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BOLIDOMONAS: A NEW GENUS WITH TWO SPECIES BELONGING TO A NEW ALGAL CLASS, THE BOLIDOPHYCEAE (HETEROKONTA)[1]

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A new algal class, the Bolidophyceae (Heterokonta), is described from one genus, *Bolidomonas*, gen. nov., and two species, *Bolidomonas pacifica*, sp. nov and *Bolidomonas mediterranea*, sp. nov., isolated from the equatorial Pacific Ocean and the Mediterranean Sea, respectively. Both species are approximately 1.2 μm in diameter and have two unequal flagella; the longer flagellum bears tubular hairs, whereas the shorter is smooth. The flagellar basal apparatus is restricted to two basal bodies, and there is no transitional helix. Cells are naked, devoid of walls or siliceous structures. The internal cellular organization is simple with a single plastid containing a ring genophore and a girdle lamella, one mitochondrion with tubular cristae, and one Golgi apparatus close to the basal bodies. The Mediterranean and the Pacific species differ in the insertion angle between their flagella and their pattern of swimming, these differences possibly being linked to each other. Analyses of the SSU rDNA gene place the two strains as a sister group to the diatoms. Moreover, pigment analyses confirm this position, as fucoxanthin is found as the major carotenoid in both lineages. These data strongly suggest that the ancestral heterokont that gave rise to the diatom lineage was probably a biflagellated unicell.

Key index words: **Bolidophyceae; diatoms; Heterokonta; marine picoeukaryotes; oligotrophic ocean; Stramenopiles**

Abbreviations: **SSU, small subunit**

Nearly two decades ago, phytoplankton in the central part of the oceans was found to be dominated by cells smaller than 2–3 μm: the picophytoplankton (Li et al. 1983, Platt et al. 1983). This discovery triggered intensive research concerning the species composition and physiology of this size class. Most attention has been devoted to the prokaryotic component, which comprises mainly two genera: *Synechococcus* (Johnson and Sieburth 1979, Waterbury et al. 1979) and *Prochlorococcus* (Chisholm et al. 1988, 1992). In contrast, the eukaryotic picophytoplankton is much more diverse and apparently composed of organisms that belong to several algal divisions, including the Heterokonta, Chlorophyta, Prasinophyta, and Haptophyta (Thomsen 1986, Potter et al. 1997). Within these algal lineages, many new taxa, at the genus, order, or even class level, have been described from picoplankton in the past 10 years (Booth and Marchant 1987, Eikrem and Throndsen 1990, Guillard et al. 1991, Andersen et al. 1993, Miyashita et al. 1993, Hasegawa et al. 1996). For example, Andersen et al. (1993) erected a new algal class, the Pelagophyceae Andersen and Saunders (Heterokonta), on the basis of a new picoplanktonic species, *Pelagomonas calceolata* Andersen et Saunders. Five years later, the order Sarcinochrysidales has been included in this new class (Saunders et al. 1997) as well as *Aureococcus anophagefferens* Hargraves et Sieburth, which is responsible for ''brown tides'' in coastal waters (De Yoe et al. 1995). These newly discovered taxa clearly point to the oceanic picoeukaryotes as a large reservoir of unexplored biodiversity.

The isolation and characterization of algal strains in this size class is thus an important task even though only a low percentage of the picoplanktonic species can probably grow in culture, as repeatedly demonstrated for bacteria (e.g. Giovannoni et al.

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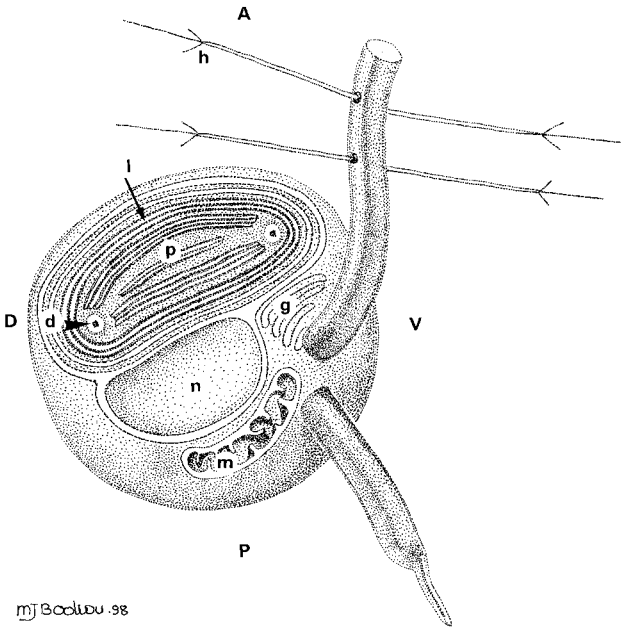


FIG. 1. Schematic drawing of *Bolidomonas* (not to scale) illustrating the main features of the genus. It possesses two laterally inserted flagella: the longer one bears tubular hairs, the shorter one is naked and acronemated. The flagellar hairs (h) are tubular and appear bipartite because their basal section is reduced to a basal disk. The cell contains one plastid (p), one nucleus (n), one Golgi body (g), and one mitochondrion (m). The plastid has a ring DNA genophore (d) and a girdle lamella (l). Cell orientation is arbitrary. A = anterior, D = dorsal, P = posterior, V = ventral. Drawing by M. J. Bodiou.

1990). The description of new taxa should further our understanding of algal taxonomy and phylogeny. Some might represent missing links between established groups (Saunders et al. 1997). Moreover, little is yet known on the species composition of marine picoeukaryotes despite the significant contribution of these communities to primary production in oligotrophic waters (Li 1994). Species adapted to these extreme environments with very low nutrient levels might have intriguing physiological adaptations.

In this study, we describe two new picoplanktonic flagellates, one isolated from the equatorial Pacific Ocean (*Bolidomonas pacifica*) and the other from the Mediterranean Sea (*Bolidomonas mediterranea*). Ultrastructural data, pigment composition, and phylogenetic analyses based on the SSU rDNA sequence confirm that these two isolates belong to the Heterokonta phylum (Cavalier-Smith 1986; or Stramenopiles *sensu* Patterson 1989) but also show that they cannot be placed in any of the presently described heterokont algal classes. A new heterokont class is proposed to include these two picoplanktonic species. Phylogenetic and ecological consequences of the discovery of these new taxa are discussed.

MATERIALS AND METHODS

Cultures. *Bolidomonas pacifica* (OLI31SE3 strain) was isolated from the equatorial Pacific Ocean (150° W, 5°30′ S, 15 m depth, 15 November 1994) during the OLIPAC cruise on board of the NO Atalante. *Bolidomonas mediterranea* (MINB11E5 strain) was collected in the eastern Mediterranean Sea (18° E, 34° N, 20 m depth, 18 June 1996) during the MINOS cruise on board of the NO Suroit. Strains were further purified by serial dilutions and maintained in K medium (Keller et al. 1987) at 19° C and 100 μmol photons·m^{-2}·s^{-1} under a 12:12 h LD (light:dark) regime. Light was provided by Sylvania Daylight fluorescent bulbs. Under standard growth conditions, cultures are pale brown at stationary phase and reach a maximum cell concentration of approximately 10^5 cells·mL^{-1}. The mean swimming speed was estimated under an inverted microscope from the observation of 10 individuals (one observation per individual). Cells swimming in a straight line were observed for 5 s, and the distance covered was measured.

Transmission electron microscopy. For whole-mount preparations, cells were fixed for 15 min in a fixative solution containing 1%–1.4% glutaraldehyde, 0.4 M cacodylate buffer (pH = 7.2), and 0.7% saccharose (final concentrations). A drop of fixed cells was deposited onto formvar-coated grids. After 10 min, most of the fluid was removed from the grids by capillarity. Cells either were stained with 1% uranyl acetate for 5 min and rinsed with distilled water or were allowed to dry for negative staining. For thin sections, 250 mL of cultures were fixed with the solution described previously. Cells were harvested by centrifugation at 4000 × *g*, and the pellet was included into 1.5% purified agarose (Appligene, ref: 130021, Illkirch, France). Agarose blocks were then rinsed in 0.5 M cacodylate buffer and postfixed with 1% OsO_4 and 0.5 M cacodylate buffer for 2 h. Cells were progressively dehydrated in ethanol and propylene oxide and then embedded into Spurr's resin. Photomicrographs were taken with a JEOL JEM-1200EX electron microscope. To harvest the cells during cell division, we followed the cell cycle by DNA staining and flow cytometry analysis (Marie et al. 1997). Five hundred microliters of a 50 mL OLI31SE3 culture were collected every hour during 24 h and fixed with 1% paraformaldehyde for 20 min. The cells were stained with 0.01% (final) SYBR Green I (Molecular Probes, Eugene, Oregon) in the presence of 0.01% (final) Triton X100 for 10 min. Analyses of the cell cycle were performed with a FACSort flow cytometer (Becton Dickinson, San Jose, California) equipped with an air-cooled laser (488 nm excitation). Green DNA fluorescence was collected as a linear signal. DNA replication (visualized by the occurrence of a second peak on DNA histograms representing the G_2 cell cycle phase) occurred mostly between 10 and 12 h after light was turned on. Most TEM micrographs of cell division were taken from samples collected during this period.

Pigment analysis. Samples from unialgal cultures were filtered onto GF/F filter (Whatman, Maidstone, England). Pigments were extracted in 3 mL of cold methanol with a known amount of Zn(II) pyropheophorbide octadecyl ester added as an internal standard (Mantoura and Repeta 1997). Extraction efficiency was improved by sonication for 30 s. The extract was then clarified by filtration (Whatman GF/C) and injected into the HPLC system through an AS-3000 TSP (Thermo Separation Products) automatic injector, which ensured mixing of the extract in 1 M ammonium acetate buffer (extract: ammonium acetate, 2:1 v/v). The HPLC system and the chromatographic conditions have been described in detail by Vidussi et al. (1996). Pigment identification was performed by comparison of absorption spectra collected online through a 991 photodiode array detector (Waters Corp., Milford, Massachussets) with those of a library of spectra established from SCOR reference algal cultures (Jeffrey and Wright 1997). Pigment quantification was performed using internal and external calibration. The internal standard, Zn(II) pyrophaeophorbide a octadecyl ester, was kindly provided by Dr. Repeta (Woods Hole Oceanographic Institution, Woods Hole, Massachusetts), whereas external standards were either commercially available (chl *a,* ß-carotene from Sigma Chemical Co., St. Louis, Missouri) or purified from reference algal cultures (e.g. fucoxanthin, diadinoxanthin).

Phylogenetic analyses. Two liters of culture were collected by centrifugation and resuspended into DNA extraction buffer (25%

sucrose, 50 mM Tris, 1 mM EDTA). Cells were incubated for 2 h with 0.4 mg·mL^{-1} proteinase K at 37° C. DNA was extracted using a standard phenol:chloroform protocol and alcohol precipitation. DNA was purified with the Geneclean II kit (BIO 101, La Jolla, California). *Bolidomonas pacifica* was sequenced in Bremerhaven (Germany) according to Chesnick et al. (1997). *Bolidomonas pacifica* and *B. mediterranea* were sequenced in Roscoff (France) with the following oligonucleotide primers: 5′-ACCTGGTTGATCCTGCCAG-3′, 5′-TGATCCTTCYGCAGGTTCAC-3′, complementary to regions of conserved sequences proximal to 5′ and 3′ termini of the 18S rRNA gene. The thermal cycle parameters were as follows: denaturation at 94° C for 1 min (initial denaturation 5 min), annealing at 55° C for 2 min, and extension at 72° C for 3 min (final extension 10 min). The reaction was cycled 30 to 35 times. PCR products were directly sequenced using the VISTRA automatic sequencer (Amersham, Les Ulis, France) using internal primers labeled with Texas Red (Amersham). Both strands of each gene were sequenced. The *Bolidomonas pacifica* sequences obtained in Bremerhaven and in the Roscoff laboratory were identical. Sequences were deposited in GenBank with the following accession numbers: *Bolidomonas pacifica,* AF123595, and *Bolidomonas mediterranea,* AF123596. The two sequences were compared with 18S rRNA gene sequences from *Achlya bisexualis* Coker (GenBank accession number = M32705), *Apedinella radians* (Lohman) Campbell (U14384), *Aulacoseira distans* (Ehrenb.) Sim. (X85403), *Aureococcus anophagefferens* Hargraves et Sieburth (DeYoe et al. 1995), *Bacillaria paxillifer* (Müller) Hendey (M87325), *Botrydiopsis intercedens* Visher et Pasher (U41647), *Botrydium stoloniferum* Mitra (U41648), *Cafeteria roenbergensis* Fenchel et Patterson (L27633), *Chromulina chromophila* Stein (M87332), *Chrysonephele palustris* Pipes, Taylor et Leedale (U71196), *Ciliophrys infusionum* CienKowski (L37205), *Corethron criophilum* Castracane (X85400), *Coscinodiscus radiatus* Ehrenb. (X77705), *Desmarestia ligulata* (Lighfoot) Lamouroux (L43060), *Developayella elegans* Tong (U37107), *Dictyocha speculum* Ehrenb. (U14385), *Ectocarpus siliculosus* (Dillwyn) Lyngbye (L43062), *Fucus distichus* Linnaeus (M97959), *Heterosigma akashiwo* (Hulburt) Taylor (L42529), *Hibberdia magna* (Belcher) Andersen (M87331), *Hyphochytrium catenoides* karling (X80344), *Labyrinthuloides minuta* (Watson et Raper) Perkins (L27634), *Lagenidium giganteum* Couch (M54939), *Mallomonas striata* Harris et Bradley (M87333), *Nannochloropsis salina* (Droop) Hibberd (M87328), *Ochromonas danica* Pringsheim (M32704), *Oikomonas mutabilis* Kent (U42454), *Paraphysomonas vestita* (Stokes) de Saedeleer (Z28335), *Pelagococcus subviridis* Norris (U14386), *Pelagomonas calceolata* Andersen et Saunders (U14389), *Phytophthora megasperma* Drechsler (X54265), *Pseudopedinella elastica* Skuja (U14387), *Pteridomonas danica* Patterson et Fenchel (L37204), *Pulvinaria* sp. (U78032), *Rhizochromulina* sp. (U14388), *Rhizosolenia setigera* Brightwell (M87329), *Sarcinochrysis marina* Geitler (U78033), *Stephanopyxis* sp. (M87330), *Synura spinosa* Korshikov (M87336), *Tessellaria volvocina* (Playfair) Playfair (U73219), *Thalassionema nitzschioides* (Grun.) V.H. (X77702), and *Tribonema aequale* Pascher (M55286). All sequences were aligned using the DCSE software (De Rijk and De Wachter 1993). Highly variable gene regions were removed, leaving 1601 sites for subsequent phylogenetic analyses (alignment available on request). Distance analysis (neighbor-joining) and maximum parsimony methods were performed using the PHYLIP package (v. 3.57c, Felsenstein 1985). The Kimura two-parameter option was employed to compute evolutionary distances (Kimura 1980). *Labyrinthuloides minuta* was used as outgroup. Bootstrap analyses (1000 replicates, Felsenstein 1985) were performed for both analyses. The percentage similarity was calculated with the BESTFIT routine from the GCG software (Genetic Computer Group, Madison, Wisconsin).

RESULTS

Bolidophyceae *Guillou et Chrétiennot-Dinet classis nova*

Cellulae libere natantes, cum duis inequalibus flagellis, ventraliter insertis. Flagellum longius porro directum, mastigonematibus tubularis obsessum, brevius sine mastigonematibus, acronematum. Radices flagellorum corpusculis basalibus contracti. Pars transitoria flagellorum sine helice. Chloroplastum unicum cum lamella cingenti, lamellis tribus thylacoidibus compositis. Nec stigmate. Distinctus annulus e desoxyribo-nuclei-acidis in chloroplasto. Mitochondria cum tubulatis cristis. Fucoxanthino pro majore carotenoido. 18S rRNA sequentia geneticae ponent classem sicut sororem classis diatomarum. Theca aut siliceae structurae desunt.

Genus typificum: Bolidomonas Guillou et Chrétiennot-Dinet

Motile cells with two unequal flagella, ventrally inserted. Long flagellum directed forward, with tubular flagellar hairs. Short flagellum naked and acronemated. Basal apparatus reduced to basal bodies. Transitional helix absent. One chloroplast with a girdle lamella, lamellae with three appressed thylakoids. No eyespot. Distinct ring-shaped chloroplast DNA genophore. Mitochondria with tubular cristae. Fucoxanthin as major carotenoid. 18S rRNA gene sequences place this class as a sister group to the diatoms, but theca or silica structures are absent.

Type genus: *Bolidomonas* Guillou et Chrétiennot-Dinet

Bolidomonadales Guillou et Chrétiennot-Dinet *ordo novus*

Sicut pro classe.

As in the class.

Bolidomonadaceae Guillou et Chrétiennot-Dinet *familia nova*

Sicut pro ordine.

As in the order.

Bolidomonas Guillou et Chrétiennot-Dinet *genus novum*

Cellulae globosae aut cordiformes, 1–1.7 μm in diametro. Duo flagella longius 4–7 μm, cum mastigonematis tubularis lateraliter insertis, porro directum, brevius 0.9–2.2 μm, nudum, acronematum. Chloroplastum dimidiam pars cellulae insidens. Pigmenta chloroplasti e chlorophyllis a, c_1, c_2 et c_3, fucoxanthino et diadinoxanthino.

Species typifica: Bolidomonas pacifica Guillou et Chrétiennot-Dinet

Round or heart-shaped cells, 1–1.7 μm in diameter. Two flagella: the long one 4–7 μm, in the forward direction, with laterally inserted tubular flagellar hairs; the short one 0.9–2.2 μm, naked, with a marked acronema. Dorsal chloroplast occupies about half the cell. Pigment composition includes chlorophyll *a*, $c_1 + c_2$ and c_3, fucoxanthin and diadinoxanthin.

Type species: *Bolidomonas pacifica* Guillou et Chrétiennot-Dinet sp. nov.

Etymology: The name refers to the rapid swimming behavior of the cells reminiscent of a racing car.

Bolidomonas pacifica Guillou et Chrétiennot-Dinet, *species nova*

Sicut genus. Cellulae celeriter natantes, itinere sine commutatione tractus. Duo flagella ad 110° inserta. Nec pyrenoide. Descriptio sequencae geneticae '18S rRNA': AF123595.

Per navigatione OLIPAC 1994, in Oceano Pacifico, (long. occident. 150° 00′, lat. austr. 5° 30′), a D. Vaulot *collectae.*

Holotypus: Fig. 2

Characters of the genus. Cells swimming rapidly, with the long flagellum pulling the cell. Two flagella inserted at 110°. No pyrenoid. 18S rRNA gene sequence: AF123595.

Collected by D. Vaulot during the OLIPAC cruise (November 1994), in the equatorial Pacific Ocean at 150° 00′ W; 5° 30′ S.

Holotype: Fig. 2

Etymology: The specific epithet refers to the geographic origin of this species.

Bolidomonas mediterranea Guillou et Chrétiennot-Dinet *species nova*

Sicut genus. Cellulae celeriter natantes, itinere cum frequente commutatione tractus. Duo flagella ad 130–150° inserta. Nec pyrenoide. Descriptio sequencae geneticae '18S rRNA': AF123596. Per navigatione MINOS 1996, in Mare Mediterraneum, (long. orient. 18°00', lat.bor 34°00'), a D. Vaulot *collectae.*

Holotypus: Fig. 3

Characters of the genus. Cells swimming rapidly, with the long flagellum pulling the cell but with frequent changes in direction. Two flagella, inserted at 130–150°. No pyrenoid. 18S rRNA gene sequence: AF123596. Collected by D. Vaulot during the MINOS cruise (June 1996), in the Mediterranean Sea at 18° 00′ E; 34° 00′ N.

Holotype: Fig. 3

Etymology: The specific epithet refers to the geographic origin of this species.

Ultrastructure. In both species, vegetative cells are spherical or ovoid, 1–1.7 μm in diameter. Whole-mount preparations show that the cells are naked and possess two unequal, ventrally inserted flagella (Figs. 2, 3). The long flagellum measures 4–7 μm in length and extends forward in a wavelike motion. The short flagellum, 0.9–2.2 μm, is not visible with light microscopy. The two species can be differentiated from one another only by the angle of their flagellar insertion and their swimming pattern, the later feature being possibly a consequence of the former. Both strains swim vigorously (approximatively 1–1.5 $mm \cdot s^{-1}$), but only *B. mediterranea* presents sudden changes in swimming direction. The long flagellum bears tubular hairs with three terminal filaments but no lateral appendages (Fig. 4). The tubular hairs appear flexible but are so fragile that they can be easily lost during fixation. Their basal fibrillar section is not visible, but their tubular structure can be seen after negative staining. Each tubular hair measures 1 μm in length and 15 nm in diameter in cross section (Figs. 4, 5). They are produced within cellular vesicles adjacent to the nucleus and the plastid (Fig. 5). The second, short flagellum is smooth and acronemated (Figs. 1, 2). The axoneme shows a classical distribution of microtubules (9 + 2, not shown). There is no paraxonemal rod.

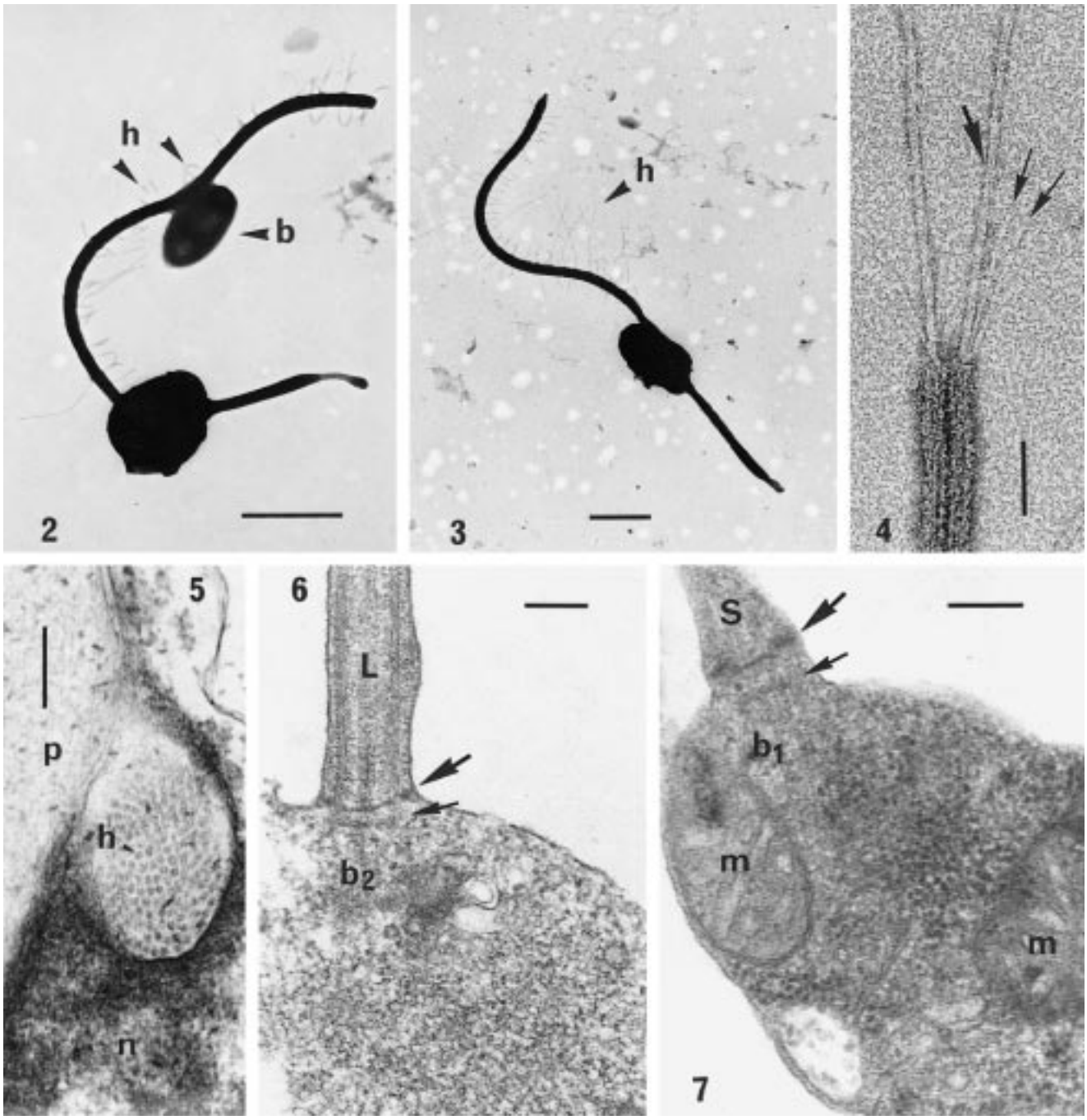
Microtubular or fibrous roots are absent, and the basal apparatus is reduced to two basal bodies. A typical transitional plate is present, located slightly above the level of the cell surface (Figs. 6, 7). A second and thinner transitional plate, more difficult to observe, is found below (Figs. 6, 7). No transitional helix has ever been observed in either species. The two basal bodies are inserted at 110° for *B. pacifica* and at more than 130° for *B. mediterranea* (Figs. 8, 9; see also Figs. 2, 3).

The Golgi apparatus is located close to the flagellar insertion (Fig. 10). An exocytosis vesicle and a mitochondrion with tubular cristae are often present in the vicinity of the Golgi body and the basal body of the short flagellum (Figs. 8–10). A single plastid occupies a dorsal position and is characterized by the presence of a girdle lamella (Fig. 11). Chloroplast lamellae consist of three (but sometimes two) adpressed thylakoids (Fig. 11). Plastid DNA is organized as a ring lying just below the girdle lamella (Figs. 11–13). An unusual microtubule-like structure of unknown composition is also always found in this ring (Figs. 11–13). The plastid is enclosed with the chloroplast endoplasmic reticulum, which is continuous with the outer membrane of the nuclear envelope (Figs. 1, 13). A dividing chloroplast is illustrated in Figure 14, showing a deep invagination of its membrane on the ventral side. Neither a pyrenoid nor an eyespot was ever seen in sections.

Flagellar duplication was observed in both strains. During this process, the long flagellum is engulfed inside the cell (Fig. 15). In transverse section, the extremity seems to emerge at the opposite side of the normal insertion point (Fig. 15). Figure 16 shows the configuration of the flagella during this stage. Transverse sections of the engulfed flagellum indicate a progressive disintegration of the microtubular structure (Fig. 17). Once both flagella are duplicated, they are diagonally opposed in each daughter cell (Fig. 18).

Pigment analyses. In addition to chl c_1 and c_2, chl c_3 is also present in both *Bolidomonas* species (Table 1). The major carotenoid is fucoxanthin. The only other important carotenoids constitute the diadinoxanthin-diatoxanthin couple, usually implicated in photoprotection. Traces of β-carotene are also found.

Phylogenetic analyses. Bolidomonas mediterranea and *B. pacifica* 18S rDNA sequences are very similar, sharing 96.1% identity. Distance and maximum par-



FIGS. 2–7. Whole mounts and TEM sections of *Bolidomonas* spp. b = bacterium, b1 = basal body of short flagellum, b2 = basal body of long flagellum, h = hairs, L = long flagellum, n = nucleus, m = mitochondrion, p = plastid, S = short flagellum. FIG. 2. *Bolidomonas pacifica.* General morphology. The flagellar hairs are fragile, and few of them are still present (arrowheads). A bacterium lies against the long flagellum. Scale bar = 1 μm, uranyl acetate staining. FIG. 3. *Bolidomonas mediterranea.* General morphology. The flagellar hairs are similar to those shown in Figure 2. Note the difference, generally observed in electron microscopy, in the relative position of the two flagella between this species and that in Figure 2. Scale bar = 1 μm, uranyl acetate staining. FIG. 4. *Bolidomonas pacifica.* Negatively stained whole mount, showing details of the upper part of two flagellar hairs. Each of them presents one long (large arrow) and two short (small arrows) terminal filaments. Lateral filaments are absent. The *B. mediterranea* hairs are similar (not shown). Scale bar = 50 nm. FIG. 5. *Bolidomonas pacifica.* The arrowhead points to a tubular hair in transverse section that is produced within an enclosed vesicle closely associated with the nucleus and the plastid. Scale bar = 200 nm. FIG. 6. *Bolidomonas pacifica.* Longitudinal section of the long flagellum and its basal body. Under the typical transitional plate (large arrow), a lower, thin transitional plate (small arrow) is present. Note the absence of a transitional helix. Scale bar = 200 nm. FIG. 7. *Bolidomonas mediterranea.* Longitudinal section through the short flagellum and its basal body. Note the absence of a transitional helix and the presence of a mitochondrion lying just beneath the basal body. The short flagellum of *B. mediterranea* shows the same characteristics of the transitional plates as the long flagellum of *B. pacifica* (Fig. 6). Scale bar = 200 nm.

simony analyses (maximum parsimony tree not shown) place both *Bolidomonas* species as sister taxa to the diatoms with 100% and 99% bootstrap support, respectively (Fig. 19). The deepest divergence among heterokont chromophytes, emerging from a heterotrophic base, is between the Bolidophyceae/diatom lineage and all other heterokont algae. The branching order within the sister clade containing all other Heterokonta algae could not be resolved because bootstrap values were low.

DISCUSSION

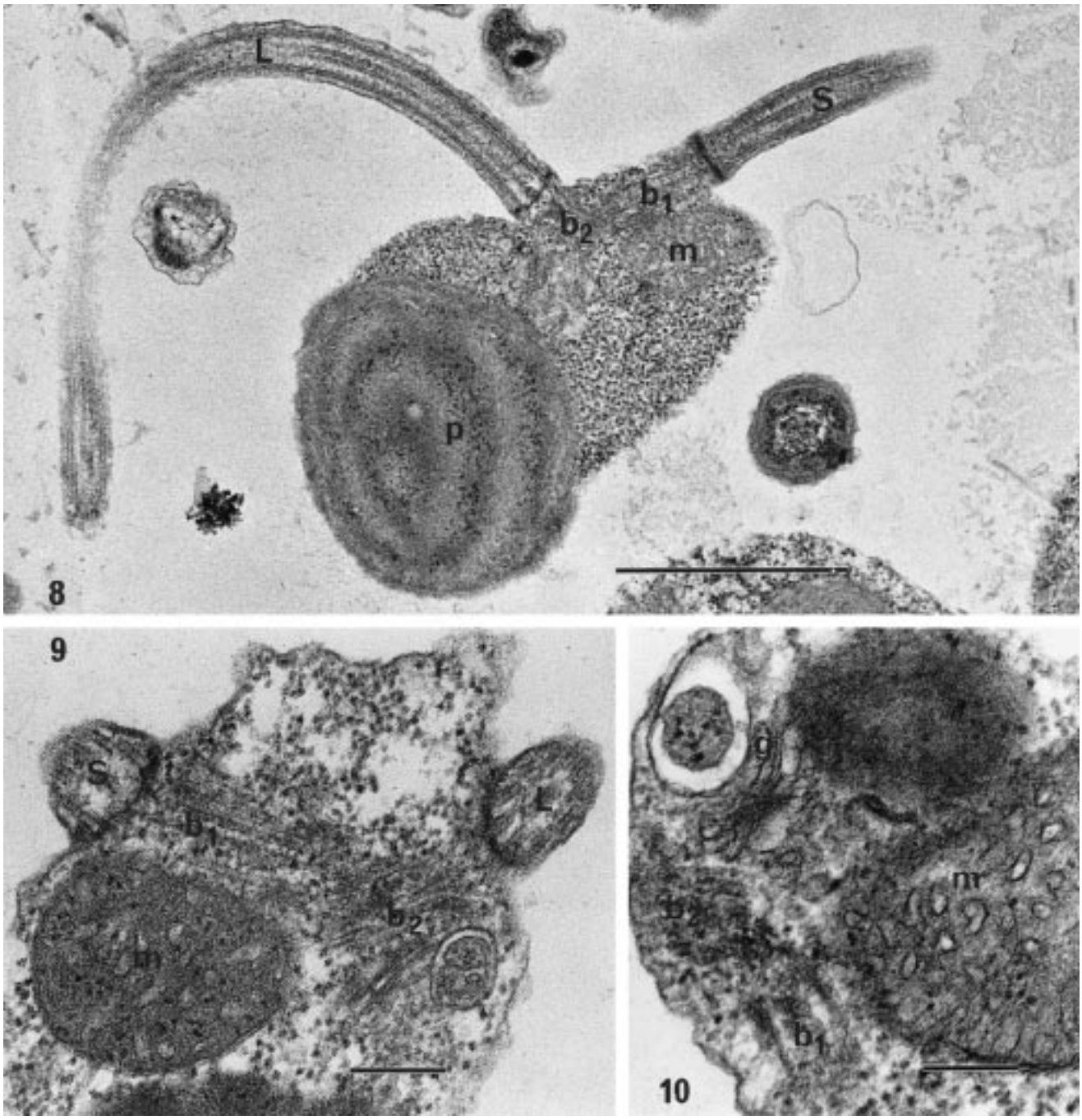
A new algal lineage. The two new *Bolidomonas* species share typical morphological characters of Heterokonta. They possess (i) two unequal flagella, the longer one bearing tubular hairs, the shorter one being smooth; (ii) a mitochondrion with tubular cristae; and (iii) a nucleoplastidial complex. Within the Heterokonta, they are positioned closer to the diatoms than to any other group on the basis of both SSU rDNA and pigment data. The position of both *Bolidomonas* species as the closest, albeit separated, lineage to diatoms suggests that the two flagellates could form a new algal lineage.

However, ultrastructural characters provide the most decisive argument in favor of the description of a new algal class. Within the Heterokonta, taxa are distinguished at the class level by the flagellar apparatus structure, the presence or absence of a transitional helix, the number of transitional plates, the chloroplast DNA organization, and the presence/absence and type of cell covering. Table 2 and Figure 20 compare 11 features (seven describing the flagellar apparatus) between all other heterokont algal classes, including the Parmales, *Sulcochrysis biplastida* Honda et al. (Honda et al. 1995), and the heterotrophic Bicosoecids. The absence of a transitional helix (Fig. 20) and a reduced flagellar apparatus (Table 2) are key elements to determining the taxonomic affinity of Bolidophyceae. *Bolidomonas* is excluded from the monophyletic clade consisting of the Chrysophyceae/Synurophyceae/Eustigmatophyceae (Bhattacharya et al. 1992) as well as the Xanthophyceae and the Chrysomeridales because species of this group typically possess a transitional helix and a well-developped flagellar apparatus. In fact, the absence of a transitional helix is rather unusual for unicellular heterokonts and has been reported only for the Raphidophyceae, diatom spermatozoids, brown algal zoids, and occasionally for some genera, such as *Ankylochrysis* Billard, belonging to the Sarcinochrysidales (Honda and Inouye 1995). The reduction of the basal flagellar apparatus (i.e. the absence of microtubular roots or of a rhizoplast) brings closer together the *Bolidomonas* spp. and the Dictyochia lineage (*sensu* Cavalier-Smith 1993, including Dictyochophyceae, Pelagophyceae, and Pedinellophyceae), the diatoms, and *Sulcochrysis biplastida.* Two transitional plates and bipartite tubular hairs, found in both isolates, were also described as particular features of the Pelagophyceae (Andersen et al. 1993, Saunders et al. 1997). Nevertheless, two transitional plates are also found in *Chrysolepidomonas dendrolepidota* Peters et Andersen (Peters and Andersen 1993). Because this structure is sometimes hard to observe, it might be difficult to use as a character to classify cells at the class level in the Heterokonta. The *Bolidomonas* hairs on the long flagellum are tubular and first assembled in the endoplasmic reticulum or nuclear envelope, but their basal section is not visible, makeing them atypical and similar to those of *Pelagomonas calceolata* (Pelagophyceae) but also of *Oikomonas mutabilis* (Chrysophyceae, Cavalier Smith et al. 1995). Such flagellar hairs were described by these authors as bipartite, although this term is usually restricted for the flagellar hairs of cryptomonads (Andersen et al. 1991). In fact, Loiseaux and West (1970) found similar hairs in the zoids of some Phaeophyceae and demonstrated that detached hairs possessed a reduced expanded base that could be considered the short basal section. The *Bolidomonas* spp. could be definitively separated from the whole Dictyochia group by the location of the basal bodies with respect to the nucleus (in a depression for all Dictyochia, above for *Bolidomonas* spp.) and the presence of a proximal helix for all Dictyochia (Table 2).

Plastid characters found in *Bolidomonas* spp.—namely, a ring chloroplast DNA, a diatoxanthin-containing chloroplast, and the absence of an eyespot—also provide good taxonomic markers, as they are found only in diatoms, a few Raphidophyceae, perhaps the enigmatic *Sulcochrysis biplastida* Honda et al., and the Parmales, for which the pigment composition is unknown. If we consider the whole set of characters, such as the location of basal bodies above the nucleus, the absence of a transitional helix, and a reduced flagellar basal apparatus, ultrastructural analysis supports phylogeny and pigment data, placing the *Bolidomonas* spp. closer to the diatoms than to any other heterokont algal class.

Nevertheless, *Bolidomonas* certainly does not belong to the diatoms sensu stricto. Silica deposition, one of the most important diagnostic features of the diatoms, is not observed in *Bolidomonas* spp. The spermatozoids, the only flagellated stages observed in diatoms, possess unusual features not found in *Bolidomonas.* They bear only the long flagellum and lack the two central microtubules in the axoneme and, perhaps, the microtubule triplet structures in the basal body (Heath and Darley 1972). There are no transitional plates in the diatoms, whereas two are found in *Bolidomonas.*

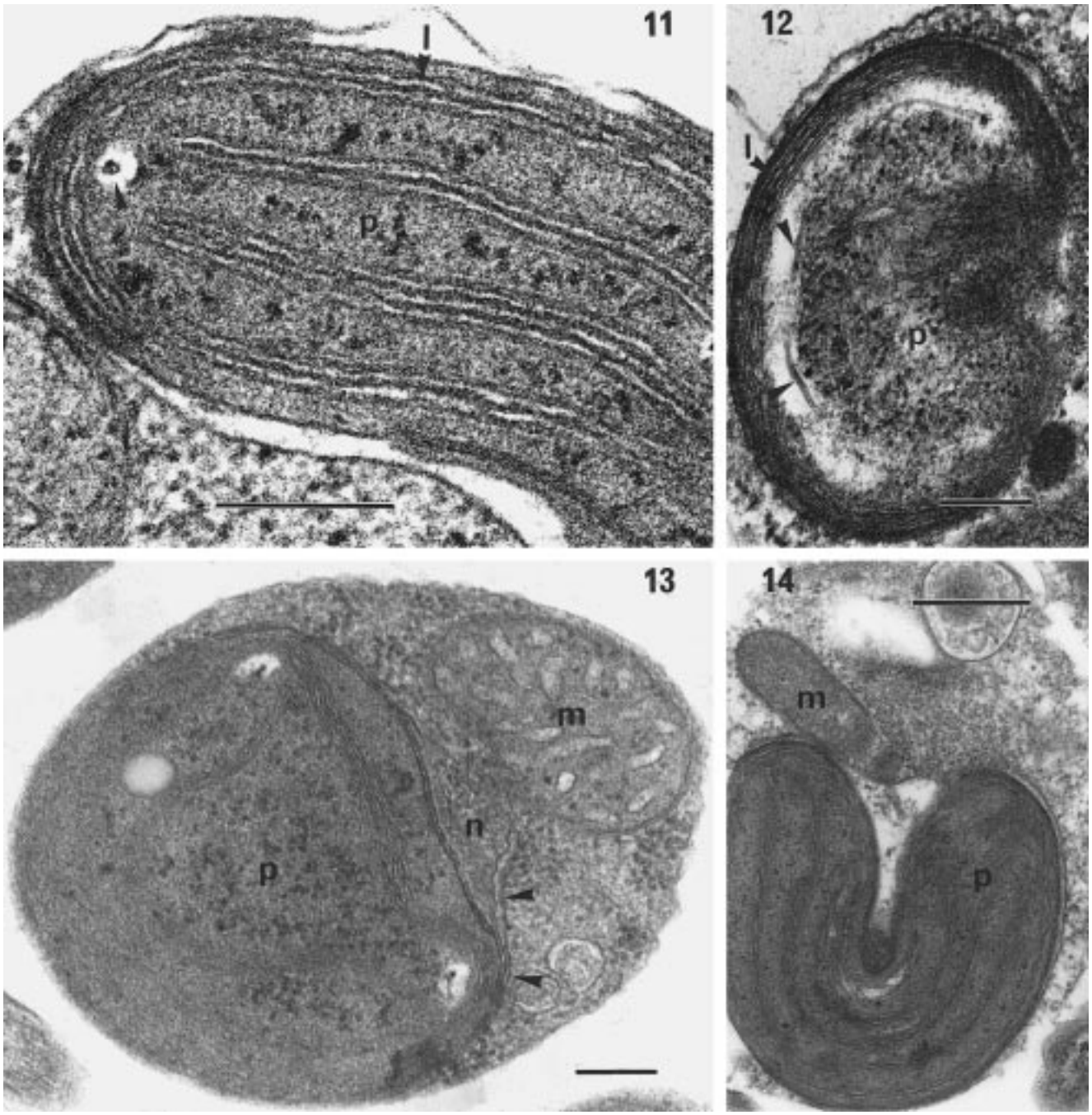
Thus, *Bolidomonas* spp. possess a unique combination of features heretofore not found in any other heterokont algal class, namely, a naked biflagellate vegetative stage with a reduced flagellar root system and a ring-DNA chloroplast but without a transitional helix or eyespot. Thus, we propose the creation



FIGS. 8–10. TEM sections showing the general ultrastructure of *Bolidomonas* spp. b1 = basal body of short flagellum, b2 = basal body of long flagellum, g = Golgi body, L = long flagellum, m = mitochondrion, p = plastid, S = short flagellum. FIG. 8. *Bolidomonas pacifica.* Longitudinal section. The two basal bodies are inserted at an angle of 110°. The basal body of the short flagellum lies just beneath the mitochondrion. The plastid occupies a large fraction of the cell. Scale bar = 1 μm. FIG. 9. *Bolidomonas mediterranea.* Oblique section showing details of the flagellar insertion and basal bodies. The mitochondrion is located next to the basal body of the short flagellum, and the microtubular roots are absent. Scale bar = 200 nm. FIG. 10. *Bolidomonas pacifica.* Longitudinal section. The Golgi body is located near the basal bodies and the nucleus. Scale bar = 200 nm.

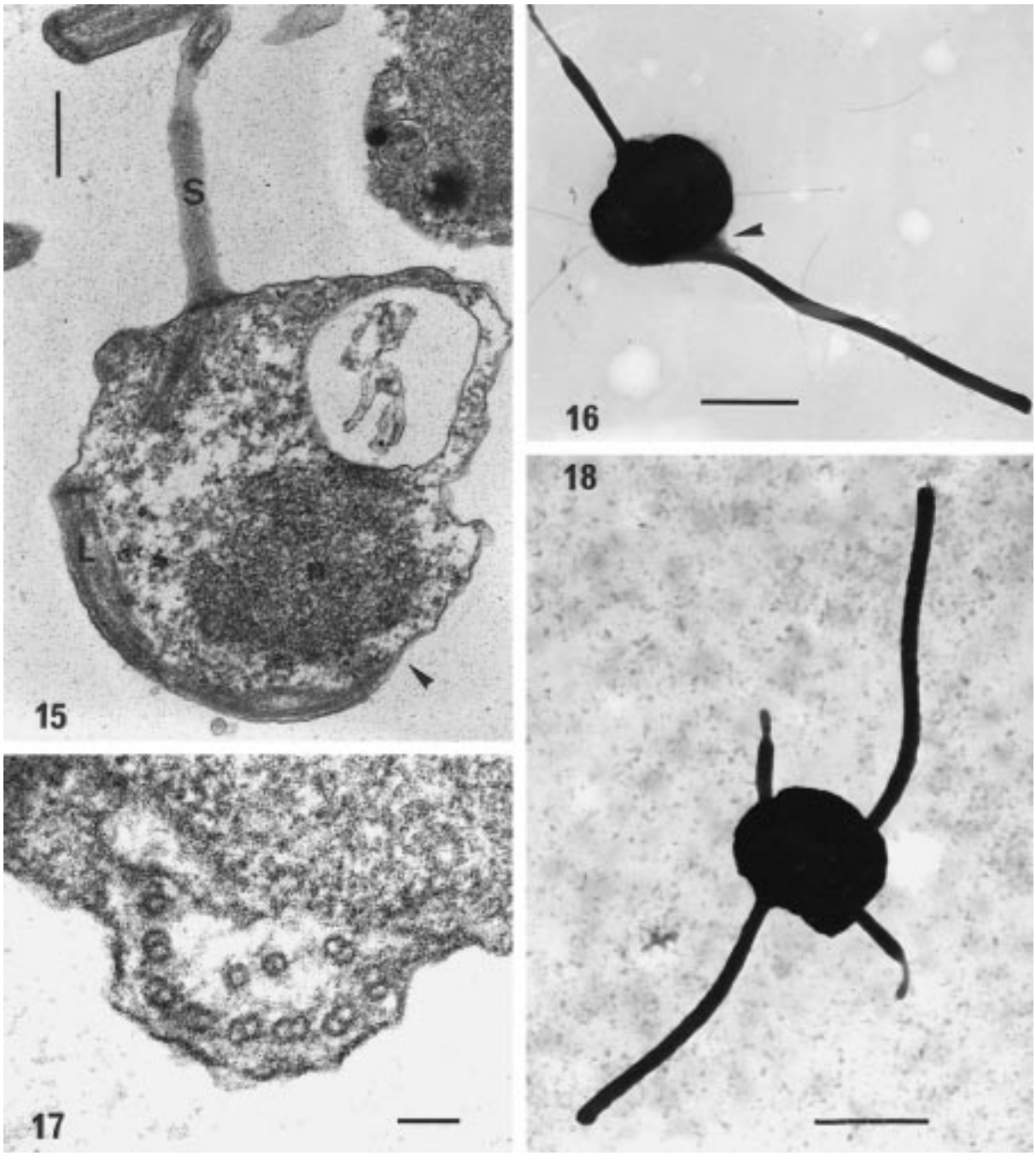
of a new heterokont algal class, the Bolidophyceae, which includes the two *Bolidomonas* species.

Flagellar propulsion in Bolidophyceae. The exceptional size of the long flagellum (about four times the cell diameter) and the rapid swimming of both *Bolidomonas* spp. is rather unusual for picoeukaryotes and must be considered as an important characteristic of these two new species. The theoretical cost of motility, at a speed of 1 $mm \cdot s^{-1}$, is much larger than the total metabolic rate for this size class (Crawford 1992). The idea generally accepted is that motility and flagella are reduced and tend to disappear



FIGS. 11–14. TEM sections showing plastid ultrastructure in *Bolidomonas* spp. l = girdle lamella, n = nucleus, m = mitochondrion, p = plastid. FIG. 11. *Bolidomonas pacifica.* Longitudinal section through the plastid (p). Note the presence of a girdle lamella (l), composed of three adpressed thylakoids, and one pole with the genophore region cut transversally (ring DNA shown by arrowhead). Scale bar = 100 nm. FIG. 12. *Bolidomonas pacifica.* Transverse section of the plastid (p). The tubular DNA ring is parallel to the girdle lamella (l). A tubular-like structure of unknown composition and function is present in the genophore (arrowheads). Scale bar = 100 nm. FIG. 13. *Bolidomonas pacifica.* Transverse section through the plastid, the nucleus, and the mitochondrion. The plastid contains a ring nucleoid and is enclosed in a layer of endoplasmic reticulum, which is continuous with the outer membrane of the nuclear envelope (arrowheads). Scale bar = 100 nm. FIG. 14. *Bolidomonas pacifica.* Longitudinal section prior to cell division. The plastid is deeply invaginated before duplication. Scale bar = 100 nm.

when cell size decreases below 3 μm (Potter et al. 1997). The existence of *Bolidomonas* suggests that motility could provide a selective advantage even for such small cells. Ultrastructural sections have shown that the long flagellum is engulfed and digested inside the cell prior duplication. Retraction of the long flagellum inside the cell before division has been observed in other heterokont algae (Beech et



FIGS. 15–18. Whole mounts and TEM sections during cell division of *Bolidomonas pacifica*. L = long flagellum, n = nucleus, S = short flagellum. FIG. 15. Longitudinal section. The long flagellum is incorporated beneath the plasmalemma prior to duplication. In several micrographs, during this incorporation, the extremity of the engulfed flagellum was observed to emerge out of the cell (arrowhead) at the opposite of its insertion point (next to the short flagellum). Scale bar = 1 μm. FIG. 16. General morphology prior to cell division. The long flagellum emerges on the opposite side of its normal insertion (arrowhead). This whole cell micrograph represents the same stage as that in the thin section shown in Figure 15. Scale bar = 1 μm, uranyl acetate staining. FIG. 17. Transverse section through the long flagellum as it is being incorporated, showing the beginning of the disorganization of its microtubular structure. Scale bar = 200 nm. FIG. 18. Uranyl acetate staining of entire cell prior to cell division. Two long and two short flagella are placed diagonally opposed. Scale bar = 1 μm.

al. 1991). A similar event has also been observed with light and electron microscopy on brown algal zoids when they attach to a substratum (Loiseaux 1973). In several cases, the long flagellum shortens and becomes the short flagellum for the next generation (Beech et al. 1991). In *Bolidomonas,* this is not the case because the longer flagellum seems to be incorporated by an invagination of the cell mem-

TABLE 1. Pigment composition of *Bolidomonas pacifica* and *Bolidomonas mediterranea*. Chlorophyll c_3–like and fucoxanthin-like pigments have the same spectral characteristics as chlorophyll c_3 and fucoxanthin, respectively, but different retention times.

Pigments	Retention time (min)	Cell content (fg·cell^{-1})	
		B. pacifica	*B. mediterranea*
Chlorophyll *a*	14.80	24.8	13.6
Chlorophyll c_1 + c_2	3.80	2.4	2.6
Chlorophyllc_3	2.61	3.4	0.8
Chlorophyll c_3-like	4.14	0.8	0.4
β-carotene	16.76	0.6	0.6
Fucoxanthin	6.81	24.6	14.4
Fucoxanthin-like	9.17	0.0	0.8
Diadinoxanthin	8.53	2.2	3.4
Diatoxanthin	9.84	0.0	0.4

brane and emerges at the opposite side of its normal insertion. This behavior, if confirmed, could be very unusual and could be a consequence of the very small organism size.

Phylogenetic and evolutionary implications. The clade comprising the Bolidophyceae and diatoms is a sister group of all other heterokont algae in SSU rDNA trees. Compared with recent published trees (Van de Peer et al. 1996, Medlin et al. 1997, Saunders et al. 1997), the addition of the Bolidophyceae defines the true sister group for the diatoms and provides strong support for the heterokont algae as a monophyletic group. Major clusters of algal classes are consistently recovered with high bootstrap support, such as the Xanthophyceae/Phaeophyceae clade and the Pedinellophyceae/Dictyochophyceae/Pelagophyceae clade. However, the Bolidophyceae addition does not improve tree topology because bootstrap values for the branching order among major clusters of heterokont algal classes remain weak. On the basis of our rDNA analyses, the Bolidophyceae and the diatoms represent the first algal group to emerge from a basal heterotrophic group, although other genes, such as *rbc*L (Daugbjerg and Andersen 1997), *tuf*A, and plastid SSU rDNA (Bhattacharya and Medlin 1995), provide different tree topologies in which the diatom radiation is not the first heterokont algal lineage to emerge.

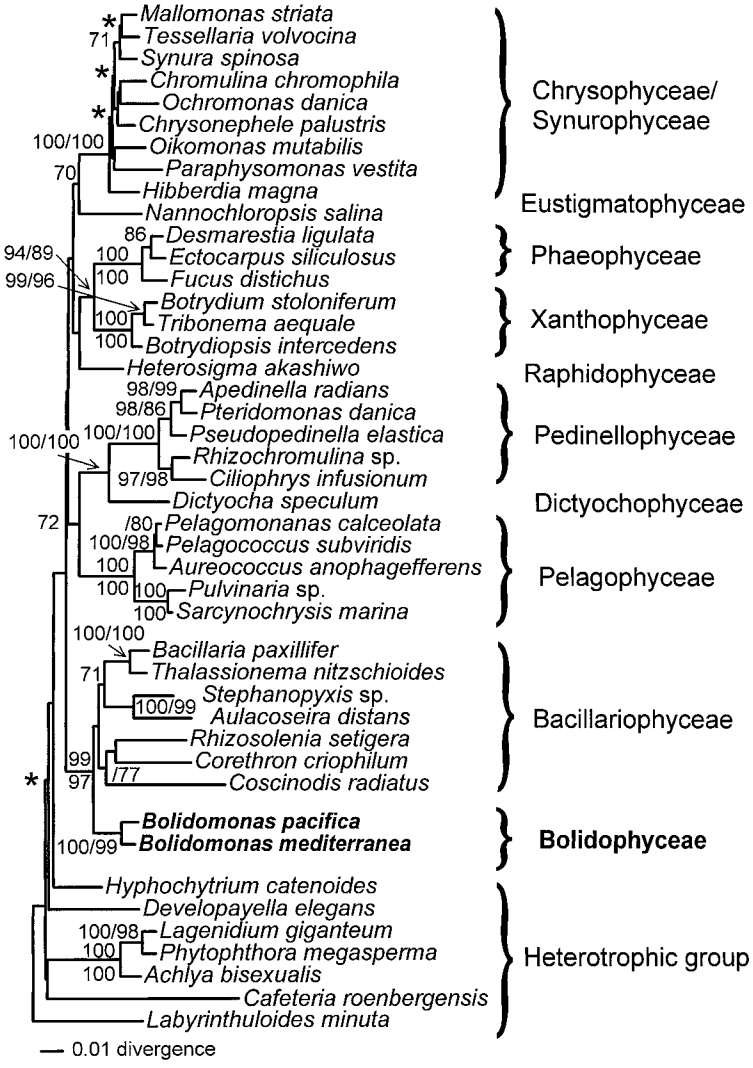


FIG. 19. Distance tree derived from an alignment of SSU rDNA sequences from different heterokonts. Bootstrap values at the internal branches (1000 replicates, values >70% displayed) corresponding to neighbor-joining (with a Kimura two-parameter correction) and maximum parsimony analysis (tree length: 1,185; residual sum squares: 0.0250786), above and below the branch, respectively. Asterisks signal where branching order is different between the two methods. Scale bar = 0.01 divergence.

TABLE 2. Comparison of selected characters in the genus *Bolidomonas* and other heterokont organisms (including all algal classes, after Manton and Von Stosch 1965, Moestrup 1970, Hibberd and Leedale 1971, Heath and Darley 1972, Hibberd and Leedale 1972, Patterson and Fenchel 1985, Marchant and McEldowney 1986, Andersen 1987, Heywood 1989, O'Kelly 1989, Moestrup and Thomsen 1990, Owen et al. 1990, Santos and Leedale 1991, Andersen et al. 1993, Honda et al. 1995, Daugbjerg 1996, O'Kelly and Patterson 1996). + = present, − = absent, ab = above the nucleus, ant = anterior to nucleus, Bico = Bicosoecids, Boli = *Bolidomonas* spp., Chryd = Chrysomeridales, Chrys = Chrysophyceae, D = diadinoxanthin containing, dep = in a depression of the nucleus, Diat = diatoms, Dict = Dictyochophyceae, Eust = Eustigmatophyceae, G = spermatozoid form, L = vegetative life form, NA = not available, ND = not determined, P = proximal position, Parm = Parmales, Pedi = Pedinellophyceae, Pela = Pelagophyceae, Phaeo = Phaeophyceae, post = posterior to nucleus, R = ring, Raph = Raphidophyceae, S = scattered, Sulc = *Sulcochrysis biplastida,* Synu = Synurophyceae, T = transitional position, V = violaxanthin containing, Xant = Xanthophyceae, Z = zoospore form.

	Bico	Boli	Diat	Parm	Sulc	Pela	Pedi	Dict	Chrys	Syn	Xant	Raph	Chryd	Phaeo	Eust
Chloroplast DNA	NA	R	R	R	R	S	S	S	R	R	R	R	R	R	S
Pigment type	NA	D	D	ND	ND	D	D	D	V	V	D	V/D	V	V	V
Golgi apparatus	ant	ant	NA	NA	ant	ant	post	scat	ant	ant	ant	ant	ant	ant	ND
Eyespot	−	−	−	−	−	−	−	−	+	−	+	−	+	+	+
Type of flagellate cells	L	L	G	L	L	L	L	L	L	L	G+Z	L	Z	Z+G	Z
Basal bodies	ab	ab	ab	NA	dep	dep	dep	dep	ab	ab	ab	ab	ab	ab	ab
Helix	−	−	−	NA	P	P	P	P	T	T	T	−	T	−	T
No. of transitional plates	1	2	0	NA	1	2	1	1	1	1	1	1	1	1	1
Flagellar roots	+	−	−	NA	+	−	−	−	+	+	+	+	+	+	+
Rhizoplast	+	−	−	NA	−	−	−	−	+	+	+	+	−	−	+
Lateral filaments of flagellar hairs	−	−	−	NA	−	−	−	−	+	+	−	−	−	−	−

However, given their sister position to the diatoms, the Bolidophyceae are an intermediate group that furthers our understanding of evolutionary relationships between the diatoms and all other Heterokonta. Diatom evolution has remained enigmatic because their silica frustule provides them with a unique morphology (Round and Crawford 1981) and because they appear suddenly in the fossil record during the Lower Cretaceous as quite elaborate organisms (Harwood and Gersonde 1990). The origin of the diatoms and their relationship to an ancestral photosynthetic heterokont are still unresolved, especially because living or fossil transitional forms reconstructing their evolution are lacking. The discovery of *Bolidomonas* and flagellated forms found in most ancestral lineages (i.e. *Developayella elegans* Tong) strongly suggests that the first heterokont that gave rise to the diatom lineage was a biflagellated unicell. Several hypotheses concerning the emergence of the diatom lineage from such an ancestor have already been proposed. Two opposite scales of a scaly flagellate ancestor could have evolved into the diatom frustule (Round and Crawford 1981). The presence of organic scales on the vegetative stages of the labyrinthulids and thraustochytrids also supports this hypothesis for the evolution of the diatom frustule (Medlin et al. 1997). However, because diatoms are diplonts, unlike many other unicellular organisms that are presumed haplonts, Mann and Marchant (1989) proposed that the diatom ancestor could have been a scaly haploid flagellate that formed scaly diploid zygotes. They suggest that mitotic division of the diploid zygote might have taken place preferentially to give rise to the diatoms. However, there are many diplontic heterokont lineages (Fucales in the brown algae, Vaucheriales in the Xanthophyceae, Oomycota, and the Raphidophyceae), and there is some evidence that

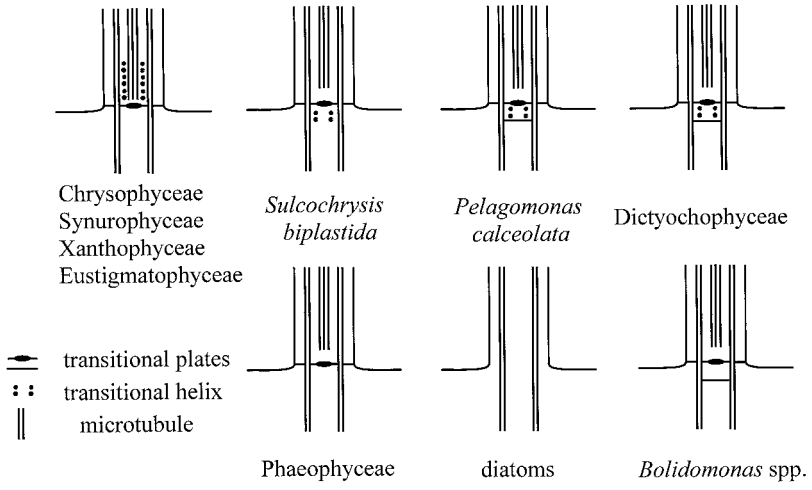


FIG. 20. Schematic drawings of the flagellar transition region in several groups including the genus *Bolidomonas*. Modified from Preisig (1989).

early heterotrophic divergences in the heterokont lineage are also diplonts (Medlin et al. 1997). Such reports suggest that the ancestral heterokont was likely diploid. Nevertheless, it is possible that life cycle stages could be decoupled so that either the haploid or the diploid stage in one group becomes the dominant vegetative form. Without knowing the ploidy level of the Bolidophyceae, one can only speculate whether the Bolidophyceae and the diatoms are representatives of two different morphologies included in an ancestral life history. The hypotheses of both Round and Crawford (1981) and Mann and Marchant (1989) could be tested if the ploidy levels were known for both groups and for the Parmales (Booth and Marchant 1987); these might also play a key role in the evolution of these groups. Key questions as to whether Bolidophyceae are diplonts or haplonts and have retained sexual reproduction and whether they can produce resistant forms with silica structures remain to be answered.

Ecological implications. In oligotrophic areas, picoeukaryotes constitute up to one-third of the total phytoplanktonic biomass, the rest being attributable to the prokaryotes *Prochlorococcus* and *Synechococcus* (Campbell et al. 1994). Because picoeukaryotes are significantly larger than the photosynthetic prokaryotes, their contribution to primary production might in fact exceed their share of the biomass (Li 1994). Despite their key role, we have very little information on the taxonomic identity of picoeukaryotes. The only technique widely used for this purpose is HPLC pigment analysis, which provides information at the class level (e.g. Claustre and Marty 1995). However, this approach relies on hypotheses that are based on pigment data from cultured strains. The fact that *Bolidomonas* strains have been isolated from several locations, both in the Mediterranean Sea and the equatorial Pacific Ocean and from both oligotrophic and mesotrophic waters, suggests that the Bolidophyceae could be widespread in the oceans. Because both the Bolidophyceae and the diatoms possess similar pigments, diatoms could well be overestimated by current pigment algorithms at the expense of the Bolidophyceae. A better knowledge of the abundance and distribution of Bolidophyceae should allow us to determine whether they are minor or major contributors to the fucoxanthin pool in the ocean and, eventually, to correct our current pigment-based estimates. The present study suggests that there might be still other major picoplanktonic lineages to be discovered in oceanic waters; thus, it is critical to continue isolating and characterizing novel strains.

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