionally assigned to the family Chlorosarcinaceae are indicated by an asterisk.

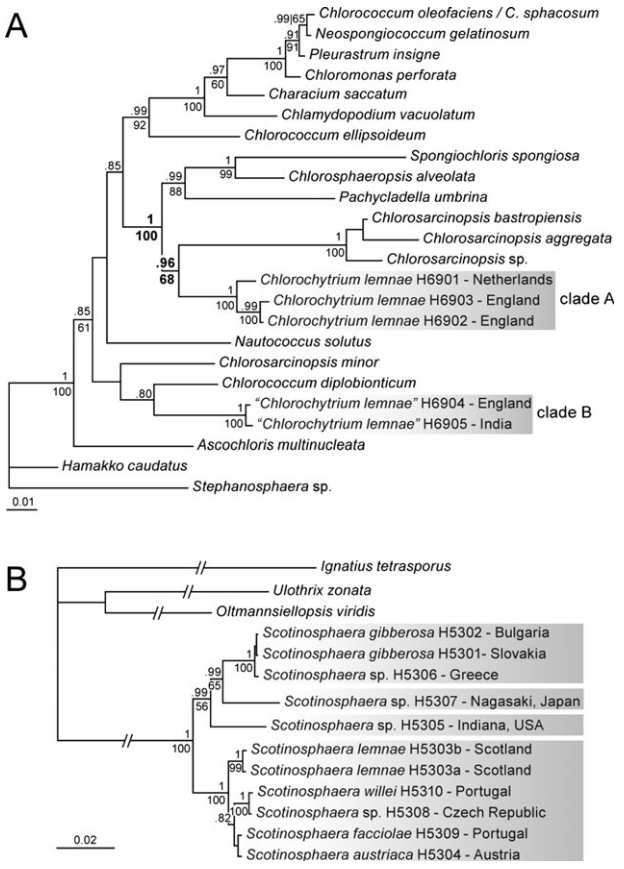

FIG. 2. Bayesian majority rule trees based on complete SSU-*rbc*L sequence alignments (i.e., with all positions included), showing the phylogenetic positions of the *Chlorochytrium* strains within the *Stephanosphaerinia* clade (A), and the genetic diversity within *Scotinosphaera* (B). Node support is given as Bayesian posterior probabilities (above branches) and maximum-likelihood (ML) bootstrap values (below branches); values <0.8 and 50, respectively, are not shown.

Clade A, including strains from England and the Netherlands (CAUP H6901, CAUP H6902, and CAUP H6903), was most closely related to *Chlorosarcinopsis aggregata*, *C. bastropiensis*, *Pachycladella umbrina*, *Chlorosphaeropsis alveolata,* and *Spongiochloris spongiosa*. The phylogenetic position of clade B, including strains from England and India (CAUP H6904 and CAUP H6905), could not be inferred with adequate support.

Scotinosphaera isolates formed a distinct, highly divergent, and strongly supported clade (Figs. 1 and 2B). Analysis of the SSU-*rbc*L alignment indicated that this clade is most closely related with three lineages of Ulvophyceae: the Ulvales/Ulotrichales clade, the *Ignatius* clade, and the Oltmannsiellopsidales. However, the relationships between these clades were not statistical supported. In addition, monophyly of the Ulvophyceae was only weakly supported in the SSU-*rbc*L tree (Fig. 1). Phylogenetic analysis of the 10-gene alignment firmly placed *Scotinosphaera* in the Ulvophyceae (PP = 1, BV = 83), but the precise phylogenetic position of the clade remained unresolved (Fig. 3 and Fig. S1 in the Supporting Information). Within the *Scotinosphaera* lineage, four clades were recovered that were separated by relatively long branches with high support: two non sister clades of European strains and two singletons including a Japanese and North American strain (Fig. 2B).

Light and confocal microscopy. Vegetative cells of *Chlorochytrium* lineage were globular, or rarely ellipsoidal or slightly irregular, 8–47 (rarely up to 66) μm in diameter (Fig. 4A). Mature cells often had a highly vacuolized cytoplasm (Fig. 4B) and partially thickened cell wall (up to 5 μm; Fig. 4C). Cells were uninucleate (Fig. 4D). Asexual reproduction took place by autospores and zoospores. Colonies of 4–16 (−32) autospores (up to 6 μm in diameter) were formed in each sporangium. Sporangia were spherical or slightly irregular, 14–23 (−37) μm in diameter (Fig. 4E). Zoosporangia were spherical, ellipsoidal or slightly irregular, 19–45 μm in diameter, at maturity containing 64–128 biflagellate zoospores (Fig. 4F). The zoospores were generally drop-shaped, with tapered anterior and rounded posterior ends. Zoospores lacked cell walls, and noticeably varied in shape and size (length 6–9 μm, width 3–4 μm). They possessed a single chloroplast with stigma. In addition to the above-mentioned sporulation, mature vegetative cells sometimes divided into pairs or tetrads, the walls of which were closely associated with the parental cell wall (Fig. 4G). The cleavage wall formation was centripetal (Fig. 4H) and the daughter cells mostly remained enclosed by the parent cell wall, forming the sarcinoid (packet-like) formations (Fig. 4I). Joined

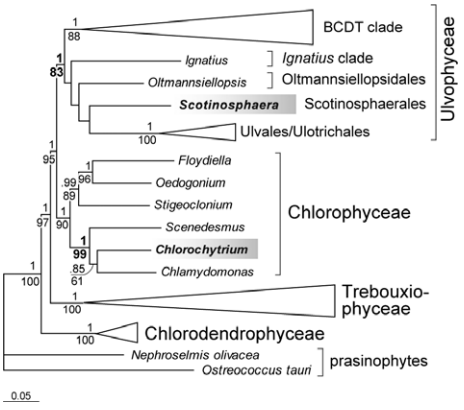

FIG. 3. Bayesian majority rule tree of the Chlorophyta based on a 10-gene alignment, showing the phylogenetic positions of *Chlorochytrium* and *Scotinosphaera*. Node support is given as Bayesian posterior probabilities (above branches) and maximum-likelihood (ML) bootstrap values (below branches); values <0.8 and 50, respectively, are not shown. BCDT clade includes the Bryopsidales, Cladophorales, Dasycladales, and Trentepohliales. The phylogram including all terminal taxa is given in the online supplementary Figure S1.

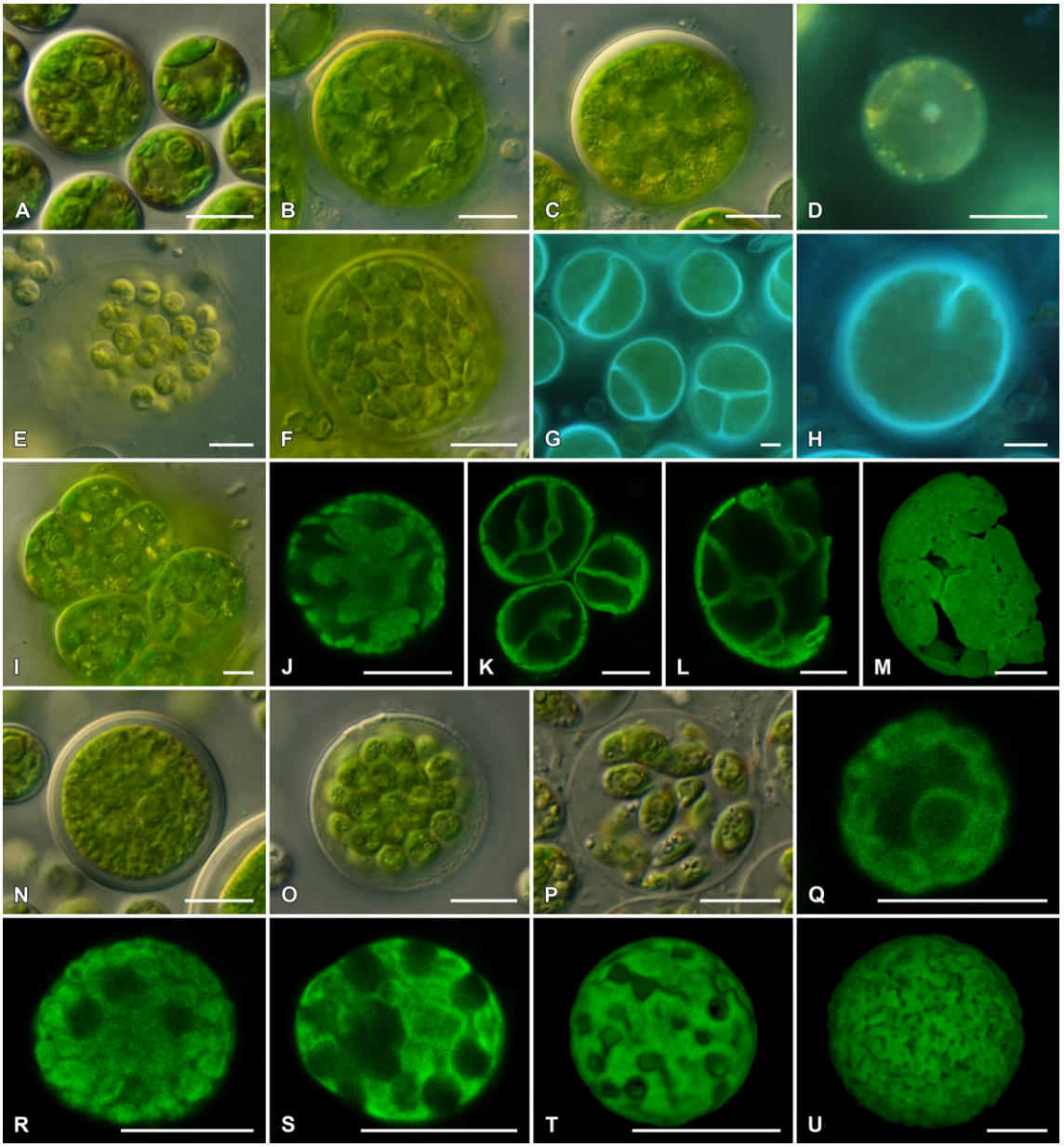

FIG. 4. Light microscopy of *Chlorochytrium*. (A–M) *Chlorochytrium* lineage A. (A) Young vegetative cells – H6901. (B) Mature vegetative cell with vacuolized cytoplasm and ribbon-like chloroplast lobes – H6903. (C) A globular cell with a flat local thickening of the cell wall (arrowhead) – H6903. (D) DAPI-stained nucleus – H6902. (E) Autosporangium – H6903. (F) Zoosporangium – H6902. (G) Division of mature vegetative cells into pairs and tetrads. Calcofluor staining – H6902. (H) The centripetal cell wall cleavage during the formation of cell tetrads. Calcofluor staining – H6902. (I) Cell packets – H6902. (J) Confocal section through the young vegetative cell – H6901. (K) Ribbon-like chloroplast lobes of mature vegetative cells. Confocal section – H6902. (L) Ribbon-like chloroplast with numerous pyrenoids. Confocal section – H6902. (M) Spatial reconstruction of a chloroplast in a mature cell – H6902. (N–U). *Chlorochytrium* lineage B. (N) A mature vegetative cell with a thickened cell wall – H6905. (O) Autosporangium – H6905. (P) Zoosporangium – H6904. (Q) The parietal chloroplast of young cells. Note a distinct pyrenoid located beneath the chloroplast layer. Confocal reconstruction – H6905. (R) Chloroplast of mature vegetative cells. Note three spherical holes inside the chloroplast. Confocal reconstruction – H6905. (S) Chloroplast of mature vegetative cells, perforated by several peripheral holes. Chloroplast reconstruction – H6904. (T) Spatial reconstruction of chloroplast. Note several peripheral holes – H6904. (U) Spatial reconstruction of chloroplasts in mature cells, showing its dense perforation by numerous pores – H6905. Scale bars: 10 μm.

daughter cells often developed into auto- or zoosporangia. Sexual reproduction was not observed.

In young vegetative cells, the chloroplast was unilayered and parietal, containing a single pyrenoid. Soon it expanded into the central cell lumen where it formed a central mass (Fig. 4J). In adult cells, the chloroplast formed a net of connected ribbonlike lobes, containing numerous (up to six) pyrenoids (Fig. 4K and L). All lobes were connected with the parietal layer, perforated by several

irregular holes and numerous very small pores (Fig. 4M).

Vegetative cells of *Chlorochytrium* lineage B were uninucleate, globular or ellipsoidal, 5–39 μm in diameter. Mature cells had evenly thickened cell walls, up to 4 μm thick (Fig. 4N). Asexual reproduction took place by autospores and zoospores. Autosporangia were spherical, (15−) 19–35 μm in diameter, containing 8–64 autospores (Fig. 4O). Zoospores were produced in spherical zoosporangia, ranging from 16 to 24 μm in diameter (Fig. 4P). In total, 16–32 biflagellate zoospores were produced per sporangium, and measured 4.5–8 μm in length and 3–5 μm in width. They possessed a rigid cell wall, and a single posterior chloroplast with stigma. Sexual reproduction was not observed. The strain CAUP H6905 was exceptional by the production of mucilaginous sheath surrounding the cells. The chloroplast of young cells was unilayered, parietal, containing a single pyrenoid located beneath the chloroplast layer (Fig. 4Q). This stage is morphologically very similar to the vegetative cells of *Chlorococcum*. In adult cells, the chloroplast formed a net of inter-connected, densely appressed tubular lobes (Fig. 4R). No ribbonlike lobes were observed. Relatively large spherical holes were often produced in the chloroplast, either completely burrowed in the chloroplast mass filling up the cell volume (Fig. 4R), or appearing along the chloroplast periphery (Fig. 4S). In the latter case, the chloroplast appeared to form several spherical holes or sockets in its surface (Fig. 4T). Apart from these large holes, the chloroplast was perforated by numerous very small pores (Fig. 4U). A single pyrenoid was observed during all stages of chloroplast ontogeny (Fig. 4N, Q and S).

Vegetative cells of *Scotinosphaera* were morphologically very variable. Young cells were generally spherical, ellipsoidal or elongated (Fig. 5A). In well growing cultures, cells were broadly ellipsoidal, pyriform or irregularly shaped (Fig. 5B and C), 5–280 μm long. Cell wall of young cells was thin. Mature cells generally possessed a single, often stratified cell wall thickening, forming an external protuberance up to 40 μm in length (Fig. 5D). Rarely, additional protuberances occurred (Fig. 5E). The cells were uninucleate (Fig. 5F). Asexual reproduction took place by autospores and zoospores. In the first stage of sporogenesis, cells synthesized secondary carotenoids which accumulated in the cell periphery, coloring it to orange (Fig. 5G). Simultaneously, the single centrally positioned nucleus migrated to the cell periphery and assumed a star-like shape (Fig. 6). In the next sporulation stage, the orange coloring disappeared and the protoplasm underwent many repeated successive cleavages followed by a quickly repeated mitosis giving the origin of a considerable number of daughter nuclei (Fig. 5H). Soon afterwards, quick simultaneous cell divisions resulted in 32–250 (−ca. 350) daughter cells. Finally, the location of the sporangium opening was predetermined by the formation of a mucilaginous hyaline vesicle arising by the local gelatinization of the sporangial cell wall (Fig. 6). Autosporangia were spherical or ellipsoidal, up to 100 μm in diameter. Up to ±400 spherical autospores were formed per sporangium (Fig. 5I). Soon after their liberation, the autospores rapidly elongated. Zoospores were formed in high numbers of 64 to ˜400. They were biflagellate, fusiform, lacking cell walls. Zoospores were 6–9 μm long and 3.5–5 μm wide, and possessed a single chloroplast with stigma. Sexual reproduction was not observed. The chloroplast of mature autospores was unilayered, parietal, containing a single pyrenoid. In young cells, it expanded into the central cell lumen and transformed into an axial chloroplast containing one pyrenoid in its center (Fig. 5J). In adult cells, the chloroplast formed a net of numerous radiating and anastomosing lobes expanding from two or more pyrenoids toward the cell periphery (Fig. 5K–M). At the chloroplast periphery, the lobes either extended into flat disks of variable shape (Fig. 5N and O) or divided into several elongated projections (Fig. 5P).

Transmission electron microscopy. An ultrastructural investigation of the *Scotinosphaera* strain CAUP H5301 was conducted to further characterize the novel clade revealed by molecular phylogenetic analyses. Young vegetative cells possessed a single nucleus and a parietal chloroplast containing a large central pyrenoid with a starch envelope (Fig. 7A). The pyrenoid was not penetrated by thylakoid membranes, but invaginated by cytoplasmic channels. In young cells, the pyrenoid was invaginated by a single, centrally located cytoplasmatic channel. In mature cells, the pyrenoid was dissected by numerous anastomosing cytoplasmatic channels, which divided the stroma into several pyramidal or irregular segments (Fig. 7B). The chloroplast of mature cells was filled with numerous, large starch grains. The nucleus of young cells was relatively large, occupying about half of the cell body. The mitochondrion profiles were scattered in the space between the nucleus and the chloroplast (Fig. 7A). The cell wall was thick and homogeneous, without prominent lamination. In the first stage of sporogenesis, the pyrenoid disappeared and the chloroplast divided into numerous parts with indiscernible thylakoids (Fig. 7C). Consequently, we observed a large number of daughter nuclei suggesting the extremely rapid nuclear division (Fig. 7C and D). Finally, the cytoplasmic cleavages were initiated by fusion of several vacuoles probably derived from dictyosomes (Fig. 7D). The cleavage proceeded without the involvement of microtubule systems.

The zoospores were naked, devoid of a cell wall. The nucleus was located in the anterior part of the cell. It was of irregular shape and contained a massive chromatine body (Fig. 7E). The chloroplast

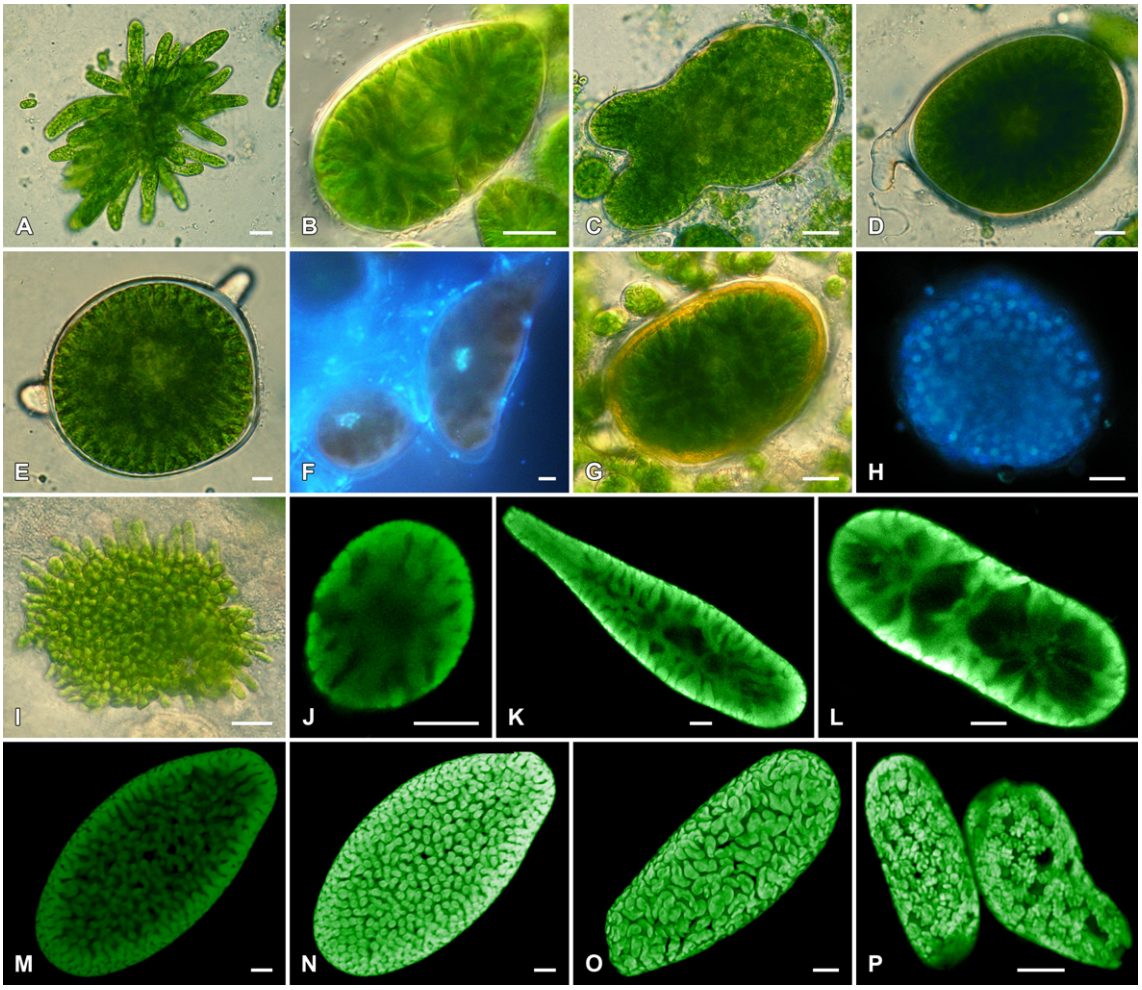

FIG. 5. Light microscopy of *Scotinosphaera*. (A) A cluster of young, elongated vegetative cells – H5305. (B) A pyriform mature cell with a radiate chloroplast containing two pyrenoids – H5302. (C) An irregular mature cell – H5305. (D) A mature cell possessing a single, stratified cell wall thickening – H5302. (E) A spherical mature cell possessing two cell wall protuberances – H5303. (F) Two mature cells with a single nucleus. DAPI staining – H5302. (G) A mature cell in the first stage of sporogenesis. A peripheral layer is colored to orange by the synthesis of secondary carotenoids – H5302. (H) Late stage of protoplasm division. Note numerous DAPI-stained daughter nuclei – H5302. (I) Autospores – H5303. (J) An axial chloroplast of young vegetative cells, containing a single pyrenoid. Confocal section – H5305. (K) A radiate chloroplast of mature vegetative cells. Note two pyrenoids in the chloroplast center. Confocal section – H5308. (L) Chloroplast of mature vegetative cells containing three pyrenoids. Confocal section – H5305. (M) A sub-peripheral confocal section through the mature vegetative cell – H5308. (N) Chloroplast with simply extended lobes. Confocal reconstruction – H5308. (O) Chloroplast with lobes extended into the flat disks. Confocal reconstruction – H5302. (P) Chloroplast with lobes divided into several elongated projections. Confocal reconstruction – H5305. Scale bars: 10 μm.

occupied the posterior or lateral region of the zoospore, and sometimes two chloroplast parts were visible in TEM sections. The inner structure of the chloroplast was electron dense, the thylakoids formed compact bodies deposited in chloroplast stroma. The chloroplast contained one to several starch grains (Fig. 7F). No pyrenoid was observed in the chloroplast. The chloroplast possessed a stigma consisting of single row of globules (Fig. 7G). Since we investigated the settled zoospores, no functional flagella were observed, but these were retracted within the cell body (Fig. 7H). The flagella persisted in the cytoplasm as a pair of coiled axonemal microtubules, arranged from the anterior kinetosomes to the posterior end of zoospore, beneath the plasma membrane (Fig. 7F, H, and I). However, the retracted flagella completely disappeared in young cells.

DISCUSSION

Our study refutes the morphology-based hypothesis that *Chlorochytrium* and *Scotinosphaera* are closely related. Instead we found that the two genera are members of different classes, Chlorophyceae and Ulvophyceae.

The five strains labelled as *Chlorochytrium lemnae* formed two distinct lineages within the *Stephanosph-*

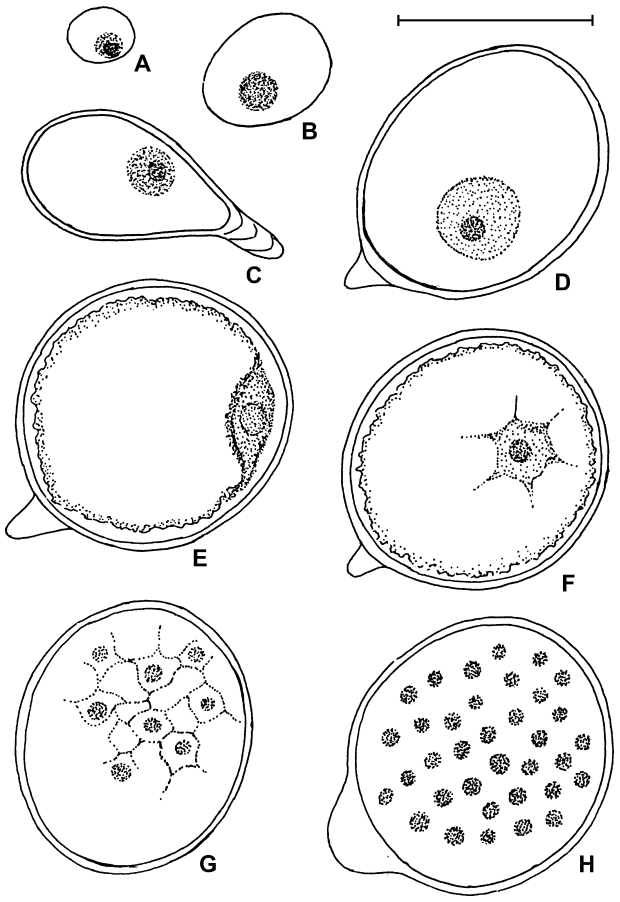

FIG. 6. Drawings of *Scotinosphaera* nuclear cycle based on material stained with aceto-iron-hematoxylin-chloral hydrate (CAUP H5301). (A–D) Vegetative cells. Large nucleus is located in the cell center. (E and F) Young sporangia with peripherally located, starlike nucleus. Secondary carotenoids are synthetized between the protoplasm and cell wall. (G) Cleavage of the protoplasm after simultaneous nuclear division. (H) Mature sporangium containing numerous nuclei of daughter cells. The daughter cell walls were not observed. The asexual spores are released by opening of the gelatinous bulge.

aerinia clade, which is one of the main clades of Chlamydomonadales in the class Chlorophyceae (Nakada et al. 2008). The two lineages did not show a sister relationship, suggesting that they in fact correspond to two different genera (Fig. 2).

Strains belonging to clade A correspond morphologically with the original description of the genus *Chlorochytrium* (Cohn 1872). Similar to Cohn's observations, the cells of all three strains were globular, ellipsoidal or irregularly shaped, occasionally grouped into pairs or tetrads. We also observed the chloroplast forming a net of connected ribbon-like lobes, containing numerous pyrenoids, a characteristic feature of the genus (Fig. 8). Like the type material, the strains of clade A were isolated from the intercellular spaces of duckweed plants (*Lemna* spp.). On the basis of these morphological and ecological features, we assume that clade A represents the genuine genus *Chlorochytrium*.

In addition to the frequent production of auto- and zoospores (where the daughter cells form a new cell wall, separate from the parental wall, known as eleutheroschisis), all three *Chlorochytrium* strains belonging to clade A reproduced asexually by a unique type of centripetal cell division, resulting in the formation of irregularly shaped, sarcinoid cell packages (Fig. 4H and I). In this type of cell division (desmoschisis) the parental wall forms a part of the cell wall of daughter cells. The presence of desmoschisis characterizes the green algal family Chlorosarcinaceae (Bourrelly 1966), encompassing several sarcinoid genera (e.g., *Chlorosarcina* Gerneck, *Chlorosarcinopsis* Herndon, *Chlorosphaeropsis* Vischer, *Desmotetra* Deason & Floyd). Based on the formation of sarcinoid cell packages in *Chlorochytrium*, the genus has been regarded as a member of the family (Lewin 1984). Our molecular phylogenetic analyses revealed a relation between *Chlorochytrium* and a clade of *Chlorosarcinopsis* species (Fig. 2). A possible affinity of *Chlorochytrium* with Chlorosarcinaceae was also proposed by Moewus (1950), who even described a population of *Chlorochytrium lemnae* as a new species of the morphologically similar, sarcinoid genus *Chlorosphaeropsis* (Wujek and Thompson 2005). Watanabe et al. (2006) recently demonstrated that the sarcinoid cell organization, and therefore the family Chlorosarcinaceae, is widely polyphyletic. This is in congruence with our phylogenetic analyses, inferring *Chlorochytrium* in a well resolved lineage together with *Chlorosarcinopsis*, *Chlorosphaeropsis*, *Pachycladella* Silva, and *Spongiochloris* Starr (Fig. 1). Whereas the former two genera form characteristic sarcinoid cell assemblages, the latter two have a solitary cell organization.

The two strains belonging to the clade B differ in some respects from the original description of the genus *Chlorochytrium*. Cells reproduced only by the formation of autospores and zoospores (eleutheroschisis), and the sarcinoid morphology was not observed. Contrary to *Chlorochytrium*, zoospores were of the *Chlamydomonas* type, possessing a rigid cell wall (Starr 1955). The chloroplast did not form a net of connected ribbon-like lobes, but it was rather composed of densely appressed tubular lobes with several spherical holes. In addition, the cells contained only a single pyrenoid during all stages of chloroplast ontogeny. Therefore, their assignment to the genus *Chlorochytrium* was obviously incorrect. Based on these morphological characteristics, in particular the chloroplast structure, the strains of clade B coincide with the circumscription of the genus *Neospongiococcum* Deason (Deason 1976), including about 15 species (Ettl and Gärtner 1988). The morphology best fits the description of *N. concentricum* (Anderson & Nichols) Deason and *N. mahleri* Deason, which slightly differ in their maximum cell sizes and zoospore dimensions (Deason 1976). The genus *Neospongiococcum* is molecularly poorly characterized. Despite a number of strains deposited

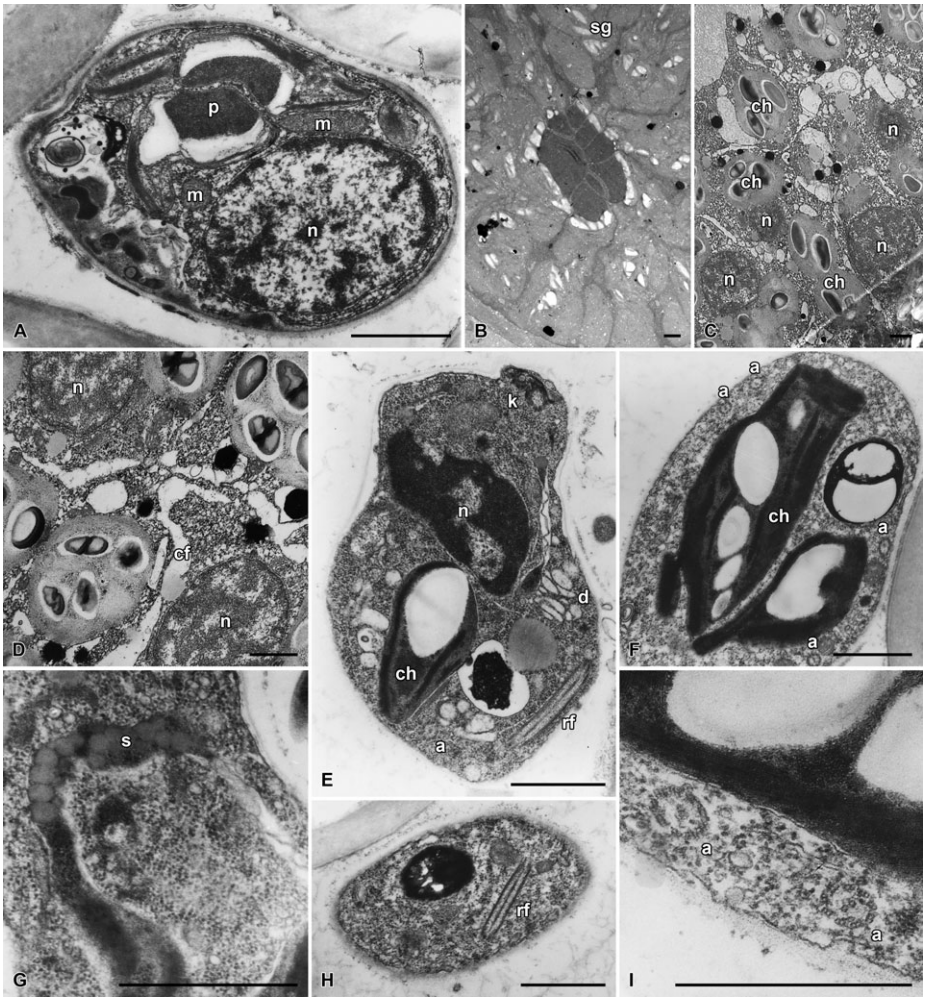

FIG. 7. TEM of *Scotinosphaera gibberosa* CAUP H5301. (A) A young vegetative cell with a single nucleus (n), mitochondrion (m), and a parietal chloroplast containing a large pyrenoid (p). (B) Pyrenoid of a mature vegetative cell invaginated by several cytoplasmatic channels. Note numerous starch grains (sg). (C) Early stage of protoplasm division. Note numerous chloroplasts (ch) and nuclei (n). (D) Late stage of protoplasm division. Note cleavage furrow (cf) formed between two nuclei. (E) Zoospore in longitudinal section. Axonema (a), chloroplast (ch), dictyosomes (d), kinetosomes (k), nucleus (n), retracted flagellum (rf). (F) Posterior region of a zoospore in longitudinal section. Note parietal chloroplast (ch) and four axonemal profiles (a). (G) Part of the zoospore chloroplast containing a stigma. (H) Section through the retracted flagellum (rf) in a sessile zoospore. (I) A pair of axonemal profiles (a) in a sessile zoospore. Scale bars: 1 μm.

in public culture collections (including the type species, *N. alabamense*), SSU rDNA sequence data are so far only available for a single species, *N. gelatinosum* (Archibald & Bold) Ettl & Gärtner (Fulnečková et al. 2012) This species is genetically allied to *Chlorococcum oleofaciens* Trainor & Bold and *C. sphacosum* Archibald & Bold, and distantly related to our strains, indicating a polyphyly of *Neospongiococcum*. This is not surprising as phylogenetic studies have shown polyphyly in many traditionally defined genera in the Chlamydomonadales (e.g., *Chlamydomonas* Ehrenberg, *Chlorococcum* Meneghini, *Tetracystis* Brown & Bold) (Nakada et al. 2008, Nakada and Nozaki 2009, Fulnečková et al. 2012).

The investigated *Scotinosphaera* strains shared several distinctive morphological characteristics, including large vegetative cells, up to 0.3 mm long, with an axial chloroplast composed of numerous anastomosing lobes. During the past century these characteristic green algae were commonly referred to as *Kentrosphaera* (e.g., Brunnthaler 1915, Reichardt 1927, Smith 1933, Korshikov 1953, Bourrelly 1966, Vodeničarov and Benderliev 1971, Punčochářová 1992). In a detailed taxonomic revision, Wujek and Thompson (2005) synonymized *Kentrosphaera* with the earlier *Scotinosphaera* (Klebs 1881c). Punčochářová (1992) was aware of the earlier description of *Scotinosphaera*, but regarded the genus name as invalid since "the structure of its vegetative cells was not described or illustrated," thereby considering Klebs's description as a mere observation of sporogenesis of an unspecified alga. We reexamined the publication of Klebs (1881c) and concur with the conclusion of Wujek and

FIG. 8. Original drawing of *Chlorochytrium lemnae* endophytic cells (Cohn 1872, Taf. II).

Thompson (2005) that *Scotinosphaera* was validly described. Even though the majority of Klebs's descriptions and drawings focused on the cell cycle and sporogenesis, the morphology of vegetative cells was sufficiently described as well. Moreover, we consider presented cytomorphological data as important for the unambiguous delimitation of *Scotinosphaera* and its differentiation from morphologically similar taxa. Accumulation of secondary carotenoids in the cell periphery and a quickly repeated mitosis without parallel cell wall synthesis are among the main diagnostic features of the genus. We observed both above-mentioned developmental stages in our studied strains (Figs. 5G, 5H and 7C), lending additional support for their assignment to the genus *Scotinosphaera*. In addition, TEM investigations showed the presence of a unique pyrenoid ultrastructure, also observed by Watanabe and Floyd (1994). The pyrenoid matrix is not penetrated by thylakoid membranes as is usual in various green algae (Pickett-Heaps 1975), but instead dissected by numerous anastomosing cytoplasmatic channels (Fig. 7A and B). To our knowledge, this pyrenoid ultrastructure was never reported for any other green algal taxa.

Our molecular phylogenetic analyses placed all investigated *Scotinosphaera* strains into the distinct, highly divergent, and strongly supported clade within Ulvophyceae (Figs. 1 and 3). Such phylogenetic position, as well as the above-mentioned unique morphological and ultrastructural features warrants the recognition of a new Ulvophycean order, Scotinosphaerales.

Scotinosphaerales Škaloud, Kalina, Nemjová, De Clerck et Leliaert, **ord. nov.**

Free-living, rarely endophytic, freshwater or aeroterrestrial algae. Cells solitary, uninucleate, variable in shape, often with one to several local cell wall thickenings. Chloroplast forming a net of numerous radiating and anastomosing lobes expanding from two or more pyrenoids toward the cell periphery. Pyrenoid matrix dissected by numerous anastomosing cytoplasmatic channels. Asexual reproduction by zoospores and autospores. Sporogenesis initiated with accumulation of secondary carotenoids in the cell periphery, followed by a quickly repeated mitosis without parallel cell wall synthesis. Zoospores biflagellate, naked, produced in high numbers.

Scotinosphaeraceae Škaloud, Kalina, Nemjová, De Clerck et Leliaert, **fam. nov.**

Characters as for order.

Genus *Scotinosphaera* Klebs 1881; Bot. Zeit. 39, p. 300, Taf. IV, Figs 55–63; type species: *S. paradoxa* Klebs.

The discovery of a new lineage of freshwater, unicellular Ulvophyceae has implications for our understanding of the evolution of the clade. The Ulvophyceae is best known for its macroscopic representatives that abound in marine coastal environments (Bryopsidales, Dasycladales, Cladophorales and Ulvales), with some members having adapted to freshwater (e.g., *Aegagropila* clade and some species of *Cladophora* Kützing and *Ulva* L.) or terrestrial habitats (Trentepohliales; López-Bautista et al. 2006, Mareš et al. 2011, Boedeker et al. 2012). These macroscopic ulvophytes encompass a wide range of thallus forms, including multicellular (Ulvales/Ulotrichales, Trentepohliales), siphonocladous (Cladophorales) and siphonous thalli (Bryopsidales and Dasycladales) (Cocquyt et al. 2010, Leliaert et al. 2012). In addition, several microscopic members from marine, freshwater or damp subaerial habitats have recently been found to form distinct lineages of Ulvophyceae (Leliaert et al. 2012). The Oltmannsiellopsidales includes a small number of flagellates, coccoids, and colonies from marine and freshwater environments (Hargraves and Steele 1980, Chihara et al. 1986, Friedl and O'Kelly 2002), and has been inferred to diverge near the base of the Ulvales-Ulotrichales clade (Nakayama et al. 1996, Cocquyt et al. 2010). Another distinct lineage, the *Ignatius* clade, includes coccoids from damp terrestrial habitats, and has been inferred as sister lineage to the Ulvales/Ulotrichales (Watanabe and Nakayama 2007) or sister to the clade containing Trentepohliales, Cladophorales, Bryopsidales, and Dasycladales (Cocquyt et al. 2010). In addition, several unicellular and sarcinoid members have been found in the Ulvales/Ulotrichales clade (e.g., *Desmochloris* Watanabe, Kuroda & Maiwa, *Halochlorococcum* Dangeard, *Pseudoneochloris* Watanabe, Himizu, Lewis, Floyd & Fuerst) (Watanabe et al. 2001, Pröschold et al. 2002, O'Kelly et al. 2004a,b).

Based on a multi-gene phylogeny of green algae it was suggested that the ancestral ulvophyte may have been unicellular and that macroscopic growth was achieved independently in various lineages (Cocquyt

et al. 2010). Even though the exact phylogenetic position of the Scotinosphaerales remains unclear, this study enforces the notion that non-motile unicellular freshwater organisms have played an important role in the early diversification of the Ulvophyceae.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Bayesian majority rule tree of the Chlorophyta based on a 10-gene alignment, showing the phylogenetic positions of Chlorochytrium and Scotinosphaera.

Table S1. List of primers used for PCR amplification and sequencing.

Table S2. PCR cycling conditions and reaction mixture composition.

Table S3. List of taxa used in the phylogenetic analysis of the concatenated *rbc*L-SSU alignment and GenBank accession numbers.

Table S4. List of taxa used in the phylogenetic analysis of the concatenated 10-gene alignment and GenBank accession numbers. Newly generated sequences are in bold.