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ZOOSPOROGENESIS, MORPHOLOGY, ULTRASTRUCTURE, PIGMENT COMPOSITION, AND PHYLOGENETIC POSITION OF *TRACHYDISCUS MINUTUS* (EUSTIGMATOPHYCEAE, HETEROKONTOPHYTA)[1]

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The traditional order Mischococcales (Xanthophyceae) is polyphyletic with some original members now classified in a separate class, Eustigmatophyceae. However, most mischococcalean species have not yet been studied in detail, raising the possibility that many of them still remain misplaced. We established an algal culture (strain CCALA 838) determined as one such species, *Trachydiscus minutus* (Bourr.) H. Ettl, and studied the morphology, ultrastructure, life cycle, pigment composition, and phylogeny using the 18S rRNA gene. We discovered a zoosporic part of the life cycle of this alga. Zoospore production was induced by darkness, suppressed by light, and was temperature dependent. The zoospores possessed one flagellum covered with mastigonemes and exhibited a basal swelling, but a stigma was missing. Ultrastructural investigations of vegetative cells revealed plastids lacking both a connection to the nuclear envelope and a girdle lamella. Moreover, we described biogenesis of oil bodies on the ultrastructural level. Photosynthetic pigments of *T. minutus* included as the major carotenoids violaxanthin and vaucheriaxanthin (ester); we detected no chl *c*. An 18S rRNA gene-based phylogenetic analysis placed *T. minutus* in a clade with species of the genus *Pseudostaurastrum* and with *Goniochloris sculpta* Geitler, which form a sister branch to initially studied Eustigmatophyceae. In summary, our results are inconsistent with classifying *T. minutus* as a xanthophycean and indicate that it is a member of a novel deep lineage of the class Eustigmatophyceae.

Key index words: **18S rRNA; autofluorescence; *Goniochloris sculpta*; lipid bodies; photosynthetic pigments; phylogeny; *Pseudostaurastrum*; *Trachydiscus minutus*; zoospores**

Abbreviations: **DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; FM, fluorescence microscopy; ML, maximum likelihood**

Forty years ago, ultrastructural investigations of motile cells of several traditional members of the heterokontophyte class Xanthophyceae revealed unique features that distinguished them from xanthophyceans sensu stricto, and led to the establishment of the class Eustigmatophyceae (Hibberd and Leedale 1970, 1971). A seminal article published by Hibberd (1981) later provided a taxonomic revision of the class, which was divided into four families, six genera, and at least 12 species. Since then, the class Eustigmatophyceae has been diversified by descriptions of four new species of the genus *Nannochloropsis* D. J. Hibberd (Lubián 1982, Karlson et al. 1996, Krienitz et al. 2000, Suda et al. 2002) and three new monospecific genera: *Botryochloropsis similis* Preisig et C. Wilh. (Preisig and Wilhelm 1989), *Pseudellipsoidion edaphicum* J. Neustupa et Němcová (Neustupa and Němcová 2001), and *Pseudotetraëdriella kamillae* E. Hegewald et J. Padisák (Hegewald et al. 2007). In addition, three more species originally placed in the

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Xanthophyceae, namely, *Monodus unipapilla* Reisigl [sometimes referred to as *Monodopsis unipapilla* (Reisigl) L. M. A. Santos, but this combination has never been validly published], *Pseudostaurastrum limneticum* (Borge) Chodat, and *Pseudostaurastrum enorme* (Ralfs) Chodat, were recognized as eustigmatophyceans based on ultrastructural, biochemical, and/or molecular phylogenetic investigations (Santos and Leedale 1995, Schnepf et al. 1996, Hegewald et al. 2007). Hence, Eustigmatophyceae formally comprise a little over 20 species in 11 genera at present. All members are unicellular coccoid algae thriving in soil, freshwater, or marine habitats and are characterized by one or more plastids lacking a girdle lamella and chl *c*. No flagellated stages are known for the genera *Monodopsis* D. J. Hibberd, *Nannochloropsis*, and *Chlorobotrys* Bohlin, whereas the remaining eustigmatophyceans can form zoospores possessing one (*Vischeria* Pascher, *Eustigmatos* D. J. Hibberd, *P. limneticum*, *P. edaphicum*, *P. kamillae*) or two (*Pseudocharaciopsis* K. W. Lee et H. C. Bold, *B. similis*) emergent flagella (Hibberd 1981, Schnepf et al. 1996, Neustupa and Němcová 2001, Hegewald et al. 2007). Uniquely among heterokontophytes, the eustigmatophycean zoospores are characterized by the presence of an extraplastidial stigma, with the known exceptions of zoospores of *P. limneticum* and *P. kamillae* lacking a stigma at all (Schnepf et al. 1996, Hegewald et al. 2007). Most eustigmatophyceans also appear to be unique among heterokontophytes by lacking continuity of the outer plastid membrane with the nuclear envelope, but members of the genera *Monodopsis* and *Nannochloropsis* were shown to have this connection preserved (Santos and Leedale 1995, Murakami and Hashimoto 2009).

Bourrelly (1951) described a new species of the genus *Pseudostaurastrum* Chodat, *Pseudostaurastrum minutum* Bourr. from a basin of the Paris Botanical Garden. The species was described as follows: cells small (8 μm in diameter), of pentagonal or quadrate shape with rounded angles, 2–3 parietal discoid plastids; the cells are compressed and are biconvex pillow shaped, with a thickness of 4–5 μm; the cell wall has hemispheric hollows regularly aligned along three axes (angles of 60°). This alga was further observed in the nannoplankton of a pool in Trappes (a commune in the western suburbs of Paris), where the cells were pentagonal, and in Bonne Mare (near Rouen, France), where cell polymorphism was notably conspicuous (Bourrelly 1951). Based on the specific pattern of cell wall ornamentation, Ettl (1964) established a new algal genus, *Trachydiscus* H. Ettl, with newly described *Trachydiscus lenticularis* H. Ettl as a type species, several other newly described species, and a new combination *T. minutus* (Bourr.) H. Ettl (*P. minutum*). Later, Bourrelly (1968) questioned the assignment of *P. minutum* into the genus *Trachydiscus* and proposed its transfer to the genus *Goniochloris* Geitler because of its scrobiculate cell wall. For years, the genera *Pseudostaurastrum*, *Trachydiscus*, and *Goniochloris* were believed to be members of the family Pleurochloridaceae, order Mischococcales, class Xanthophyceae (Ettl 1978). However, most of the known representatives of the class Eustigmatophyceae were actually recruited from traditional members of the family Pleurochloridaceae (Hibberd 1981), and only one species of this family has been so far confirmed as a xanthophycean using modern approaches, namely *Pleurochloris meiringensis* Vischer (Andreoli et al. 1999). Indeed, the structure of the vegetative cells and zoospores, life cycle characteristics, and the absence of chl *c* in *P. limneticum* led Schnepf et al. (1996) to transfer this species to the class Eustigmatophyceae. Recently, the phylogenetic position of *P. limneticum* and *P. enorme* was investigated using 18S rRNA gene sequences and these two species were found to form a sister branch to other eustigmatophyceans included in the study (Hegewald et al. 2007). These investigations suggest that the taxonomic position of many other members of Pleurochloridaceae needs to be evaluated.

Our knowledge of many members of the family Pleurochloridaceae is limited by the lack of cultures. In 2005, we found an abundant population of a unicellular alga that was identified as *T. minutus* in cooling pools of the Temelín nuclear power plant (Czech Republic). We established an axenic culture, enabling us to investigate the morphology, ultrastructure, life cycle, biochemical characteristics and phylogenetic position of this species. Herein, we report results of our studies and reveal that *T. minutus* is another entry in the growing list of eustigmatophycean species.

MATERIALS AND METHODS

Experimental material and growth conditions. *T. minutus* strain LUKAVSKY et PRIBYL 2005/1 was collected from a cooling pool at the Temelín nuclear power plant (Czech Republic, 49°11′0.585″ N; 14°22′35.893″ E). Hydrochemical characteristics in the field were measured and species composition of phytoplankton was determined as described previously (Lukavský et al. 2006). A unialgal culture was isolated from a plankton sample using an agar-plate method. The sample was diluted with distilled water to obtain cell density of ~1–5×10^5 cells · mL^{-1}, spread out on a petri plate containing agar-solidified 1/2 SŠ nutrient medium (Zachleder and Šetlík 1982) and allowed to grow under irradiance of 30 μmol · $m^{-2} \cdot s^{-1}$ and at temperature of 22°C for 7 d. Single colonies of *T. minutus* were transferred to a new agar plate and spread out again, and this procedure was repeated until the axenic culture was obtained. After isolation, the axenic strain was deposited in the Culture Collection of Autotrophic Organisms (CCALA, http://www.butbn.cas.cz/ccala) as CCALA 838. It has been maintained on agar slants under irradiance of ~23 μmol · $m^{-2} \cdot s^{-1}$ and at temperature of 12°C–15°C. *G. sculpta* Geitler, strain SAG 29.96 was provided by the Culture Collection of Algae at the University of Göttingen, Germany (SAG, http://sagdb.uni-goettingen.de/), and it has been maintained under the same conditions.

The zoosporogenesis experiments included batch precultivation of both *T. minutus* and *G. sculpta* in a 100–120 mL bubble column photobioreactor filled with sterilized nutrient

medium 1/2 SŠ. Cultures were grown under continuous light of irradiance 500 μmol · m^{-2} · s^{-1} provided by a panel of light tubes Osram L 36 W/830 Lumilux (Osram, München, Germany) and at temperature of 28 ± 0.5°C. Cultures were bubbled vigorously with air enriched with 2% carbon dioxide (CO_2, v/v). Obtained exponentially growing cultures were used for studying zoosporogenesis, as follows. Cultures were synchronized by alternating light and dark periods (3–5 cycles, 12:12 h) under conditions described above. At the onset of each light phase, the cultures were diluted with fresh medium 1/2 SŠ to obtain a cell density of ~0.3–0.5 × 10^6 cells · mL^{-1}. Synchronized cultures were then continuously shaded using aluminum foil to induce zoospores; other cultivation conditions remained unchanged. Production of zoospores was quantified as a proportion of flagellates to the whole cell population using a Bürker counting chamber (Assistent, Sondheim, Germany); at least 400 cells were counted for each treatment.

Light and fluorescence microscopy (FM). Samples of algae were withdrawn during growth at regular intervals and immediately fixed in 1% glutaraldehyde (Merck, Whitehouse Station, NJ, USA) for subsequent microscopic observations. Cell size was assessed using the digital image analysis software AnalySIS; at least 100 cells were measured in every sample. Intracellular oil droplets were observed in a fluorescence microscope due to their autofluorescence, which gave the same signal as the Nile Red staining method (Eltgroth et al. 2005). DNA was stained by 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Serva, Heidelberg, Germany) as described previously (Přibyl and Cepák 2007). Samples were observed using an Olympus BX 60 epifluorescence light microscope (Olympus, Tokyo, Japan) equipped with the filter combination U-MNU2 (360–370 nm excitation and >515 nm barrier filter) and with Nomarski differential interference contrast. Microphotographs were taken with an Olympus DP-71 digital camera (Olympus) and processed using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA, USA).

TEM. A suspension of algae was fixed using 2.5% glutaraldehyde in 0.2 M cacodylate buffer for at least 4 h. Glutaraldehyde was washed out with the same buffer (3 × 15 min) and cells were postfixed using a mixture of 0.2 M cacodylate buffer and 4% osmium tetroxide (OsO_4) solution (1:1) for 2 h. The fixative was then washed out (3 × 15 min) and cells were dehydrated in ascending sequences of acetone (30, 50, 70, 80, 90, 95, 100%), each for 15 min. Samples were then immersed into sequences of the mixture consisting of Spur's resin and 100% acetone (1:2, 1:1, 2:1), each for 1 h, and finally embedded into 100% Spur's resin and put in a desiccator for 24 h. Resin blocks with samples were then cut using an ultramicrotome Reichert Jung Ulracut E (Reichert, Depew, NY, USA) and ultrathin sections were attached to grids and contrasted using saturated solution of uranyl acetate and lead citrate. Samples were examined in a Jeol 1010 TEM electron microscope (Jeol, Tokyo, Japan); images were recorded with a Lhesa 72 W digital TV camera (Lhesa, Cergy-Pontoise, France) and processed using Adobe Photoshop 7.0 (Adobe Systems).

SEM. An algal suspension was attached to a slide using poly-L-lysine and fixed using 2.5% glutaraldehyde in 0.2 M cacodylate buffer for at least 4 h. Glutaraldehyde was washed out with the same buffer (3 × 15 min) and cells were postfixed using a mixture of 0.2 M cacodylate buffer and 4% OsO_4 solution (1:1) for 2 h. The fixative was then washed out (3 × 15 min) and cells were dehydrated in ascending sequences of acetone (30, 50, 70, 80, 90, 95, 100%), each for 15 min. Samples were then dried by the critical point method using CO_2, attached to grids and coated with gold. Samples were examined in a Jeol JSM-7401F SEM (Jeol), images were recorded with a Lhesa 72 W digital TV camera (Lhesa) and processed using Adobe Photoshop 7.0 (Adobe Systems).

Pigment extraction and HPLC. Cells were collected by centrifugation of ~10 mL of algal culture. A mixture of 0.5 mL of PBS (8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 140 mM NaCl, pH 7.4) and 1 mL of glass beads (diameter 0.5 mm) was added to the algal pellet in a glass test tube. Cells in the pellet were disintegrated using a high-speed disintegrator for 4 min in four 1 min steps interrupted by cooling the test tubes on ice. A quantity of 3 mL of methanol was added, samples were shaken briefly and centrifuged (5,000*g*, 10 min, 20°C). Extracted pigments were drained using a micropipette and the extraction procedure was repeated with 2 mL of methanol. The collected extracts were gathered and directly used for HPLC analysis. HPLC was performed using an Agilent 1200 chromatography system equipped with the diode array detector (Agilent, Santa Clara, CA, USA). Pigments were separated on a Phenomenex column Luna 3 μ C8, size 100 × 4.60 mm (Phenomenex, Torrance, CA, USA) in an ammonium acetate/MeOH gradient. Eluted pigments were monitored by their absorption at 440 nm and determined according to literature (Jeffrey et al. 1997).

DNA extraction, amplification of the 18S rRNA gene, and sequencing. Cells were harvested from an agar-solidified medium, transferred to a microtube, resuspended in distilled water, collected by centrifugation, and disrupted using a Retsch mixer mill MM 200 (Retsch, Haan, Germany) by shaking with glass balls with a diameter of 0.5 mm for 5 min. Total DNA was extracted using the Invisorb® Spin Plant Mini Kit (Invitek, Berlin, Germany) following the manufacturer's instructions. A segment of the 18S rDNA gene (1,755 bp excluding primer regions) was amplified by PCR using universal forward (F) and reverse (R) primers according to Katana et al. (2001). The PCR product was purified directly using JETQUICK PCR Product Purification Spin Kit (Genomed, Löhne, Germany). The sequence of the purified DNA fragment was obtained by sequencing reactions performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) and analyzed using the 3130 Genetic Analyzer (Applied Biosystems). Primers employed for sequencing included the amplification primers and internal sequencing primers as described in Katana et al. (2001). Sequencing reads were assembled with the CAP3 assembler server (Huang and Madan 1999; http://pbil.univ-lyon1.fr/cap3.php), and manually edited by visual inspection of sequencing chromatograms. The newly obtained sequence, excluding the primer regions, was deposited at GenBank with accession number HQ007250.

Phylogenetic analyses. A BLASTN search against the nonredundant nucleotide sequence database at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) indicated that the 18S rRNA gene sequence of *T. minutus* is most similar to sequences from eustigmatophyceans and other stramenopile algae (heterokontophytes). To determine the actual phylogenetic position of *T. minutus*, the newly determined sequence was aligned to 53 other 18S rRNA gene sequences from the DDBJ/EMBL/GenBank database selected to represent all known major heterokontophyte lineages. The selection rationale was to include 2–3 representative sequences for each formally described heterokontophyte class as defined in Kai et al. (2008), plus some additional sequences representing unplaced taxa (*Olisthodiscus luteus* NIES-15, *Leukarachnion* sp. ATCC PRA-24, *Chlamydomyxa labyrinthuloides*, *Tetrasporopsis fuscescens* SAG 20.88, *Pleurochloridella botrydiopsis* CCMP 1665, ''*Chloromorum toxicum*'' strain C. Tomas Delaware). Eustigmatophyceae were sampled more densely to include one sequence of each nominal species represented in the public database. Sequences from the hyphochytriomycete *Hyphochytrium catenoides* ATCC 18719 and the heterotrophic stramenopile flagellate *Developayella elegans* ATCC 50518 were included as a suitable outgroup for heterokontophytes. The multiple alignment was built using ClustalX (Thompson et al. 1997) and manual editing with

GeneDoc (K. B. Nicholas and H. B. Nicholas, http://www.nrbsc.org/gfx/genedoc/) guided by the secondary structure model of the *Bacillaria paxillifer* 18S rRNA available from the European Ribosomal RNA Database (http://bioinformatics.psb.ugent.be/webtools/rRNA/secmodel/Bpax_SSU.html; Wuyts et al. 2000). The sequence alignment is available from the authors upon request. Regions that could not be aligned with confidence were excluded, leaving 1,650 positions that were subjected to phylogenetic analyses. A maximum-likelihood (ML) tree was inferred using RAxML 7.0.4 run at the Cyberinfrastructure for Phylogenetic Research (CIPRES) Portal (http://www.phylo.org/sub_sections/portal/) employing a strategy that was recently demonstrated as especially effective in finding a tree with the highest likelihood. This strategy combines a new rapid bootstrap algorithm to infer 100 bootstrap trees with subsequent thorough ML inference on the original dataset utilizing the bootstrap trees for further optimization (Stamatakis et al. 2008). The substitution model employed for the final ML search was set to the most general GTR+Γ+I model. A Bayesian inference was performed using MrBayes 3.1 (Huelsenbeck and Ronquist 2001) and also run at the CIPRES portal. Two parallel MCMC runs were carried out for 1 million generations each with one cold and three heated chains employing the GTR+Γ+I+COV substitution model (with parameters estimated from the data). Trees were sampled every 100 generations. The initial 2,501 trees of each run were discarded as ''burn-in'' and posterior probabilities of tree bipartitions were calculated on the basis of the consensus of the remaining 15,000 trees.

RESULTS

Ecology. *T. minutus* was found in September 2005 in the plankton of one of the three cooling pools at the Temelín nuclear power plant (Czech Republic). This was the first record of the species from the Czech Republic area. During autumn 2005 it was a dominant species in all three cooling pools among 12 species of Chlorophyceae, Bacillariophyceae, Trebouxiophyceae, Euglenophyceae, and Cyanobacteria. The total phytoplankton density was $\sim 10^5$ cells $\cdot$ mL^{-1}. Water in these pools was oligotrophic with mean yearlong temperature of 19.3°C–20.9°C, pH 8.13–8.64 and conductivity 0.39–0.40 mS $\cdot$ cm^{-1}. Moreover, *T. minutus* was rarely found in the crude water from the river Vltava used for filling the cooling system of the Temelín nuclear power plant. *T. minutus* was also collected in all cooling pools during the year 2006, although not as a dominant species, and from 2007 until 2010 it was not observed in any of the study locations. An algal species tentatively determined as *T. minutus* was rarely found during March–April, 2010 in the phytoplankton of the fishpond Svět near the town Třeboň, 35 km away from the Temelín locality.

Life cycle. *T. minutus* possessed a simple life cycle, which is summarized in Figure 1. Usually, the alga reproduced by endogenous division of the protoplast of a parent cell (Fig. 1, a and b) into two (Fig. 1c) or four progeny (aplanospores), which eventually achieved the size of the parent cell (Fig. 1d). Under standard conditions zoospores were observed neither in natural samples nor in culture. However, under specific conditions zooids were produced (Fig. 1e), from which new population of parent cells arose (Fig. 1, f and g). The sexual process was never observed and the zooids behaved solely as zoospores. Compared to, for example, *Chlamydomonas* zoospores, the zoospores of *T. minutus* moved relatively slowly; in the light microscope individual zoospores could be trailed for ~10 min until they stopped moving, cast off the flagellum, and became rounded. Figure 2 demonstrates zoospores and vegetative cells of a *T. minutus* culture.

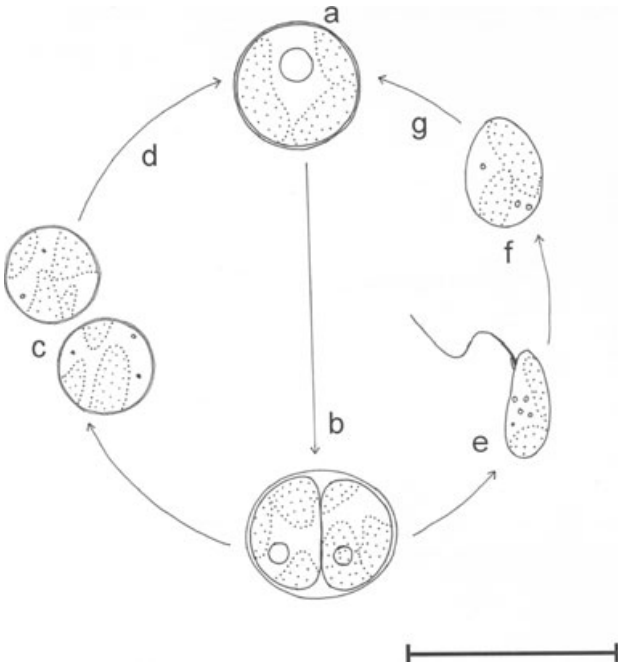

FIG. 1. Interpretative drawing of the life cycle of *Trachydiscus minutus*, representing a summary of many individual observations. (a) Parent cell, (b) endogenous division of the protoplast, (c) progeny aplanospores, (d) growth, (e) zoospore, (f) loss of flagellum and rounding of the zoospore, (g) growth. Scale bar, 10 μm.

Zoosporogenesis. The culture of *T. minutus* was grown and synchronized as described in the ''Materials and Methods'' and afterward transferred to the dark. Zoospores started to be released after 3–4 d of cultivation in darkness and their maximal count was recorded within 6–7 d in dark conditions (Fig. 3). Light/dark synchronization was not an essential condition for zoospore production; zoospores in asynchronized cultures were still released, but in lower number. Zoospore production was temperature dependent; at a low temperature (15°C) zoospore production was not higher than 10% of the total cell population; at a higher temperature (28°C) zoospore production was reduced (3%–5%). The optimal temperature was found ~20°C, when motile zoospores represented about one-third of the total cell population. Nitrogen starvation during cultivation inhibited the release of zoospores (not shown). When transferred from the dark back into the light, the number of zoospores in the cultures fell to nearly zero regardless of the temperature.

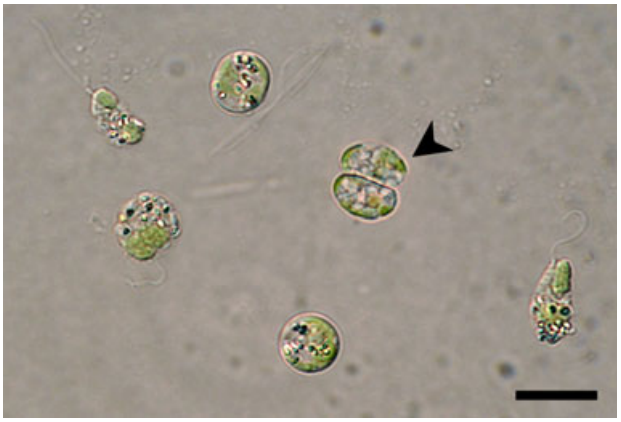

FIG. 2. Overview of a population of *Trachydiscus minutus* cells after 5 d of cultivation in darkness at 20°C. Both aplanospores and zooids are visible; arrowhead points to a dividing cell. Scale bar, 10 μm.

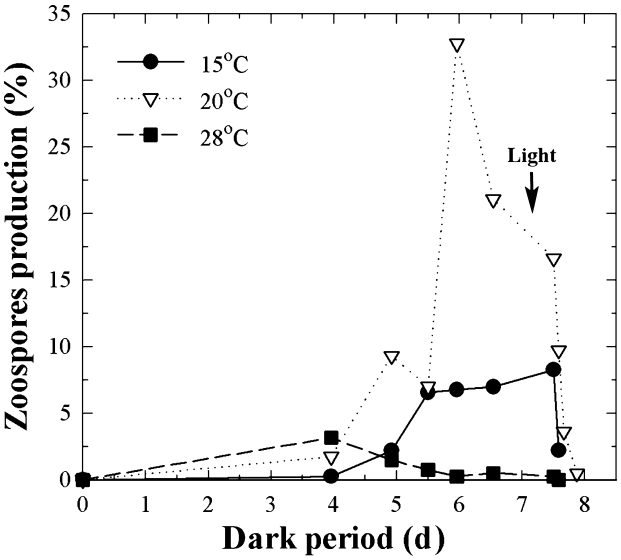

FIG. 3. Time course of zoospore induction in *Trachydiscus minutus* using dark cultivation at three different temperatures. The zoospore production is expressed as % of the total cell number in the culture. The arrow shows the time of transfer of cultures into the light.

Morphology and ultrastructure. Vegetative cells of *T. minutus* were discoid or fusiform, from the wider side usually rounded or pentagonal with rounded ends (Fig. 4a). The size of the wider side of the cell ranged between 7 and 9 μm. The cell wall was relatively thick (0.15–0.25 μm) with numerous smallish papillae (Fig. 4b). Cells were mononuclear, the nucleus was 0.8–1.4 μm in diameter (Fig. 4, b and c), sometimes the single nucleolus and condensed chromatin was visible (Fig. 4c). Mitochondria were oblong shaped, 0.7–1.2 μm long, with tubular cristae (Fig. 4d); a pyrenoid was observed neither in the light microscope nor TEM. The cells contained several (usually 3–4) lens-shaped parietal plastids (Fig. 4, a and b) without a peripheral ring of DNA. The outer plastid membrane was never continuous with the nuclear envelope (Fig. 5, a and b) and plastids lacked a girdle lamella (Fig. 5b). Numerous electron-transparent vesicles were found in the cytoplasm of vegetative cells (Fig. 5c). These vesicles had distinct lamellate structure (Fig. 5, c and d), lamellae were oriented parallel to their long axis with spacing of 6–7 nm. Each vesicle was surrounded by two membranes (Fig. 5d).

The presence of easily visible yellowish oil droplets within cells (Fig. 4a) was a conspicuous attribute of *T. minutus*. Generally, neutral lipids of *T. minutus* were localized in spherical lipid bodies of various numbers, as revealed by both FM and TEM. Tiny lipid bodies took form as numerous plastoglobuli among thylakoid lamellae in the plastid stroma (Fig. 6, a–c), later they were released but remained attached to the plastid surface (Fig. 6, c–f). Afterward lipid bodies gradually fused to form larger droplets (Fig. 6, f and g), finally creating one large lipid inclusion released in the cytoplasm (Fig. 6, h and i), often in the central position (Fig. 6i). Lipid droplets were observed in the cells during the entire growth phase, but only during stationary phase they were apparently larger. Older cells usually had one large lipid body 1–2 μm in diameter (Fig. 6i). This was also readily apparent owing to lipid autofluorescence (Fig. 6, d and h), which gave observable fluorescence signal within a wide range of wavelengths (520–640 nm) with a maximum of ~600 nm.

Zoospores of *T. minutus* were ovate or fusiform in shape, naked, without a visible eyespot (Fig. 7, a and c) and always mononuclear (Fig. 7c). The size of zoospores was 3.5–6 × 8.5–11 μm; they lacked pyrenoid and contained usually two plastids. The zoospores bore only a single flagellum inserted slightly subapically (Figs. 7a and 8), covered by mastigonemes and exhibited a putative basal swelling (Fig. 8) characteristic of eustigmatophyceans. The length of the flagellum slightly exceeded the length of the zoospore body (Figs. 7, a and c; 8). *T. minutus* showed distinctive morphological similarities to *G. sculpta*, strain SAG 29.96. Both species had identical plastid shape and arrangement and contained a conspicuous oil droplet in the cytoplasm of vegetative cells. The pattern of cell wall ornamentation was similar as well. They differed in the cell shape and size; *G. sculpta* was predominantly triangular in shape and cells were about two times larger (10–20 μm) compared to *T. minutus*. The morphology of *G. sculpta* zoospores was also similar to those of *T. minutus* (Fig. 7, a and b). A similar pattern of zoosporogenesis was observed also in *G. sculpta*, although zoospores were released with lower efficiency at 20°C (up to 1%).

Photosynthetic pigment composition. We found violaxanthin as the predominant carotenoid representing 56.6% of total detected carotenoids in

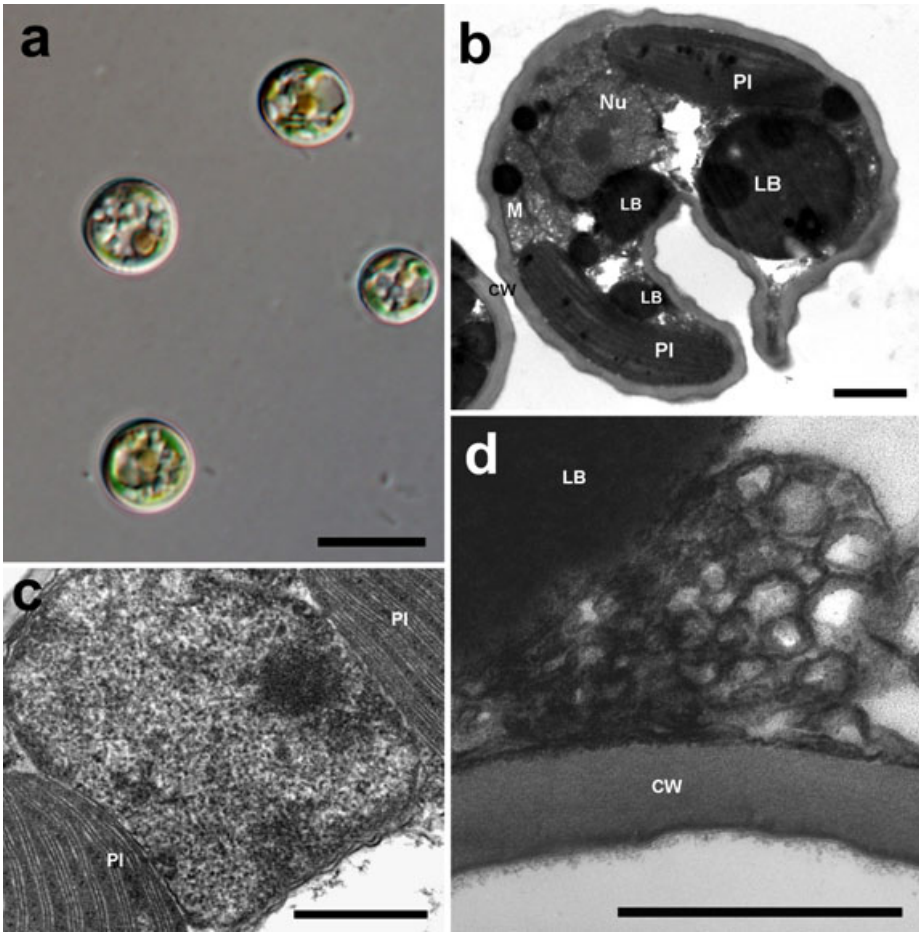

FIG. 4. Morphology and ultrastructure of *Trachydiscus minutus* vegetative cells. (a) LM image of a population of old cells. (b) TEM image of a whole cell, Nu = nucleus, Pl = plastids, CW = cell wall, M = mitochondrion, LB = lipid bodies. (c) Detail of a nucleus; nucleolus and condensed chromatin are visible, Pl = plastids with thylakoid lamellae. (d) A detailed view of a mitochondrion with tubular cristae. Scale bars: (a) 10 μm, (b) 1 μm, (c and d) 0.5 μm.

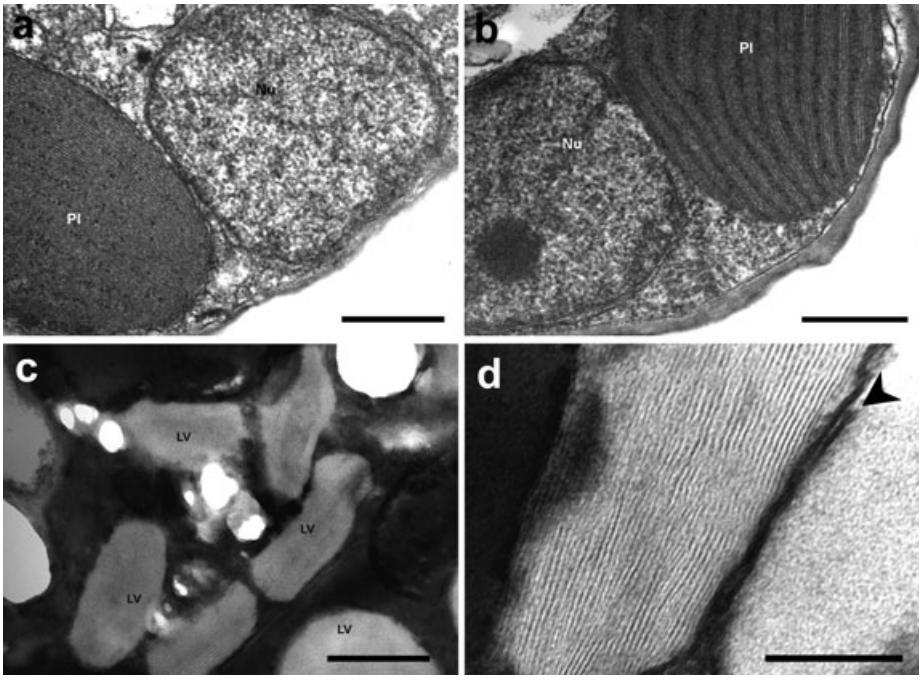

FIG. 5. Ultrastructure of *Trachydiscus minutus* cells, TEM images. Nu = nucleus, Pl = plastids with thylakoid lamellae, LV = lamellate vesicles. The lack of connection between the plastid membrane and the nuclear envelope (a and b) and the absence of a girdle lamella of a plastid (b) are demonstrated. (c) Lamellate vesicles of an old cell. (d) Detail of two adjacent lamellate vesicles, arrowhead points to surrounding membranes. Scale bars: (a–c) 0.5 μm, (d) 0.2 μm.

T. minutus. Other main carotenoids were vaucheriaxanthin (ester) (25.9%) and β-carotene (9.7%); only a low amount of zeaxanthin (3.2%) was detected. Moreover, we identified another xanthophyll species, probably neoxanthin (4.7%). Chl *a* was determined to be the only chlorophyll species,

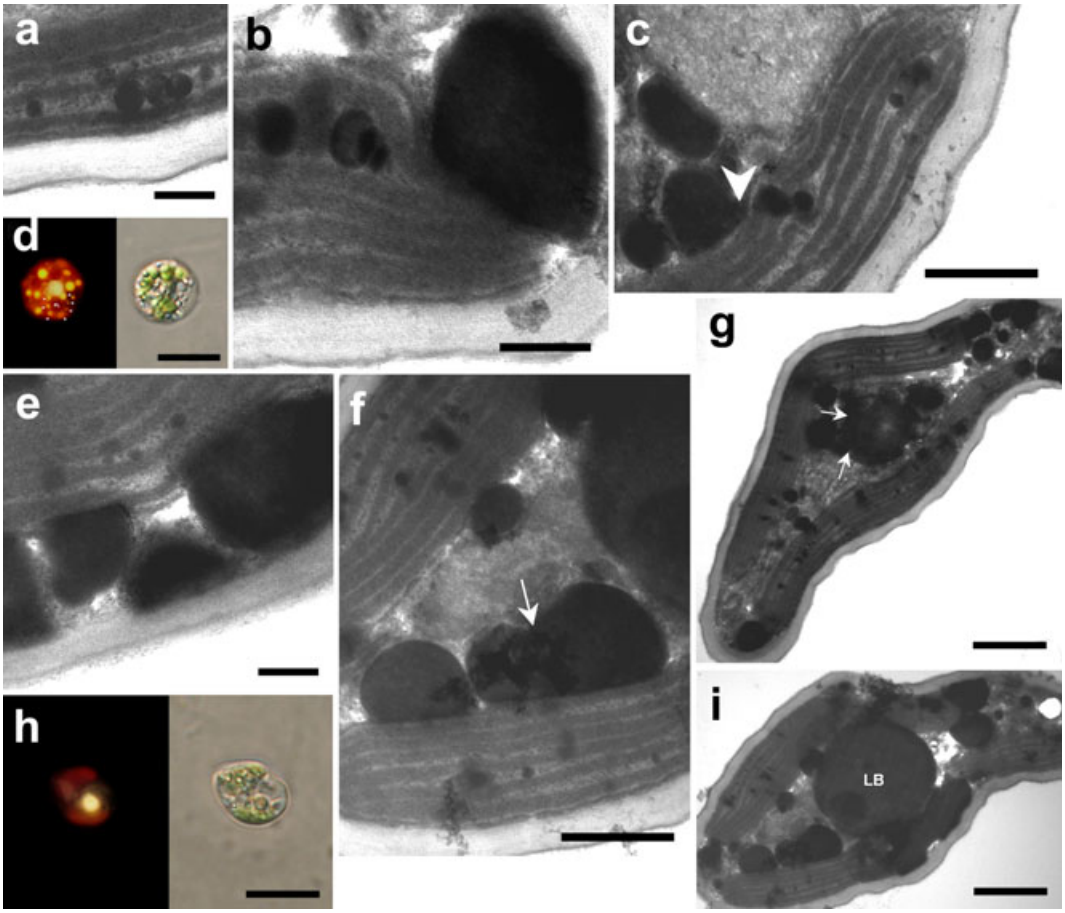

FIG. 6. Biogenesis of lipid bodies in *Trachydiscus minutus* vegetative cells, TEM, fluorescence microscopy (FM), and LM images. The autofluorescence of plastids (red) and lipid bodies (yellow) is visible in FM; lipid bodies are observable as droplets of high electron density in TEM. (a) Lipids arise as tiny plastoglobuli inside the plastid among its thylakoid lamellae. (b–d) Larger droplets are excluded from the surface of the plastid (arrowheads). (e) Growth of released droplets. (f) Droplets fuse forming larger inclusions (arrow). (g) A transverse section of a whole cell showing the fusion of lipid bodies into a larger one (arrows). (h) Single lipid body (LB) visible in both FM and LM. (i) A transverse section of a whole cell showing the central LB. Scale bars: (a, b, e) 0.2 μm, (c, f) 0.2 μm, (g, i) 0.1 μm, (d, h) 10 μm.

representing 71% of total detected photosynthetic pigments in *T. minutus* (Table 1).

Molecular phylogeny. The phylogenetic position of *T. minutus* was investigated using the 18S rRNA gene sequence. A BLASTN search against the non-redundant nucleotide database at NCBI revealed that the *T. minutus* sequence is most similar to a sequence from *G. sculpta* (FJ858970.1) among all sequences currently available in the database, with 96% identity in the pair-wise alignment. The phylogenetic provenance of *G. sculpta* has not yet been formally published and in the NCBI taxonomy database it is classified as ''Xanthophyceae; Mischococcales; Pleurochloridaceae,'' in accordance to the traditional assignment. The following top BLASTN hits represented sequences from known members of the class Eustigmatophyceae, starting with *P. enorme* SAG 11.85 (EF044312.1) and *P. limneticum* SAG 14.94 (EF044313.1).

Phylogenetic analyses with sequences representing all known heterokontophyte classes revealed that *T. minutus*, *G. sculpta*, and the two *Pseudostaurastrum* species form a common clade with maximal statistical support. This new lineage is sister to a lineage comprising all ''traditional'' eustigmatophyceans (Fig. 9). These two clades were allied, again with maximal support, to form the monophyletic class Eustigmatophyceae. All other (nonmonotypic) classes were recovered as monophyletic, with strong support in most cases (except the poorly supported class Phaeothamniophyceae). Higher order relationships among some classes were congruent with the results of other similar 18S rDNA-based analyses (Kai et al. 2008, Patil et al. 2009) and were also recovered with high statistical support, but the deep nodes of the heterokontophyte tree, including the position of the Eustigmatophyceae clade, remained unresolved.

DISCUSSION

T. minutus (originally assigned as *P. minutum*) was described from a basin of the Paris Botanical Garden, and it was further observed in phytoplankton

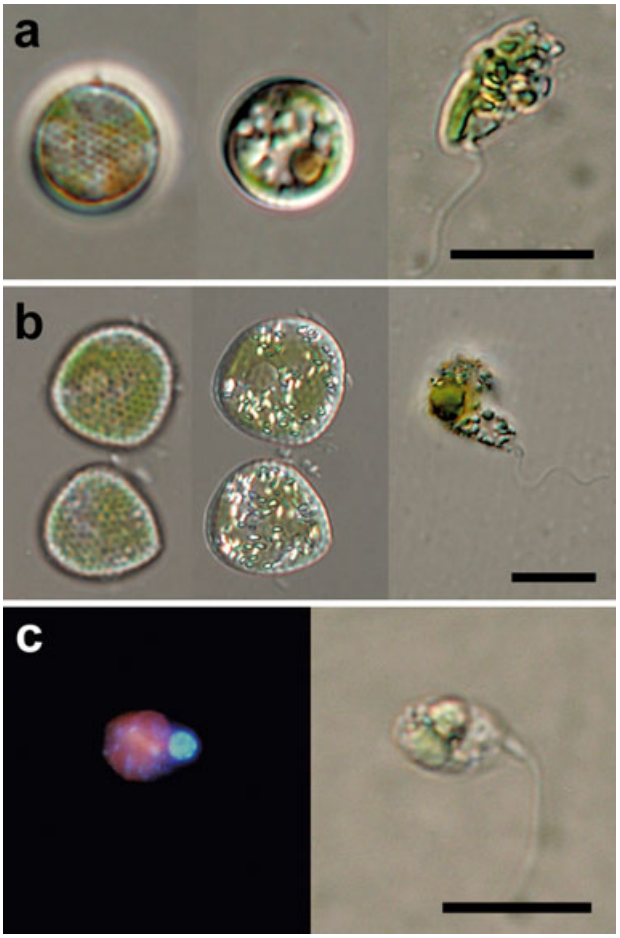

FIG. 7. Morphology of *Trachydiscus minutus* zoospores and comparison with *Goniochloris sculpta*, LM and fluorescence microscopy (FM) images. (a and b) Comparison of morphology of *T. minutus* and *G. sculpta* vegetative cells and zoospores. Plastids, cell wall ornamentation, and an oil droplet of vegetative cells are visible; naked zoospores have one flagellum, an eyespot (stigma) is missing. (c) A zoospore of *T. minutus* with one visible flagellum, DNA stained using 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (blue). Scale bars, 10 μm.

from several localities in France (Bourrelly 1951). More recently, *T. minutus* was found in Ukrainian swamps of the biospheric reserve Srebarna (Stoyneva 1998) and in a subtropical lake in Argentina (27°29′ S; 58°45′ W) (Zalocar et al. 1998). Herein, we present the first observation of this species in the Czech Republic. Generalizing available studies, we may conclude that *T. minutus* is widespread in the freshwater of both temperate and subtropical zones, although usually present in low abundance amid other phytoplankton species. This could contribute to the fact that this species can be easily overlooked using standard ecological methods. However, our isolate was present in unusual abundance representing a dominant phytoplankton species for two consecutive years and then disappeared from the locality.

The morphology of our strain corresponds well to the original description of *P. minutum* (Bourrelly 1951), later reclassified as *T. minutus* (Ettl 1964). *T. minutus* differs from other *Trachydiscus* species by both cell shape and size. Cells from the wider side are pentagonal with rounded ends and the diameter is usually up to 8 μm (Bourrelly 1951). Nevertheless, taking into account that the cell shape usually becomes rounded in culture, the characteristic feature that remains is the relatively small cell size, which can help to distinguish *T. minutus* from other species quite reliably (Ettl 1964).

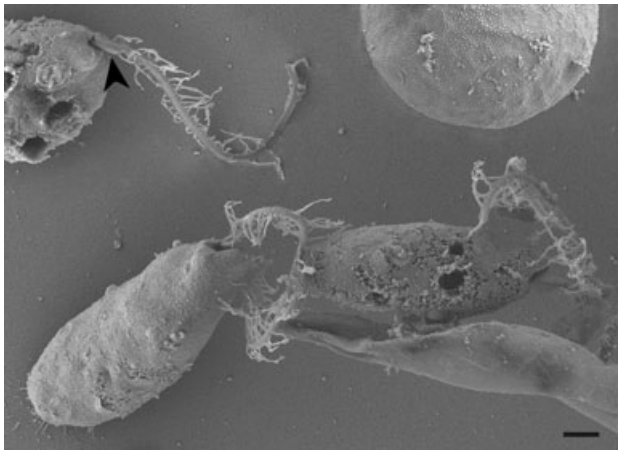

FIG. 8. Morphology of *Trachydiscus minutus* zoospores, SEM image. Flagellum insertion is slightly subapical and it is covered with mastigonemes, a putative flagellar swelling is marked with the arrowhead. Scale bar, 1 μm.

Lipids in *T. minutus* cells were easily observed using a fluorescence microscope owing to the autofluorescence of lipid droplets within a wide range of wavelengths (520–640 nm). This interesting phenomenon was described very recently (Řezanka et al. 2010); however, it does not seem to be unique for *T. minutus*, because we observed similar autofluorescence of lipids in 12 other species of Xanthophyceae and Eustigmatophyceae (data not shown). In contrast, we have never observed lipid autofluorescence in any of the 118 tested strains of Chlorophyceae, Trebouxiophyceae, or Bacillariophyceae from CCALA. This newly discovered phenomenon raises possibilities that should be explored further with respect to algal biotechnology and taxonomy; nevertheless, a more detailed research is necessary. Numerous medium-sized lipid globules similar to those shown in Figure 6 were observed by TEM in the starchless mutant strain STL-PI of *Chlorella pyrenoidosa* cultivated under nitrogen limitation (Ramazanov and Ramazanov 2006). After 5 weeks of growth, the nitrogen-starved green alga *Parietochloris incisa* produced numerous large cytoplasmic oil bodies, which occupied most of the cell volume (Merzlyak et al. 2007). We showed biogenesis of numerous small lipid-containing plastoglobuli inside the plastid of *T. minutus*; lipids were subsequently released to the cytoplasm and fused together, forming larger lipid bodies associated with the plastid

TABLE 1. Main photosynthetic pigments of *Trachydiscus minutus* detected by HPLC.

Retention time (min)	Absorption maxima (nm)	Photosynthetic pigment	Total pigments (%)	Total carotenoids (%)
10.33	416, 439, 469	Violaxanthin	16.2	56.5
15.01	450	Zeaxanthin?	0.9	3.2
15.36	421, 443, 471	Vaucheriaxanthin (ester)	7.4	25.9
17.31	417, 439, 465	Neoxanthin?	1.3	4.7
23.59	430, 619, 664	Chl *a*	71.4	–
26.84	450, 475	β-Carotene	2.8	9.7

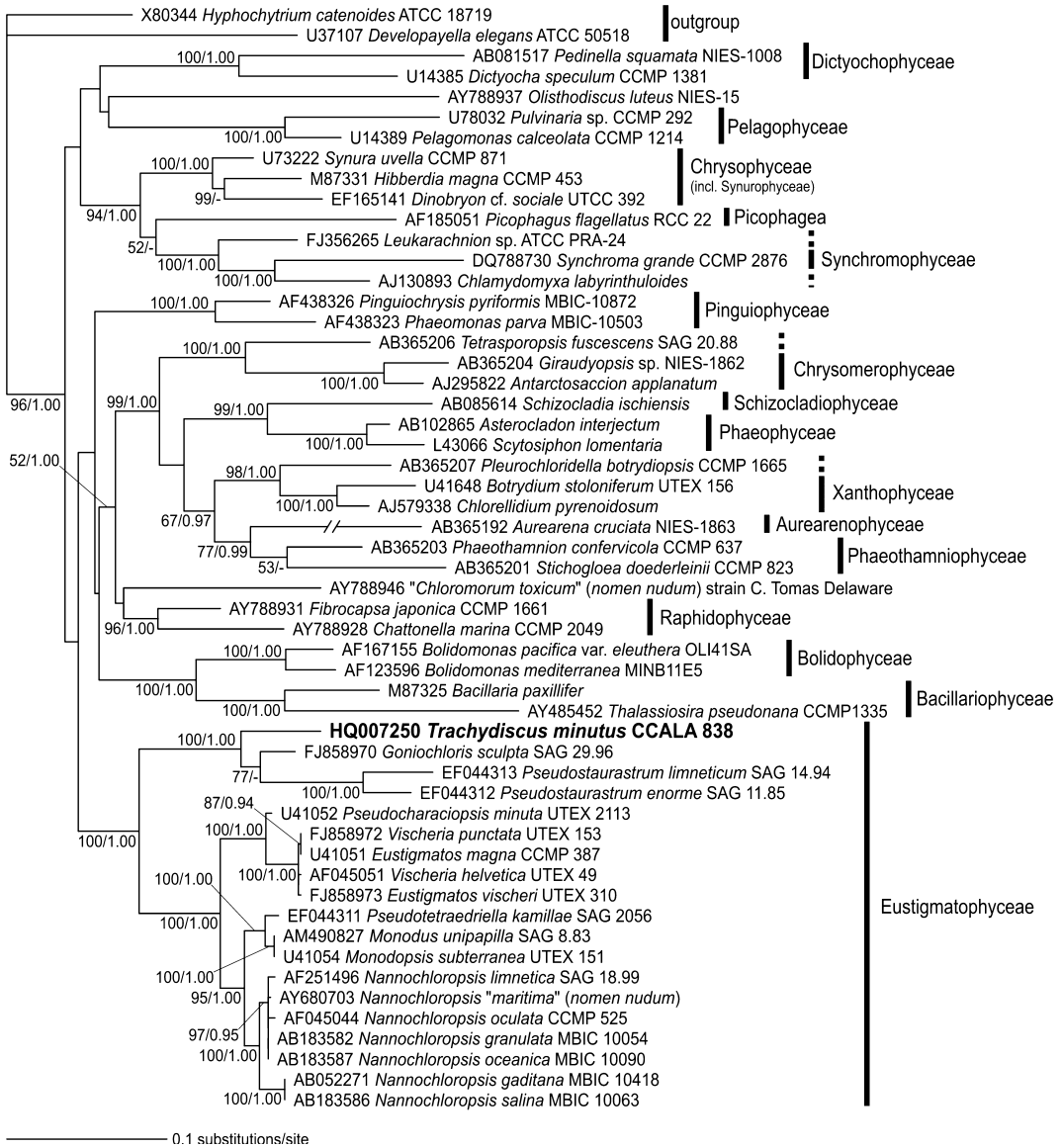

FIG. 9. The phylogenetic position of *Trachydiscus minutus* based on the 18S rRNA gene sequence. The phylogeny shown is a maximum likelihood (ML) tree inferred using RAxML (employing the GTR+Γ+I substitution model). Numbers at branches represent bootstrap support values from the RAxML rapid bootstrapping search/Bayesian posterior probabilities (only values >50/0.90 are shown). Labels at terminal leaves comprise a DDBJ/EMBL/GenBank accession number of the sequence, source organism, and a strain number (if known). The terminal branch leading to *Aurearena cruciata* NIES-1863 (AB365192) was reduced to a half to fit it into the picture. Vertical lines on the right delimit individual heterokontophyte classes as accepted at present. Possible expansion of Synchromophyceae, Chrysomerophyceae, and Xanthophyceae to embrace species currently placed elsewhere is indicated by dashed vertical lines. *Olisthodiscus luteus* NIES-15 (AY788937) and ''*Chloromorum toxicum*'' strain C. Tomas Delaware could not be placed with confidence into any class. *Hyphochytrium catenoides* ATCC 18719 (X80344) and *Developayella elegans* ATCC 50518 (U37107) are treated as a out group to root the tree.

and, finally, one central lipid body. Similar formation of lipid bodies was described previously in the haptophyte *Isochrysis* sp. CCMP 1324 (Liu and Lin 2001) and in the eustigmatophycean *Monodopsis subterranea* (J. B. Petersen) D. J. Hibberd (=*Monodus subterraneus* J. B. Petersen) (Liu and Lin 2005), where the lipid bodies occurred in the thylakoid space of the plastid. Their size varied with growth phase stages and they finally assumed a rounded shape (Liu and Lin 2001). The presence of plastoglobuli inside the plastid stroma was reported in other heterokontophyte algae, including the eustigmatophycean *Nannochloropsis oculata* (Droop) D. J. Hibberd (Antia et al. 1975) or the raphidophycean *Haramonas pauciplastida* (Yamaguchi et al. 2008). Moreover, multiple lipid bodies associated with the plastid membrane were found in stationary-phase cells of the haptophyte *Isochrysis galbana* (Eltgroth et al. 2005). It seems that even evolutionary distant algal taxa share a similar pattern of formation of storage lipid bodies.

Our culture of *T. minutus* allowed us to conduct a series of investigations that indicate beyond any doubt that it is a member of the class Eustigmatophyceae specifically related to the genera *Pseudostaurastrum* and *Goniochloris*. Firstly, plastids of vegetative *T. minutus* cells have no girdle lamella and the plastid membrane is not continuous with the nuclear envelope, which is characteristic for most Eustigmatophyceae but in contrast to the situation in Xanthophyceae (Hibberd and Leedale 1972). By analogy to other eustigmatophyceans, we expect the absence of both a girdle lamella and a connection of the outer plastid membrane to the nuclear envelope in *T. minutus* zoospores as well, but this is yet to be confirmed by an ultrastructural study of the zoospores. The eustigmatophycean affinities of *T. minutus* are also strongly suggested by the presence of lamellate (storage) vesicles, one of the most characteristic ultrastructural features of this class (Santos 1996). Secondly, *T. minutus* fits the characteristics of the class Eustigmatophyceae rather than Xanthophyceae in its carotenoid composition (violaxanthin as the predominant carotenoid and diatoxanthin/diadinoxanthin missing) and in the absence of even traces of chl *c* (Antia et al. 1975, Volkman et al. 1993, Karlson et al. 1996, Schnepf et al. 1996). Strikingly, using spectrofluorometry of a total pigment extract, chl *c* (~0.2% of dry weight) was previously reported in our isolate of *T. minutus* (Iliev et al. 2010). However, we do not consider this method as suitable for assessing the presence and quantity of chl *c* and we prefer the results of our HPLC analysis demonstrating absence of any traces of chl *c* in *T. minutus*.

Finally, the eustigmatophycean affinities of *T. minutus* are clearly indicated by the results of our phylogenetic analysis of the 18S rRNA gene. These results demonstrate the existence of a strongly supported clade that can be recognized as the class Eustigmatophyceae and that splits into two strongly supported subclades, one comprising initially studied eustigmatophyceans (i.e., those serving as ''founding members'' of the class) and the other comprising *T. minutus* together with at least two species of the genus *Pseudostaurastrum* (*P. limneticum* and *P. enorme*) and *G. sculpta* SAG 29.96. A clade comprising *G. sculpta* and *Pseudostaurastrum* species is being described as the family Goniochloridaceae (J. Craig Bailey, University of North Carolina, Wilmington, USA, manuscript under revision, personal communication). The interrelationships among *T. minutus*, *G. sculpta*, and the *Pseudostaurastrum* lineage remain unresolved in our analysis shown in Figure 9. Increasing the number of characters (from 1,650 to 1,761) by restricting the analysis to sequences from Eustigmatophyceae only did not improve the resolution within the *Trachydiscus/Goniochloris/Pseudostaurastrum* clade (data not shown), so further sampling of both additional taxa, especially the type species of the genus *Trachydiscus* (*T. lenticularis*), and additional molecular markers (such as *rbc*L) may be needed to establish the phylogenetic history and taxonomy within this emerging clade. These additional analyses would help to decide the actual relationship between *T. minutus* and *G. sculpta* and whether or not *T. minutus* should be regarded as a species of the family Goniochloridaceae or even the genus *Goniochloris*, as suggested by Bourrelly (1968). Interestingly, a recent phylogenetic study employing *rbc*L sequences from a number of mostly unidentified eustigmatophycean strains revealed a deep divergence within this class (Prior et al. 2010) that may correspond to the two principal clades shown up in the 18S rRNA gene tree. Sequencing the *rbc*L gene from *T. minutus* is needed to check whether or not it is specifically related to any of the strains reported by Prior et al. (2010).

A salient aspect of our study is the discovery of the zoosporic reproductive cycle in *T. minutus*, which was not noted in the previous studies on this species. We also induced zoosporogenesis in *G. sculpta* and observed that zoospores of both species are morphologically very similar on the LM level. Our SEM observations revealed that the zoospores of *T. minutus* have one long flagellum covered with mastigonemes and a putative flagellar swelling, which is typical for other zoosporic Eustigmatophyceae (Santos and Leedale 1991, Santos 1996). The zoospores of both *T. minutus* and *G. sculpta* remained motile for 8–10 min, compared to those of *P. limneticum*, which were motile only for 3–5 min (Schnepf et al. 1996). A unique character that gave the name to the whole class Eustigmatophyceae is the presence of an extraplastidial (true) stigma in zoospores (Hibberd and Leedale 1970, 1971). However, zoospores of *T. minutus* lack a stigma, as previously found for zoospores of *P. limneticum* (Schnepf et al. 1996) and *P. kamillae* (Hegewald et al. 2007).

Considering the distribution of the extraplastidial stigma in the context of the phylogeny as reconstructed using the 18S rRNA gene, it is actually possible that it evolved only in the lineage comprising the genera *Eustigmatos*, *Vischeria*, and *Pseudocharaciopsis* and its absence from zoospores of the genera *Trachydiscus*, *Pseudostaurastrum*, and *Pseudotetraëdriella* is primary. However, an extraplastidial stigma occurs also in *B. similis* (Preisig and Wilhelm 1989) and *P. edaphicum* (Neustupa and Němcová 2001), whose phylogenetic position within Eustigmatophyceae remains to be established.

We showed that the main factor affecting the release of both *T. minutus* and *G. sculpta* zoospores was the absence of light, because zoospores were produced only in darkness. Similarly, production of gametes in the green alga *Chlorococcum echinozygotum* occurred in darkness (O'Kelley 1983) and inhibition of zooid (gamete and zoospore) production in the presence of light was described in the green alga *Botryosphaerella sudetica* (Přibyl and Cepák 2007). In many green algae, nitrogen is the main factor affecting zooid production. Nitrogen depletion was found to be a prerequisite for gametogenesis in species such as *Chlamydomonas reinhardtii* (Sager and Granick 1954, Weissig and Beck 1991, Beck and Acker 1992, Rodriguez et al. 1999), *Chlamydomonas monoica* (Vandenende 1995), *Oedogonium hatei* (Chaudhary and Singh 1988), and *C. echinozygotum* (O'Kelley 1983, 1984). In contrast, nitrogen starvation inhibited production of zoospores in *T. minutus*, which indicates that the processes of zoospore development in this species are regulated differently as compared to the gametogenesis in Chlorophyceae. Conditions for zooid production in the green alga *Scenedesmus obliquus* are specific in comparison to the above-named species and represent a transitional type. Light enhances zooid production, which occurs in the dark as well. In addition, zooid production is inhibited by an excess of nitrogen, but zooids are not induced by nitrogen depletion itself (Cain and Trainor 1976). Temperature also influences the release of zooids. In *S. obliquus* the optimal temperature is 15°C (Cain and Trainor 1976) and in *T. minutus* 20°C. Increasing or decreasing the temperature brings about a reduction in zooid numbers in both species.

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