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CHARACTERIZATION AND PHYLOGENETIC POSITION OF THE ENIGMATIC GOLDEN ALGA *PHAEOTHAMNION CONFERVICOLA*: ULTRASTRUCTURE, PIGMENT COMPOSITION AND PARTIAL SSU rDNA SEQUENCE[1]

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ABSTRACT

The morphology, ultrastructure, photosynthetic pigments, and nuclear-encoded small subunit ribosomal DNA (SSU rDNA) were examined for Phaeothamnion confervicola *Lagerheim strain SAG119.79. The morphology of the vegetative filaments, as viewed under light microscopy, was indistinguishable from the isotype. Light microscopy, including epifluorescence microscopy, also revealed the presence of one to three chloroplasts in both vegetative cells and zoospores. Vegetative filaments occasionally transformed to a palmelloid stage in old cultures. An eyespot was not visible in zoospores when examined with light microscopy, but small droplets, similar to eyespot droplets, were apparent beneath the shorter flagellum when cells were viewed with electron microscopy. Zoospores had two flagella that were laterally inserted in the cell approximately one-third of the cell length from the apex. The longer flagellum was directed anteriorly and the shorter flagellum was directed posteriorly. Electron microscopy revealed the presence of tubular tripartite flagellar hairs on the longer flagellum, but no lateral filaments were found on the tripartite hairs. The general organization of the flagellar root system was similar to that of zoospores belonging to the Xanthophyceae and Phaeophyceae. However, the transitional region of the flagella contained a transitional helix with four to six gyres. Microtubular root R_1 consisted of six microtubules at its proximal end and one microtubule at its distal end. Roots R_2 and R_4 consisted of one microtubule each and root R_3 consisted of two microtubules. No rhizoplast was found. Thin-layer chromatography revealed the presence of fucoxanthin, diadinoxanthin, neoxanthin, and heteroxanthin as well as chlorophylls* a, c_1 *and* c_2*. High-performance liquid chromatography revealed the presence of fucoxanthin, diadinoxanthin, diatoxanthin, heteroxanthin, and β,β-carotene as well as chlorophylls* a *and* c*. The complete sequence of the SSU rDNA could not be obtained, but a partial sequence (1201 bases) was determined. Parsimony and neighbor-joining distance analyses of SSU rDNA from* Phaeothamnion *and 36 other chromophyte algae (with two Öomycete fungi as the outgroup) indicated that* Phaeothamnion *was a weakly supported (bootstrap = <50%, 52%) sister taxon to the Xanthophyceae representatives and that this combined clade was in turn a weakly supported (bootstrap = <50%, 67%) sister to the Phaeophyceae. Based upon ultrastructural observations, pigment analysis, and SSU rDNA phylogenetic analysis,* Phaeothamnion *is not a member of the Chrysophyceae and should be classified as* incertae sedis *with affinities to the Xanthophyceae and Phaeophyceae.*

Key index words: 18S rRNA; algae; carotenoids; chlorophylls; Chrysophyceae; flagellar apparatus; heteroxanthin; microtubular roots; Phaeophyceae; Phaeothamnion; *phylogeny; Xanthophyceae*

Abbreviations: bp, boiling point; HPLC, high-performance liquid chromatography; SSU rDNA, nuclear-encoded small subunit ribosomal DNA

Phaeothamnion is a genus of freshwater filamentous algae currently classified in the Chrysophyceae (e.g. Bourrelly 1957, Preisig 1995). However, in the past, *Phaeothamnion* has been placed in the Phaeophyceae (West 1904) and the Cryptophyceae (Pascher 1914). *Phaeothamnion confervicola* Lagerheim is the type species (Lagerheim 1884, as *P. confervicolum*), and it is typified by specimens distributed as no. 608 in the *Algae Aquae Dulcis Exsiccatae* (Wittrock and Nordstedt, fasc. 13). There are two described varieties, the nominate variety and *P. confervicola* var. *brittanica* Godward. *Phaeothamnion,* in turn, is the type genus for the family Phaeothamniaceae and order Phaeothamniales (Bourrelly 1957) or suborder

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Phaeothamnineae (Bourrelly 1981, Starmach 1985). Four additional species have been described: *P. articulatum* Ettl, *P. borzianum* Pascher, *P. dichrysis* Villaret, and *P. polychrysis* (Geitler) Pascher. These species are separated on the basis of chloroplast number, presence or absence of an eyespot in the zoospore, cell size, basal cell morphology, cell wall thickness, branching patterns of the thalli, and other morphological features. The type description for *P. confervicola* indicates that it has one chloroplast (Lagerheim 1884), whereas others report more than one chloroplast in at least some cells (Godward 1933, Geitler and Schiman-Czeika 1970). Also, an erroneous cell measurement has made its way into the literature; Starmach (1985) gives the cell length for *P. confervicola* as 20–30 μm even though Lagerheim (1884) clearly reports the cell length to be no more than 12 μm.

Several additional golden-colored algae with filamentous or sarcinoid habits have, at one time or another, been assigned to the Phaeothamniales. Many of these algae, especially those placed in the Sarcinochrysidales by Gayral and Billard (1986), are no longer assignable to the Chrysophyceae (O'Kelly 1989a, Preisig 1995, Saunders et al. 1997). Preisig (1995) refers only the genera *Chrysoclonium, Phaeothamnion, Sphaeridiothrix,* and *Tetrachrysis* to the Phaeothamniaceae and places this family in the Chromulinales. *Sphaeridiothrix* has been considered a palmelloid stage of *Phaeothamnion* by Geitler and Schiman-Czeika (1970), but others have considered it to be separate (e.g. Pascher and Vlk 1943, Bourrelly 1963, Andrews 1970, Dop and Vroman 1976, Santos 1976, Cambra 1989). To further complicate matters, the xanthophyte genus *Heterodendron,* with two species, may also be referable to *Phaeothamnion* (Ettl 1959). Species of *Heterodendron* are otherwise nearly indistinguishable from *Phaeothamnion* except for their yellow-green plastids, and they have been assigned to the Xanthophyceae as a morphological parallel to *Phaeothamnion* (Steinecke 1932, Pascher 1939). However, Ettl (1959) suggests that this color difference reflects growth conditions, not differences in diagnostic chloroplast pigments.

Unlike most typical Chrysophyceae sensu stricto, *Phaeothamnion* species have branching filamentous habits and lamellate cell walls but lack siliceous cysts (= statospores, stomatocysts). Our initial examinations of zoospore morphology and pigment composition also indicated that *Phaeothamnion* is not a typical chrysophycean algae. In this paper, we report observations on a cultured isolate of *P. confervicola,* including light and electron microscopy, pigment composition, and partial nucleotide sequence of the nuclear-encoded small subunit rDNA gene (SSU rDNA). These observations suggest that *Phaeothamnion* does not belong to the Chrysophyceae but instead is a member of the clade that also includes yellow-green algae (Xanthophyceae) and brown algae (Phaeophyceae).

MATERIALS AND METHODS

Phaeothamnion sp. strain SAG119.79 was obtained from the Sammlung von Algenkulturen at the University of Göttingen, Germany (Schlösser 1994). Cells were grown in DYIII (Lehman 1976) or DYIV (Andersen et al. 1997) culture media at 12–25° C under ca. 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ visible light irradiance and either a 14:10 h or a 12:12 h LD photocycle. The vegetative features of this strain were compared with isotype material of *P. confervicola* obtained from the Farlow Herbarium of Harvard University (no. 608 in the *Algae Aquae Dulcis Exsiccatae,* Wittrock and Nordstedt, fasc. 13).

Free-swimming zoospores were obtained by placing filaments from an old culture (at least 30 days old) into fresh culture medium. During the light cycle on the second and third day following the culture medium change, zoospore release occurred from most filaments. For electron microscopy, whole mount preparations were prepared by collecting zoospores with centrifugation and drying cells directly on grids coated with pioloform (Ted Pella Inc., Redding, California). Cells were negatively stained with a saturated aqueous solution of uranyl acetate. Zoospores prepared for electron microscopic thin sectioning were fixed for 30–60 min at room temperature (ca. 20° C) in a cocktail consisting of 1 mL 5% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7, 0.5 mL 4% aqueous osmium tetroxide, and 8.5 mL zoospores in DYIII medium. The cells were harvested by gentle filtration onto cellulose acetate filters (3.0-μm pore size), enrobed in 3.0% aqueous ultralow-temperature gelling agarose, dehydrated in an acetone series at 4° C, and embedded in Polybed-Araldite resin (Ted Pella Inc.).

For pigment analysis by thin layer chromatography (TLC), cells were concentrated by centrifugation, and pigments were extracted from cell pellets using a Turrax macerator (Thomas Scientific, Swedesboro, New Jersey) and various combinations of acetone, methanol, acetone:methanol (1:1), and DMSO. The extract was mixed with one-half volume of diethyl ether followed by an excess of 10% aquaeous NaCl (w/v) to concentrate the pigments in the epiphase. The epiphase volume was reduced with a stream of nitrogen (N_2) gas. The concentrated extract was stored in darkness at −15° C over anhydrous NaCl to remove water. Six TLC systems were used. System 1 was a silica gel G/borax plate (Keast and Grant 1976) and a solvent solution of petroleum ether (60°–80° C bp):ethyl acetate:diethylamine (58:30:12) (Riley and Wilson 1965). System 2 was a silica gel G/borax plate and a solvent solution of 20% acetone in *n*-hexane (Chapman 1965). System 3 was a silica gel G/borax plate and a solvent solution of 40% acetone in *n*-hexane (Chapman 1965). System 4 was a silica gel G/borax plate and a solvent solution of 30% acetone in petroleum ether (60°–80° C bp) (Yokahama 1983). System 5 was a polyethylene (Polysciences Inc.) plate and a solvent solution of 100% acetone (Jeffrey 1972). System 6 was a reversed-phase RP-8 F_{254} plate and a solvent solution of 90% methanol (Jeffrey 1987).

For pigment analysis by high-performance liquid chromatography (HPLC), cells were collected on a 25-mm Whatman GF/F glass fiber filter and stored under liquid nitrogen prior to analysis to improve extraction efficiency and minimize pigment alterations. The filter was disrupted in 3 mL acetone by sonication (0° C, in the dark) and allowed to extract for 24 h (0° C, in the dark). Prior to analysis, the pigment extract was vortexed and centrifuged to separate cellular and filter debris. Samples (200 μL) of a mixture of 0.3 mL H_2O plus 1.0 mL extract were injected onto a Varian 5000 HPLC system equipped with a Varian autosampler, a Timberline column heater (26° C), and Spherisorb 5-μm ODS2 analytical (4.6 × 250 mm) and guard (4.6 × 50 mm) columns. Pigments were detected with a ThermoSeparation UV2000 detector (λ = 436 nm). Separations were performed with a ternary solvent system: eluent A (MeOH:0.5 M ammonium acetate, 80:20), eluent B (acetonitrile:water, 85:15), and eluent C (ethyl acetate). The linear gradient used for pigment separation was a modified version of the Wright et al. (1991) method: 0.0′ (100% A), 2.0′ (100% B), 2.6′ (90% B, 10% C), 12.6′ (65% B, 35% C), 18.0′ (31% B, 69% C), 28.0′ (31% B, 69% C), and 30.0′ (100% B). HPLC-grade solvents (Fisher) were used to prepare eluents

A, B, and C. The eluent flow rate was held constant at 1 $mL \cdot min^{-1}$. Peaks were identified by (i) on-line absorbance spectroscopy (400–700 nm) and (ii) comparison of retention times with those of pure standards (monovinyl chlorophyll *a*, fucoxanthin, diadinoxanthin, diatoxanthin, and β,β-carotene) and extracts prepared from plant materials of known pigment composition (*Phaeodactylum tricornutum* Bohlin, diatom; *Nannochloropsis salina* Hibberd, eustigmatophyte; *Botrydium stoloniferum* Mitra, xanthophyte; and green grass). The HPLC method employed was not capable of separating chlorophyll c_1 from chlorophyll c_2. Pigment concentrations ($ng \cdot mL^{-1}$ extract) were calculated using internal standards provided as part of the U.S. JGOFS pigment intercalibration exercise (see Latasa et al. 1996).

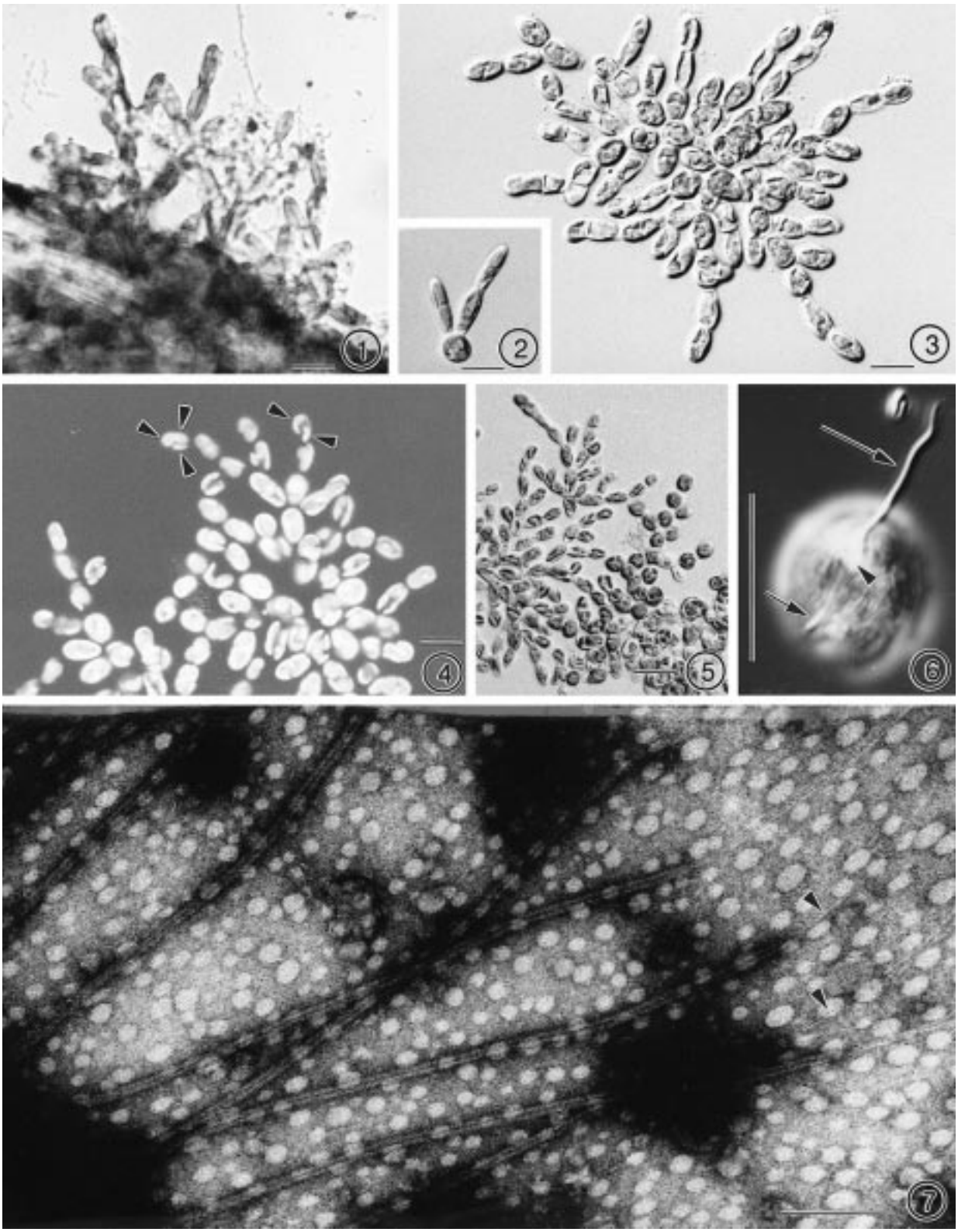
For gene sequence analysis, DNA was extracted using a modified CTAB extraction protocol (Doyle and Doyle 1987). The SSU rDNA was amplified via PCR using the Perkin Elmer Gene Amp II kit and two primers [5′ → 3′ = CGGTAATTCCAGCTCC; 3′ → 5′ = GATCCTTCTGCAGGTTCACCTAC]. PCR products were isolated using a 0.8% agarose gel, and the DNA was separated from the agar using a Gene Clean II kit (Bio 101). Nucleotide sequences of PCR products were determined in both directions using the Perkin Elmer AmpliTaq Cycle Sequencing Kit with 5′-biotinylated sequencing primers. Sequencing reaction products were separated on 6% Long Ranger (AT Biochem) acrylamide gels and transferred to nylon membranes (Millipore). Sequences were visualized by treating the membranes with the New England Biolabs Phototope Detection Kit and exposing them to x-ray film. Sequences were read by eye and the partial sequence was deposited in GenBank (accession number AF044846). The *Phaeothamnion* partial sequence combined with complete sequences of representative organisms of other chromophyte groups (GenBank accession number or literature source follow each scientific name and authority): *Achlya bisexualis* Coker (M32705), *Alaria marginata* Postels et Ruprecht (from Saunders and Druehl 1992), *Apedinella radians* (Lohmann) Campbell (U14384), *Aureococcus anaphagefferens* Hargraves et Sieburth (from DeYoe et al. 1995), *Aureoumbra lagunensis* (from DeYoe et al. 1995), *Botrydiopsis intercedens* Vischer et Pascher (U41647), *Botrydium stoloniferum* Mitra (U41648), *Chattonella subsalsa* Biecheler (U41649), *Chromulina chionophila* Stein (M87332), *Costaria costata* (C. A. Agardh) Saunders (X53229), *Cylindrotheca closterium* (Ehr.) Reimann et Lewin (M87326), *Dictyocha speculum* Ehr. (U14385), *Fucus distichus* L. (M97959), *Giraudyopsis stellifera* Dangeard (U78034), *Heterosigma akashiwo* (Hada) Hada (U41650), *Hibberdia magna* (Belcher) Andersen (M87331), *Lagenidium giganteum* (M54939), *Mallomonas papillosa* Harris et Bradley (M55285), *Mallomonas striata* Harris et Bradley (Bhattacharya et al. 1992), *Nannochloropsis granulata* Karlson et Potter (U38902), *Nannochloropsis oculata* (Belcher) Hibberd (U38903), *Nitzschia apiculata* (Greg.) Grunow (M87334), *Ochromonas danica* Pringsheim (M32704), *Pelagococcus subviridis* Norris (U14386), *Pelagomonas calceolata* Sanders et Andersen (U14389), *Pseudopedinella elastica* Skuja (14387), *Pulvinaria* sp. (U78032), *Rhizochromulina* sp. (U14386), *Rhizosolenia setigera* Brightwell (M87329), *Sarcinochrysis marina* Geitler (U78033), *Stephanopyxis* cf. *broschii* (M87330), *Synura spinosa* Korshikov (M87336), *Scytosiphon lomentaria* (Lyngbye) C. A. Agardh (L43066), *Tribonema aequale* Pascher (M55286), *Vacuolaria virescens* Cienkowski (U41651), *Vaucheria bursata* (O. F. Müller) C. A. Agardh (U41646), *Xanthonema debile* (Vischer) Silva (U43277), and an undescribed coccoid strain CCMP1144 (U40929). The missing nucleotide bases of the partial *Phaeothamnion* sequence were coded as "N." Sequences were aligned using MALIGN (Wheeler and Gladstein 1993) and refined by eye. Ambiguously aligned sites were excluded from phylogenetic analyses. Phylogenetic analyses were conducted using test version 4.0.0d60 of PAUP*, written by David L. Swofford. Bootstrapping (1000 replicates) was employed with both parsimony analysis and Tamura-Nei distance analysis (neighbor-joining search option) to assess relative support for branches (Felsenstein 1985).

RESULTS

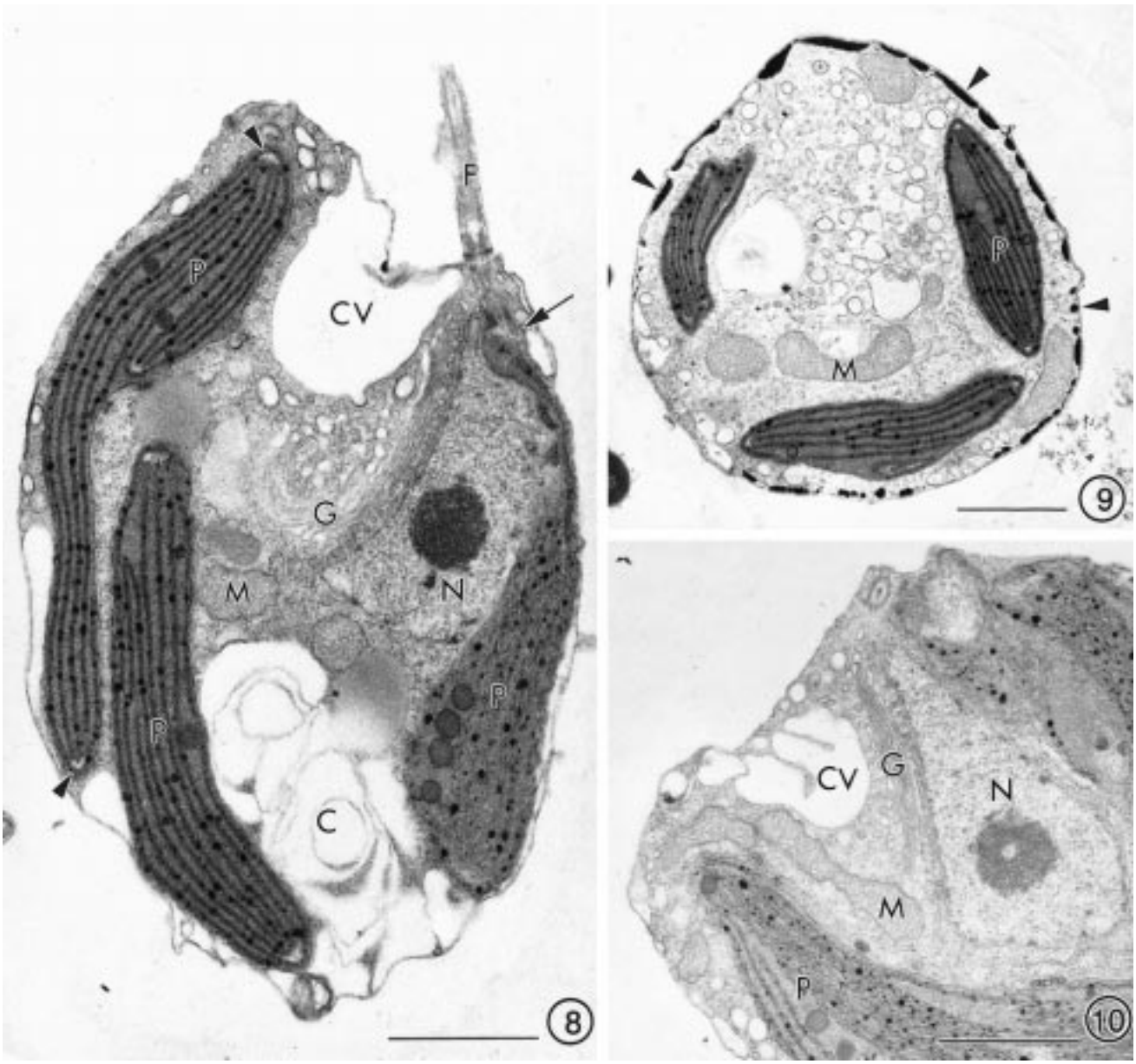
Light microscopy. Type material of *Phaeothamnion confervicola* was examined and, for comparative purposes, a photograph from the type material is provided (Fig. 1). Vegetative cells of *Phaeothamnion* strain SAG119.79 were typically 4–8 μm wide and 6–11(15) μm long (Figs. 2, 3). Cells appeared to have one parietal chloroplast that was frequently lobed, but examination with epifluorescence microscopy revealed that some cells had two (rarely three) chloroplasts (Fig. 4). Rapidly growing cells were more elongate (Fig. 2), whereas those growing more slowly were wider (Fig. 3). Chloroplasts lacked a pyrenoid, and small granular bodies were often visible in the cytoplasm. The living cells were always golden brown in color. In older cultures, filaments were occasionally transformed into palmelloid forms where the cell arrangement generally maintained a filamentous appearance (Fig. 5). Zoospore formation was common following the transfer of older filaments into fresh culture medium, and the zoospores were released through a pore on the lateral cell wall (not shown). Zoospores had a long, anteriorly directed, hairy flagellum (immature flagellum) and a short, posteriorly directed, smooth flagellum (mature flagellum) (Fig. 6). The flagella were inserted laterally, about ⅓ of the way down from the anterior end. Zoospores contained one to three chloroplasts.

Electron microscopy. Whole mounts of negatively stained zoospores revealed that the long (immature) flagellum possessed tripartite hairs that lacked short and long lateral filaments (Fig. 7). Ultrastructural examination indicated that the zoospores lacked scales or a conspicuous surface coat on both the cell body and flagella (Figs. 8, 9). Small vesicles, many with electron-opaque contents, sometimes formed an almost continuous layer just inside the plasma membrane (Fig. 9). The zoospores shown in Figures 8 and 9 had three chloroplasts: one lined the ventral surface of the cell, and the other two were located nearer the dorsal surface (Figs. 8, 9). The chloroplasts had ring-shaped nucleoids and lacked pyrenoids. The single nucleus usually was found near the middle of the cell, close to the ventral surface, and the outer nuclear membrane was continuous with the chloroplast endoplasmic reticulum of the ventral chloroplast (Fig. 8). The anterior end of the ventral chloroplast approached the basal body complex (Figs. 8, 10). One side of the nucleus, facing the dorsal surface of the cell's anterior end, was flattened and was associated with a single large Golgi body (Figs. 8, 10). A large contractile vacuole was present anterior to the nucleus–Golgi complex and the basal bodies (Figs. 8, 10). Vesicles bearing tripartite tubular hairs were not observed. Mitochondrial profiles were present throughout the cell. The mitochondria had tubular cristae lacking intracristal inclusions (Figs. 8, 9). Large vacuoles, presumably containing a carbohydrate storage product, were located posterior to the nucleus (Fig. 8).

The flagellar apparatus in free-swimming zoospores was centered on basal bodies located in the



FIGS. 1–7. *Phaeothamnion confervicola*. FIG. 1. Filaments from the isotype, dried on a mica sheet and photographed *in situ* without wetting. Scale bar = 10 μm. FIGS. 2–7. Strain SAG119.79. FIG. 2. Young filament showing basal cell (spherical) and three elongate cells. Nomarski interference microscopy. Scale bar = 10 μm. FIG. 3. Filamentous mass (flattened by coverslip) typical in cultures. Nomarski interference microscopy. Scale bar = 10 μm. FIG. 4. Filaments viewed with epifluorescence microscopy. Chloroplasts indicated by arrowheads. Scale bar = 10 μm. FIG. 5. Typical filamentous cells (left) and palmelloid cells (right). Scale bar = 5 μm. Nomarski interference microscopy. FIG. 6. Zoospore showing longer anteriorly directed flagellum (long arrow), shorter posteriorly directed flagellum (short arrow), and lateral insertion of flagella (arrowhead). Scale bar = 5 μm. FIG. 7. Transmission electron micrograph of negatively stained flagellar hairs showing the lack of lateral filaments on the shaft and the single terminal hair (arrowheads). Scale bar = 200 nm.



FIGS. 8–10. Zoospore ultrastructure of *Phaeothamnion confervicola* strain SAG 119.79. Scale bars = 1 μm. FIG. 8. Longitudinal section. C, putative carbohydrate storage material; CV, contractile vacuole; F, anteriorly directed flagellum; G, Golgi body; M, mitochondrion; P, chloroplast. Note the chloroplast lobe adjacent to the basal body of the posteriorly directed flagellum (arrow) and the presence of a ring genophore (arrowheads) in the chloroplast. FIG. 9. Transverse section. Note the vesicles with electron-opaque material (arrowheads) immediately beneath the plasma membrane. FIG. 10. Oblique section showing the contractile vacuole, Golgi body, and nucleus in close proximity. Note also the chloroplast lobe closely associated with the flagellar basal body.

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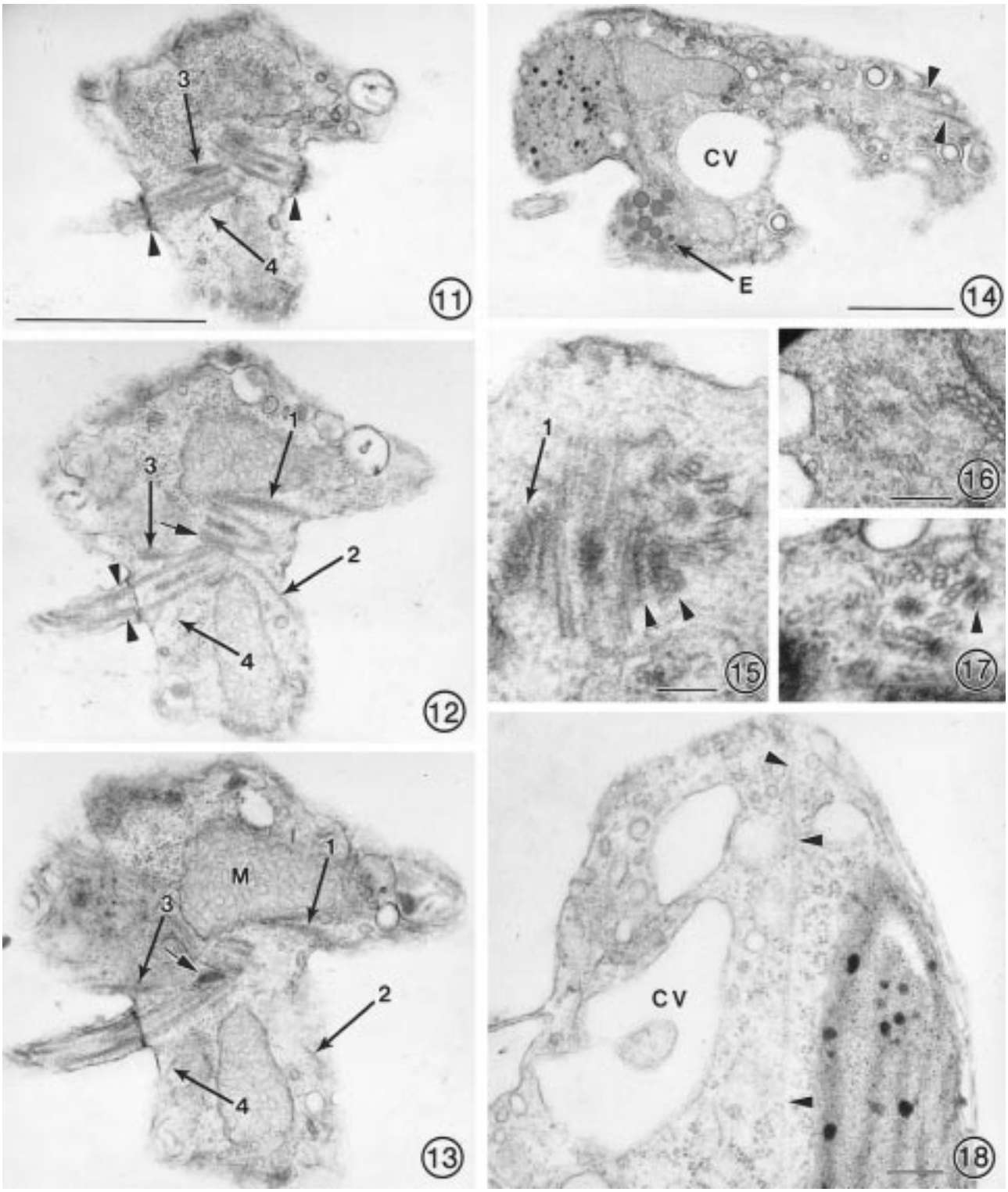
FIGS. 11–18. Zoospore ultrastructure of *Phaeothamnion confervicola* strain SAG 119.79. FIGS. 11–13. Adjacent serial sections showing the origins of microtubular roots. The anterior is toward the right side of the micrograph and the left side of the cell is toward the bottom. The series progresses from near the ventral surface toward the dorsal surface. Scale bar = 1 μm. FIG. 11. The two basal bodies are oriented at approximately 145°. The origins of microtubular roots R_3 (3) and R_4 (4) are from the surface of the mature basal body. Note the dense ring (arrowheads) on each flagellum at the plane of the transitional plate. FIG. 12. Next adjacent section showing the proximal regions of the four microtubular roots (R_1 = 1, R_2 = 2, R_3 = 3, R_4 = 4). Note the transitional helix between the arrowheads and the



striated band (short arrow), which connects the basal bodies. FIG. 13. Next adjacent section showing the four microtubular roots (1–4) and the striated band (short arrow) connecting the basal bodies. FIG. 14. Nonadjacent section from the same series shown in Figs. 11–13. Scale bar = 1 μm. Note the droplets of the putative eyespot (E) and the contractile vacuole (CV). Microtubules associated with root R_1 are present (arrowheads). Scale bar = 1 μm. FIG. 15. Obliquely longitudinal section through the immature basal body showing the proximal end of the R_1 root and a transverse section through the mature basal body. Note the striated band (arrowheads) connecting the two basal bodies. Scale bar = 100 nm. FIG. 16. A nearly transverse section through the immature basal body showing the six microtubules of the R_1 root. Scale bar = 100 nm. FIG. 17. Transverse section through the mature basal body showing the two microtubules of the R_3 root (arrowhead). Scale bar = 100 nm. FIG. 18. Longitudinal section showing two cytoskeletal microtubules (arrowheads) that originate from the R_1 root. Scale bar = 200 nm.

anterior third of the cell on the ventral surface (Fig. 8). As an overview, it consisted of two basal bodies, a single small band connecting the basal bodies to each other, and four microtubular roots, two associated with each basal body. The basal bodies formed a large obtuse angle, typically 145° (Figs. 11–13). The long axis of the anteriorly directed immature basal body was parallel with the long axis of the cell (Fig. 8), whereas the long axis of the posteriorly directed mature basal body projected obliquely to the right, at an angle of approximately 30° relative to the plane tangential to the ventral surface of the cell and in the vicinity of the ventral chloroplast. The ventral chloroplast usually had lipid globules concentrated near the insertion of the posterior flagellum (Fig. 14).

The transitional region of each basal body consisted of a single transitional plate with a nondiscrete axosome lying close to the plane of the plasma membrane (Figs. 11, 12). Distal to the basal plate was an indistinct structure, interpreted as a transitional helix with four to six gyres (Fig. 12). A single coarsely striated band connected the proximal ends of the two basal bodies with each other (Figs. 12, 13, 15). No rhizoplast was observed and no other bands connecting the basal bodies to each other or to any other structure were observed.

Microtubular root R_1 originated on the left side of the immature basal body near its proximal end (Figs. 11–13, 15). The root path was along the left ventral surface of the cell, extending anteriorly to the cell apex while skirting the left edge of the contractile vacuole complex (Fig. 14) and terminating on the right side of the cell near the apex. Six microtubules were present at the proximal end of the root (Fig. 16), dwindling to a single microtubule distally (not shown). Root R_2 originated in the cleft between the two basal bodies. Its path was along the ventral surface of the cell, extending anteriorly and to the right and terminating in the vicinity of the contractile vacuole (Figs. 11–13). It consisted of a single microtubule throughout. Root R_3 arose near the proximal end of the mature basal body on the side facing the cell posterior (Figs. 11–13). It extended posteriorly and to the right of the cell on its ventral surface and terminated immediately posterior to the flagellar insertion. It had two microtubules throughout (Fig. 17). Root R_4 also arose near the proximal end of the mature basal body, but on the side facing the ventral surface of the cell (Figs. 11–13). It extended up to the right of the cell, and passed over the lipid globules (= eyespot, when present) in the chloroplast (Fig. 14) before turning to the posterior of the cell and terminating some distance away from the flagellar insertion. It consisted of a single microtubule throughout.

A few secondary cytoskeletal microtubules appeared at the distal end of the R_1 root, extending dorsally into the cell (Fig. 18). No other secondary cytoskeletal microtubules were observed. The main features of the flagellar apparatus are illustrated diagrammatically (Figs. 19, 20).

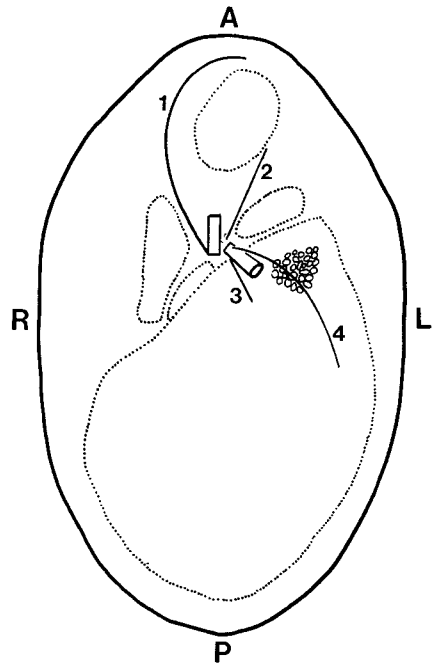


FIG. 19. Diagrammatic illustration of the flagellar apparatus showing the paths of the microtubular roots in the cell (R_1 = 1, R_2 = 2, R_3 = 3, R_4 = 4). The anterior (A) of the zoospore is at the top, posterior (P) at the bottom, the left surface (L) is at the right, the right surface (R) at the left, the ventral surface is toward the viewer, and the dorsal surface is toward the page. Note the eyespot-like droplets in the chloroplast underneath the R_4 root.

Pigment Analysis. The TLC systems preferentially separated some but not all of the pigments. System 1 separated all of the xanthophylls: fucoxanthin, diadinoxanthin, neoxanthin, and heteroxanthin. Isofucoxanthin appeared using System 4, but this may have been an artifact because it did not appear in the alkaline System 1. Systems 2 and 3 did not resolve neoxanthin and heteroxanthin. System 5 revealed the presence of chlorophylls c_1 and c_2, whereas System 6 indicated that chlorophyll c_3 was absent.

HPLC analysis revealed the presence of chlorophylls *a* and *c,* fucoxanthin, diadinoxanthin, diatoxanthin, and β,β-carotene (Fig. 21). In addition, a minor carotenoid, whose retention time was identical to that of heteroxanthin, eluted as a shoulder of the primary carotenoid fucoxanthin. The retention times and weight ratios (relative to chlorophyll *a*) are reported in Table 2.

SSU rDNA. The entire SSU rDNA would not amplify with PCR, but we were able to amplify a portion of the gene that began at a position that corresponded to nucleotide 613 of *Pelagomonas calceolata* (Andersen et al. 1993) and ended at the 3′ end of the gene, for a total of 1201 bases. Maximum parsimony analysis produced one most parsimonious tree (tree length = 1463, consistency index = 0.552, retention index = 0.689). However, bootstrap analysis re-

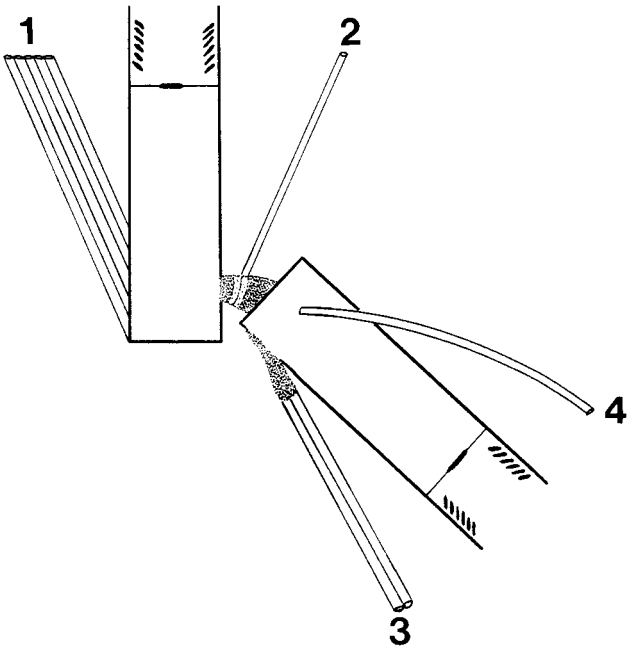


FIG. 20. Enlarged diagrammatic illustration of the basal bodies and the proximal ends of the microtubular roots (R_1 = 1, R_2 = 2, R_3 = 3, R_4 = 4). Orientation is the same as Fig. 19. Note the transitional helix above the transitional plate and the striated connecting band that links the two basal bodies.

vealed little support for deep branches (Fig. 22). *Phaeothamnion* had a weak (bootstrap = <50%) sister relationship to the Xanthophyceae, and this combined clade, in turn, was sister to the Phaeophyceae (bootstrap = <50%). The Chrysophyceae were paraphyletic with respect to the Synurophyceae, and the Eustigmatophyceae were weakly (bootstrap = <50%) aligned with the Chrysophyceae/Synurophyceae clade. An unusual and undescribed alga (strain CCMP1144) formed the deepest branch within the chromophyte algae. A parsimony analysis

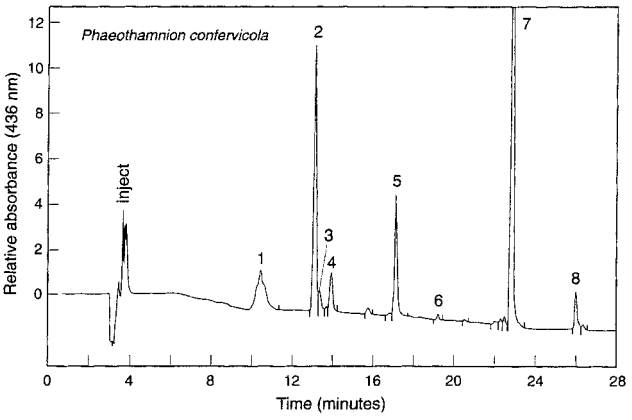


FIG. 21. Reversed-phase HPLC chromatogram (λ = 436 nm) of an acetone extract prepared from *Phaeothamnion confervicola* strain SAG119.79. Peak identities: (1) chlorophyll *c*-related pigments, (2) fucoxanthin, (3) heteroxanthin (shoulder), (4) *cis*-fucoxanthin, (5) diadinoxanthin, (6) diatoxanthin, (7) monovinyl chlorophyll *a*, and (8) β,β-carotene.

TABLE 1. *Morphological features of* Phaeothamnion *and* Heterodendron *taxa. When exact measurements were not provided, they were determined using the scale bar provided in the paper; these values are indicated with a ~ before them. Data not available are indicated with na.*

Taxon	No. of plastids	Cell length (μm)	Cell width (μm)	Palmelloid stage	Zoospore eyespot	Zoospore plastids	Branch pattern	Branches on the basal cell	Source
P. confervicola strain SAG119.79	1(–3)	6–11(15)	4–8	yes	no	3?	wide	yes	this study
P. confervicola	1	6–12	4–8	yes	no	1	wide	yes	Lagerheim (1884)
P. confervicola	1	~9–15	~5–7	yes	na	na	wide	yes	Geitler & Schiman-Czeika (1970)
P. confervicola	1	~12–18	~5–9	yes	no	1	wide	yes	Pascher (1925)
P. confervicola var. *britannica*	1–3	10–20	3–6	no	na	na	narrow	no	Godward (1933)
P. articulatum	1	8–12	3–5	no	yes	1	narrow	yes	Etl (1959)
P. borzianium	1	~9–14	~3–7	yes	yes	1	narrow	no	Pascher (1925)
P. borzianium	1	~10–20	~5–10	yes	yes	na	narrow	na	Dop & Vroman (1976)
P. dichrysis	2	10–15	~3–6	no	na	na	wide	yes	Villeret (1951)
P. pobychrysis	3–5	~15–20	~5–10	yes?	na	na	wide	yes	Pascher (1925)
H. paschери	1–3	8–12	3–5	no	no	3	wide	yes	Steinecke (1932)
H. squarrosum	1	9–18	≤6	no	yes	1	wide	yes	Pascher (1932)

TABLE 2. *Pigment composition of the freshwater alga* P. confervicola *strain SAG119.79 as determined by reversed-phase HPLC. Peak numbers refer to those given in Figure 1.*

Peak no.	Retention time (min)	Pigment identity	Pigment : chl *a* ratio (w/w)
1	10.43	chl *c*-related pigments	0.076
2	13.12	fucoxanthin	0.245
3	13.36	heteroxanthin[a]	0.013
4	13.98	*cis*-fucoxanthin[b]	0.041
5	17.11	diadinoxanthin	0.091
6	19.28	diatoxanthin[a]	0.004
7	22.86	monovinyl chlorophyll *a*	1.000
8	26.05	β,β-carotene	0.027

[a] Concentration calculated using the diadinoxanthin response factor.

[b] Concentration calculated using the fucoxanthin response factor.

was also conducted using only the aligned partial sequences for all taxa, and the position of *Phaeothamnion* as well as the position of most other taxa was unchanged (not shown). The distance analysis provided a very similar tree, with *Phaeothamnion* occupying a weak (bootstrap = 52%) sister relationship to the Xanthophyceae, and this combined clade, in turn, was sister to the Phaeophyceae (bootstrap = 67%, Fig. 23).

DISCUSSION

Examination of isotype material revealed no morphological features that could be used to separate *Phaeothamnion* sp. strain SAG119.79 from *Phaeothamnion confervicola,* and we conclude that this strain is *P. confervicola.* The type description for *P. confervicola* is clear and detailed (Lagerheim 1884), and the

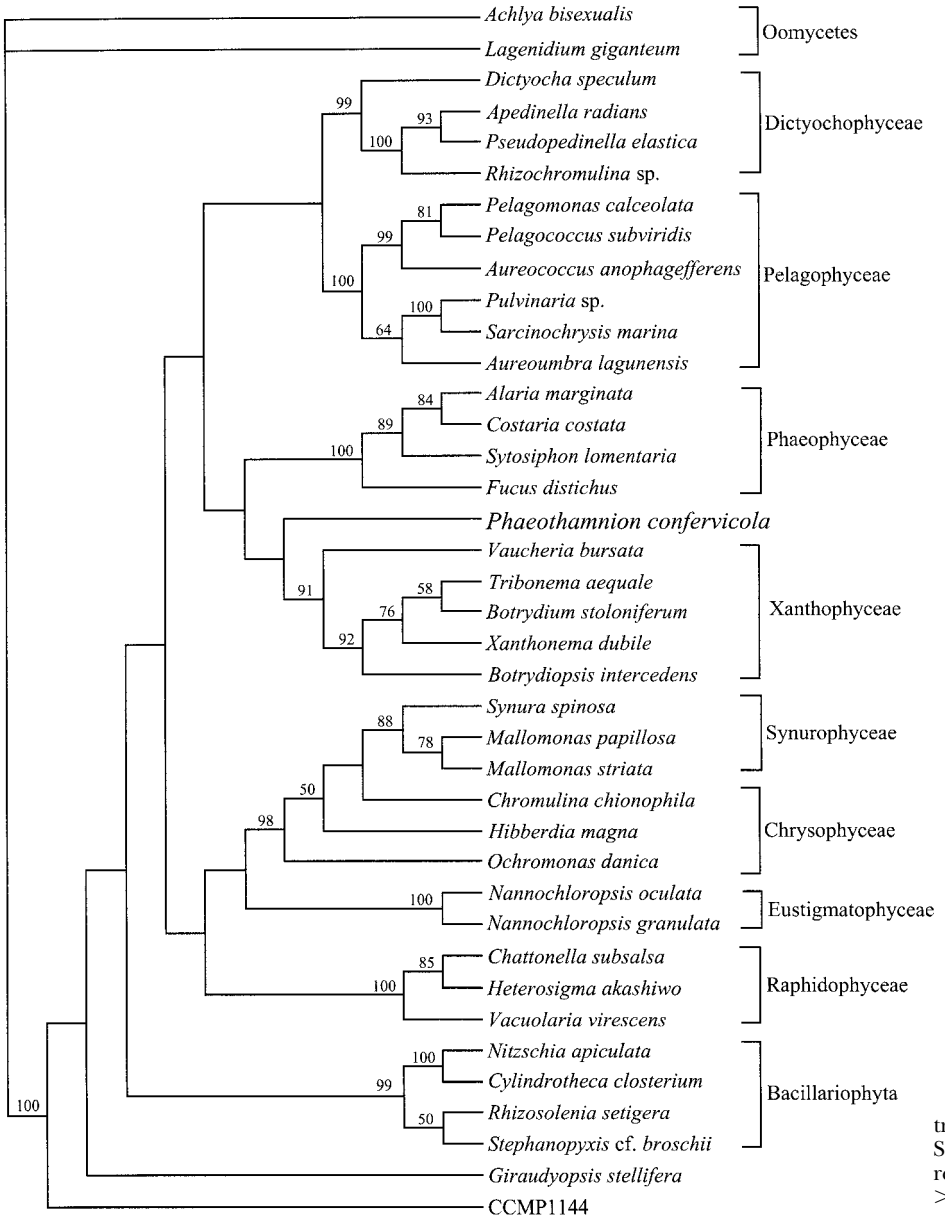


FIG. 22. Single most parsimonious tree based on a cladistic analysis of the SSU rDNA. Bootstrap values (% of 1000 replicates) are given when support is >50%.

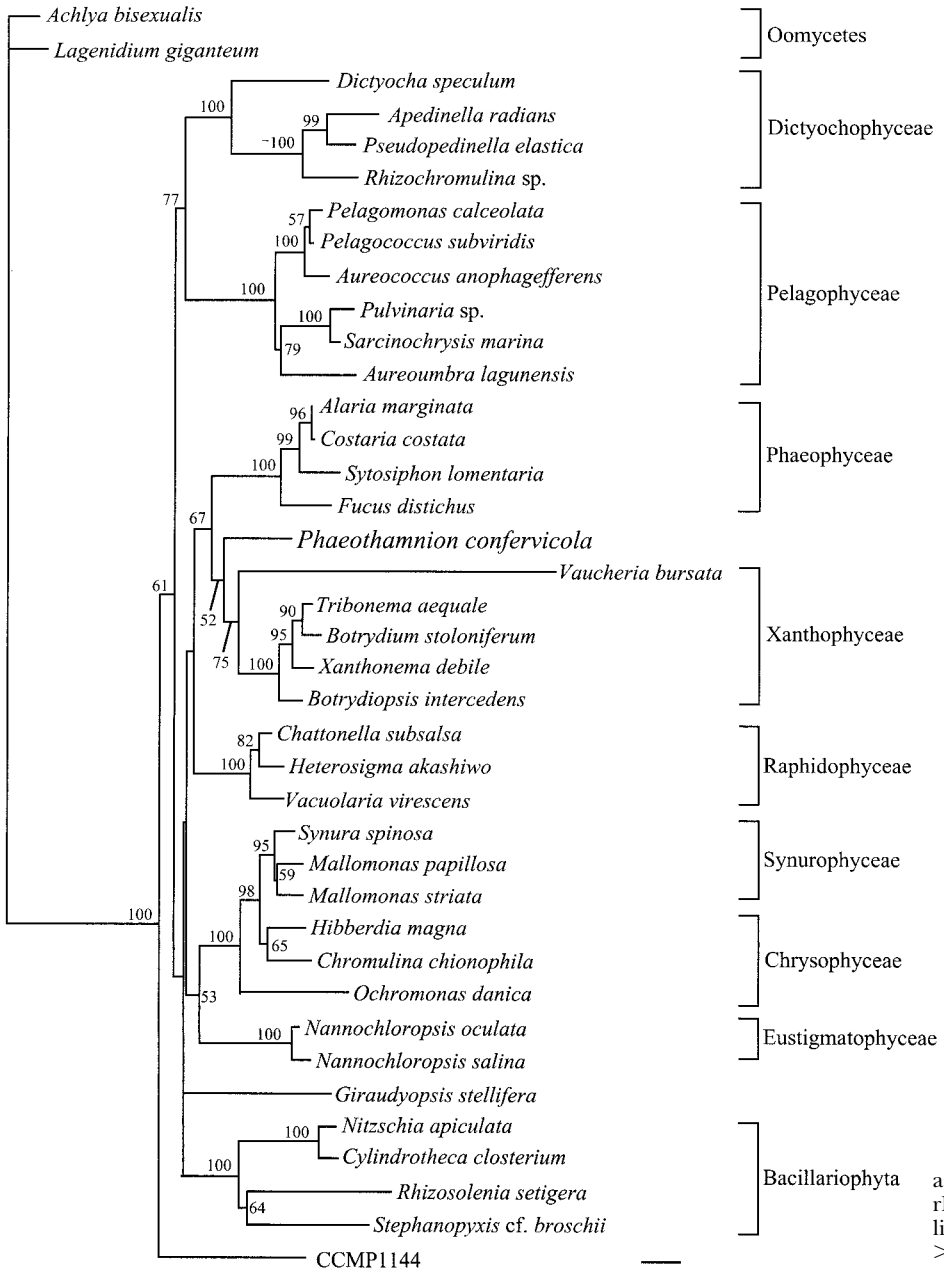


FIG. 23. Distance tree derived from a neighbor-joining analysis of the SSU rDNA. Bootstrap values (% of 1000 replicates) are given when support is >50%. Scale bar = 1% divergence.

Exsiccatae are well preserved. Our cell size measurements agree closely with those of Lagerheim (1884), although it should be noted that longer cells have been reported for specimens identified as *P. confervicola* (Table 1) (Pascher 1925, Geitler and Schiman-Czeika 1970). A minor discrepancy is the number of chloroplasts. We found one to three plastids per zoospore but others report only one (Lagerheim 1884, Pascher 1925). Also, we found occasional vegetative cells with two or three chloroplasts in our material but the number could not be demonstrated convincingly without epifluorescence. Epifluorescence microscopy was not available to earlier workers, and therefore they may have missed or ignored occasional cells with more than one chloroplast.

A number of taxa have been described for *Phaeothamnion,* and certain features of the strain we studied agree with descriptions for other taxa (see Table 1). These taxa, as described, can be distinguished based upon the characters listed (see Table 1), but careful examination of these taxa using modern techniques (epifluorescence and electron microscopy, DNA analysis) may be beneficial in evaluating them. Of interest, *Heterodendron pascheri* (Xanthophyceae) is similar in many respects to the strain we studied. Unfortunately, *Heterodendron* is not maintained in any culture collection, and we were unable to examine it. *Heterodendron* is characterized by a greenish color (hence its classification as a xanthophyte), and its zoospores are released at the tip of

cells, whereas those in our isolate of *Phaeothamnion* are released through lateral pores as described by Lagerheim (1884). Ettl (1959) considers *Heterodendron* to be synonymous with *Phaeothamnion* based upon his observations of both brown- and green-colored filaments; a similar color range was also reported by Godward (1933) for *P. confervicola* var. *britannica*. The possible synonymy of *Heterodendron,* as suggested by Ettl (1959) is not resolved by the results of this study. A thorough study of *Phaeothamnion* under controlled growth and illumination conditions may be required to determine the possible variation in individual carotenoid concentrations and thus the possible variability in cell color. During the present study, we never observed green-colored cells. One might speculate that *Heterodendron* evolved from *Phaeothamnion* and thereby gave rise to the Xanthophyceae, but preliminary studies using SSU rDNA and *rbc*L sequences strongly suggest that the siphonous *Vaucheria* is the most basal taxon of the Xanthophyceae and that the filamentous taxa are more derived (Daugbjerg and Andersen 1997b, Potter et al. 1997b).

The class-level classification of *Phaeothamnion* is not clear, but there is no support for its classification in the Chrysophyceae. Algae belonging to the class Chrysophyceae typically contain the carotenoid vioxanthin (Bjørnland and Liaaen-Jensen 1989, Rowan 1989), their swimming cells have a flagellar apparatus with two flagella oriented in an approximately perpendicular fashion (Andersen 1991), their flagella have tripartite flagellar hairs with short and long lateral filaments (Bouck 1971, Leadbeater 1989), and they produce siliceous cysts (Bourrelly 1957). *Phaeothamnion* has none of these features: it has the carotenoids diadinoxanthin, diatoxanthin, and heteroxanthin; its zoospore has two approximately antiparallel flagella; it has tripartite flagellar hairs that lack lateral filaments; and it does not produce silica cysts. Although Pascher (1925) reports the presence of cysts for *P. borzianium* when it is in the palmelloid stage, these ''cysts'' are almost certainly empty spherical cells having a pore through which the zoospores have escaped. Pascher did not describe cysts that contained protoplasm, he did not report the presence of an organic plug that is typical for chrysophycean cysts, and he did not demonstrate the presence of silica. No other worker has reported the presence of silica cysts or any other type of cysts for *Phaeothamnion*.

In addition to these differences, our phylogenetic analysis of the partial SSU rDNA sequence does not place *Phaeothamnion* in the Chrysophyceae; rather, it weakly aligns *Phaeothamnion* at the base of the Xanthophyceae. Although the SSU rDNA data suggest *Phaeothamnion* may have ties to the Xanthophyceae, it differs from the current definition for the Xanthophyceae. For example, *Phaeothamnion* contains fucoxanthin and no member of the Xanthophyceae is known to have fucoxanthin (Bjørnland and Liaaen-Jensen 1989, Rowan 1989). *Phaeothamnion* appears to have a single-gyred transitional helix in its flagella (Fig. 12) but the Xanthophyceae appear to have an atypical double-gyred helix if a helix is present (Hibberd 1979, O'Kelly 1989b). The flagellar root arrangement in *Phaeothamnion* resembles that found in the Xanthophyceae, but similar patterns are also present in the Phaeophyceae and the enigmatic *Giraudyopsis* (see O'Kelly and Floyd 1985, O'Kelly 1989a, Andersen 1991). Fucoxanthin is present in the Phaeophyceae (Bjørnland and Liaaen-Jensen 1989, Rowan 1989) but a transitional helix is absent (Hibberd 1979, O'Kelly 1989a, Preisig 1989). Thus, the position of *Phaeothamnion* between the phaeophyte and xanthophyte clades in the SSU rDNA tree is not unexpected considering the variously shared characters of *Phaeothamnion* and these two algal classes. It is interesting to note that Blackman (1900) placed *Phaeothamnion* at the base of the Phaeophyceae. However, a wealth of new data has been reported during the past 100 years, and the exact classification of *Phaeothamnion* is not yet clear. It seems likely that *Phaeothamnion* is more closely related to the Xanthophyceae and Phaeophyceae than to any other existing classes of algae, but it cannot be placed in either class given their current definitions. For the time being, we suggest *Phaeothamnion* be classified as *incertae sedis*.

The relationship of the Raphidophyceae to the *Phaeothamnion*/Xanthophyceae/Phaeophyceae clade is not supported in our phylogenetic analysis (Fig. 22). However, in a previous study based on SSU rDNA, a weak relationship of the Raphidophyceae to the Xanthophyceae/Phaeophyceae clade was reported (Potter et al. 1997b), and in a study based on the *rbc*L gene, very strong support for this relationship was found (Daugbjerg and Andersen 1997a). The inclusion of *Phaeothamnion* in a phylogenetic analysis based on the *rbc*L gene may help resolve the relationships of these taxa.

The weak or unresolved relationships of the deep branches of the chromophyte algae shown in our phylogenetic analysis (Fig. 22) have been found in other studies (e.g. Bhattacharya et al. 1992, Andersen et al. 1993, Leipe et al. 1994, Van de Peer et al. 1996, Potter et al. 1997b, Saunders et al. 1997). The ability of SSU rDNA data to resolve deep branches in chromophytes by itself has been questioned (Potter et al. 1997b). Studies that combine SSU rDNA data and traditional data (morphology, pigment data) have provided some additional resolution (Saunders et al. 1995, 1997, Potter et al. 1997b), but even when *rbc*L gene sequence data are added to these datasets, complete resolution is not achieved (Andersen et al., unpubl.). The reason(s) for this lack of resolution is (are) unclear. Leipe et al. (1994) have suggested there may have been a rapid radiation early in the evolutionary history of the chromophytes, and in this case it may never be possible to resolve the relationships. It is also possible

that one or more as yet undescribed groups (extant or extinct) may provide the essential data for complete phylogenetic resolution once they have been studied. The undescribed organism, CCMP1144 (see Potter et al. 1997a), is an example; however, this taxon alone did not improve the resolution in our analysis (Fig. 22) except that it was the most basal taxon of the chromophytes.

The order Phaeothamniales sensu Bourrelly (1957) is generally discounted today because it contained marine members currently classified in the Chrysomeridales (see O'Kelly 1989a) and the Haptophyceae. Neither of these groups has a close relationship to *Phaeothamnion* (see *Giraudyopsis* in Fig. 22 and in Saunders et al. 1997, Haptophyceae in Daugbjerg and Andersen 1997b). The family Phaeothamniaceae sensu Preisig (1995) includes *Chrysoclonium, Sphaeridothrix* and *Tetrachrysis,* but no pigment data, ultrastructural observations of the zoospores, or DNA analyses are available for these taxa. Interestingly, none of these taxa produce silica cysts. *Tetrachrysis* has zoospores with flagella inserted approximately one third the way down the cell, one anteriorly directed and the other posteriorly directed, appearing very similar to zoospores of *Phaeothamnion* (Dop 1980). Thus, it is suggestive that these genera are related to *Phaeothamnion* rather than being members of the Chrysophyceae. Thus far, all modern examinations of filamentous ''chrysophytes'' have shown clearly that they are not members of the Chrysophyceae (e.g. Gayral and Billard 1986, O'Kelly 1989a, Saunders et al. 1997), and the continued inclusion of *Chrysoclonium, Sphaeridothrix,* and *Tetrachrysis* in the Chrysophyceae is becoming more speculative.

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