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Rediscovery of *Tetraëdriella subglobosa* Pascher, a member of the Eustigmatophyceae

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Abstract: An algal strain from an acidic pool near the shore of Lake Mácha, Czech Republic, was identified as *Tetraëdriella subglobosa* Pascher by light microscopy. Phylogenetic analysis of nuclear 18S rDNA and plastid *rbc*L sequence data indicated that this alga is a member of the Eustigmatophyceae, rather than the Xanthophyceae as presently classified. This is the first report of *T. subglobosa* since Pascher's description of the species in 1930.

Key words: Eustigmatophyceae, Goniochloridales, phylogeny, *rbc*L, *Tetraëdriella*, 18S rDNA

Introduction

One of the many heterokont algae that were described by Pascher in the early 20th century is *Tetraëdriella subglobosa*, which Pascher found in acidic pools near Františkovy Láznê and Lake Mácha, Czech Republic (Pascher 1930). To our knowledge, there are no other records for this species. *Tetraëdriella subglobosa* is listed in a database of Czech algae (Poulíčková et al. 2004), but with no data except the original description. AlgaeBase (Guiry & Guiry 2015) does not list *T. subglobosa* under the genus *Tetraëdriella*, despite the fact that it was one of the two original species in the genus (Pascher 1930).

Tetraëdriella subglobosa is a small (8–13 µm), nearly spherical organism with a finely sculpted cell wall. However, some of the cells display a slight pyramidal or tetrahedral shape, with the walls highly convex so as to be nearly spherical. The cell walls have thickened bands that form the edges of the tetrahedron. The edges are easily seen in the old mother cell walls that result from autospore formation. The small size and simple morphology of *T. subglobosa* make it likely that this species has been overlooked or identified as other similar taxa, such as some species of *Trachydiscus* Ettl or even *Pleurochloris* Pascher.

We collected water samples from acidic pools at the Lake Mácha site in April, 2014 and cultured several Eustigmatophyceae strains from these samples. We have identified one of these strains as *Tetraëdriella subglobosa.* Here we present the results of phylogenetic analysis of nuclear 18S rDNA and plastid *rbc*L sequence data for this organism, as well as new insights on its morphology.

Materials and Methods

Strain isolation. A tychoplankton sample was collected from an acidic pool near the shore of Lake Mácha, Czech Republic, (approximately 50.577° N, 14.699° E) on 24 April, 2014. The sample was kept chilled and transported to our laboratory in Monticello, Arkansas, USA, and processed on 2 May, 2014. *Tetraëdriella subglobosa* was isolated from a sample spread on an agar plate of M5.5 medium and incubated at room temperature under continuous cool–white fluorescent light. The M5.5 medium is made by diluting WH+ medium (Fawley et al. 2013) 1:9 with distilled water, adding 0.1 $g.l^{-1}$ MES buffer (Fisher Scientific BP300, Thermo Fisher Scientific, Hampton, New Hampshire, USA) and adjusting the pH to 5.5 with sodium hydroxide. *Tetraëdriella subglobosa* strain F4 4/24–10m was maintained on M5.5 agar. This strain is now also held by the Culture Collection of Algae at Charles University in Prague as CAUP Q 601.

Light Microscopy. A Nikon NiU microscope (Nikon, Melville, New York, USA) equipped with a Plan Apochromat 100x objective (numerical aperture 1.45) and differential interference contrast optics was used for light microscopy. Images were captured with a Nikon DS–Fi2 digital camera and Nikon Elements BR software. Strain F4 4/24–10m was grown in M5.5 liquid medium at 20 °C with illumination of about 50 $\mu M.m^{-2}.sec^{-1}$ and a 12:12 light:dark cycle and examined within 10 days of inoculation. Three different techniques were used to stimulate zoospore production. An

exponentially growing culture in liquid medium was placed in darkness (wrapped in aluminum foil) for at least 18 h at either 20 °C (Lee & Bold 1973) or at 10 °C (Trzcińska et al. 2014), or fresh liquid medium was applied to the surface of a culture on agar medium that was over 1 month old which was then placed in darkness for at least 5 days (Santos & Leedale 1991).

Phylogenetic analysis. Strain F4 4/24–10m was grown in liquid M5.5 medium as above. Cells were collected by centrifugation and DNA was isolated according to Fawley & Fawley (2004). Previously collected genomic DNA samples from other Eustigmatophyceae strains were also used to generate new *rbc*L sequences to complete a data set for that locus similar to the taxon set used in Fawley et al. (2014). Conditions for polymerase chain reaction amplification were as described in Fawley & Fawley (2004) for the nuclear 18S rDNA region and Fawley et al. (2015) or Prior et al. (2009) for the plastid *rbc*L gene. DNA sequencing was performed as described in Fawley et al. (2015), with sequencing done by Sequetech (Mountain View, California, USA). Sequence reads were joined using the Staden Package 2.0.0b8. New 18S rDNA sequences from F4 4/24–10m (KX373531) and additional new sequences published in GenBank (Table 1) were added to the alignment of Fawley et al. (2014) and aligned by eye in MacClade 4.08 (Maddison & Maddison 2000). A concatenated 18S rDNA and *rbc*L alignment including the new *rbc*L sequences for *T. subglobosa* (KX354388) and additional strains (Table 1) was produced in MacClade 4.08. Taxa lacking *rbc*L sequences were excluded **f**rom the concatenated alignment except for *Vacuoliviride crystalliferum*, *Trachydiscus* sp. LCR–AWA–9–2, strain Itas 9/21 S––11w, *Pseudostaurastrum enorme* and *Pseudostaurastrum limneticum*, all members of the clade Goniochloridales.

Maximum Parsimony (MP) analyses were performed with PAUP* 2.0b (Swofford 2002) and Maximum Likelihood (ML) analyses employed GARLI 2.01 (Zwickl 2006), under the GTR +I + Γ model of substitution (Tavaré 1986) with parameters selected by GARLI. The ML analysis of the concatenated data set had partitions for 18S rDNA and each codon position of the *rbc*L sequences. Twenty replicates with different starting trees were used for ML analyses. Maximum Parsimony analyses were bootstrapped with 1000 replicates of the data and ML analyses were bootstrapped with 200 replicates, with each replicate evaluated for 2 random starting trees.

Outgroup taxa for phylogenetic analyses with GenBank accession numbers for 18S rDNA and *rbc*L were *Aurearena cruciata* (AB365192, AB365193), *Botrydium stoloniferum* (U41648, AFR064743), *Chromulina nebulosa* (AF123285, AF155876), *Pylaiella littoralis* (AY032606, X55372) and *Synchroma grande* (DQ788730, DQ788731). The concatenated alignment included the following taxa from the Eustigmatales for which both 18S rDNA and *rbc*L sequences are available: strain BogD 9/21 T–2d (KF757230, GQ405004); *Eustigmatos magnus* CCMP 387 (U41051, AF015575); strain Mary 6/3 T–1w (KF757240, GQ405005); strain Mary 8/18 T–3d (KF757238, KX354375); strain Mary 8/18 T–4d (KF757239, KX354376); *Monodus unipapilla* SAG 8.83 (AM490827, HQ710608); *Microchloropsis gaditana* MBIC10123 (AM052270, AB052734); *Microchloropsis salina* CCAP 840/2 (AF045046, AB052288); *Nannochloropsis granulata* CCMP1662 (AF045041, AB052280); *Nannochloropsis limnetica* SAG 18.99 (AF251496, AF251496); *Nannochloropsis oceanica* MBIC10090 (AB183587, AB052283); *Nannochloropsis oculata* CCMP525 (U38902, HQ710609); strain Tow 8/18 T–6d (KF757249, KX354384); strain Tow 8/18 T–12d (KF757250, KX354385), strain Tow 9/21 P–2w (KF757253, KX354386) and strain WTwin 8/18 T–5d (KF757254, GQ405007).

For the figures resulting from all analyses, the generic name *Monodus* was used with the GenBank accessions of *Monodus* and *Monodopsis* because the taxonomy of *Monodus* and *Monodopsis* is quite confused and uncertain (Ott et al. 2015).

Results and Discussion

Light microscopy indicated the presence of a prominent orange lipid body in the cytoplasm of strain F4 4/24–10m, along with a finely sculpted cell wall (Figs

Table 1. GenBank accession numbers of new and updated DNA sequences of Eustigmatophyceae used in this study. All unnamed strains are from Itasca State Park (ISP) in Minnesota, USA, except Chic 10/23 P–6w and Chic 10/23 P–37w from Lake Chicot, Arkansas, USA. See Fawley et al. (2004) for descriptions of sites in ISP and Fawley et al. (2013) for a description of Lake Chicot.

Eustigmatophyceae strain	Locus and GenBank accession number
Chic 10/23 P–6w	*rbc*L KX354371
Chic 10/23 P–37w	*rbc*L KX354372
Itas 6/3 T–8w	*rbc*L KX354373
Itas 8/18 S–5d	*rbc*L KX354374
Itas 9/21 S–8W	*rbc*L GQ405009 update
Mary 8/18 T–3d	*rbc*L KX354375
Mary 8/18 T–4d	*rbc*L KX354376
Mary 8/18 T–4w	*rbc*L KX354377
Pic 8/18 P–2d	*rbc*L KX354378
Pic 8/18 P–13d	*rbc*L KX354379
Pic 8/18 T–15d	*rbc*L KX354380
Pic 8/18 T–19w	*rbc*L KX354381
Tow 2/24 P–2d	*rbc*L KX354382
Tow 8/18 T–2d	*rbc*L KX354383
Tow 8/18 T–6d	*rbc*L KX354384
Tow 8/18 T–12d	*rbc*L KX354385
Tow 8/18 T–4w	*rbc*L GQ405008 update
Tow 8/18 T–8w	*rbc*L GO405010 update
Tow 9/21 P–2w	*rbc*L KX354386
WTwin 8/18 T–15d	*rbc*L KX354387
T. subglobosa F4 4/24–10m	*rbc*L KX354388
T. subglobosa F4 4/24–10m	18S rDNA KX373531

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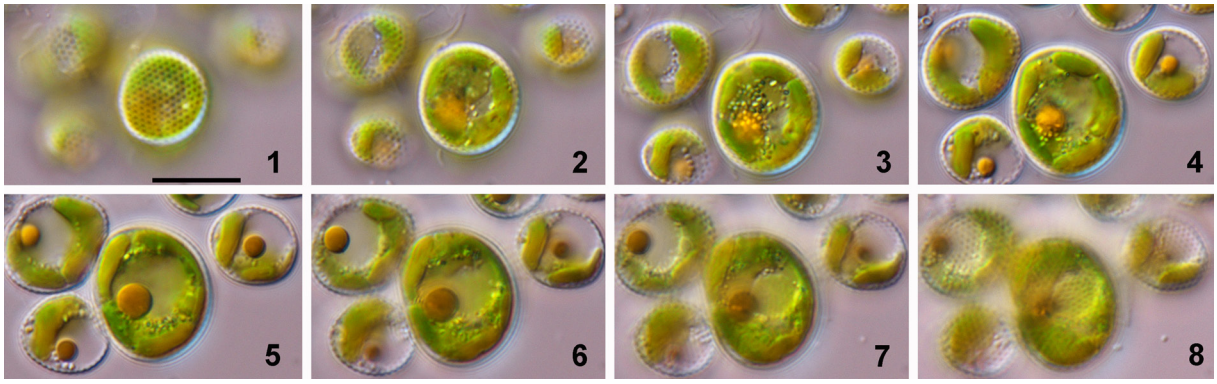
1–8). These features are indicative of some clades in the Eustigmatophyceae (Fawley et al. 2014). The cells ranged from about 3.0 µm for autospores to 10.0 µm for large vegetative cells. Occasional giant cells 20 µm or larger were found, especially in older cultures (not shown). Cells often appeared spherical, but careful observation revealed that they are typically somewhat irregular, oblong or roughly elliptical. Ridges on the cell walls were sometimes visible, but were best seen on old mother cell walls that had released autospores. One to four (or more in giant cells, not shown) plastids formed smooth parietal sheets without pyrenoids. Some plastids had small indentations or lobes, but most were rounded or had slightly crenate edges. Numerous small granules or vesicles were present in the cells, but the lamellate granules or highly refractive bodies that are sometime found in eustigmatophycean cells were not present. The "sculpted" cell wall was comprised of ridges that form small hexagonal pits about 400 nm across. Reproduction was by the production of 2 or 4 autospores; no zoospores or other flagellate cells were observed. When autospores were formed, the orange body was retained without division in one of the daughter cells.

The characteristics of F4 4/24–10m are nearly identical to those of *Tetraëdriella subglobosa* as described by Pascher (1930). The major feature of *T. subglobosa* is the presence of four ridges along the sculpted cell wall that are similar to those found on other tetrahedral coccoid algae, such as *T. acuta* Pascher. However, in *T. subglobosa*, the normally planar features of the cells are "inflated" such that the four sides of the cell are rounded. Figures 9–13 show a comparison of Pascher's (1930) original illustrations



Figs 1-8. Z-stack light microscopy of vegetative cells of *T. subglobosa*: (1) shows the top surface of one cell, with the hexagonal sculpting visible; (2-7) are optical sections progressing through 4 cells that clearly show the irregular shapes of the cells and the presence of 2 plastids and the large orange lipid bodies; (8) shows the lower surface of the cells, which are also sculpted. Scale bar 10 µm.

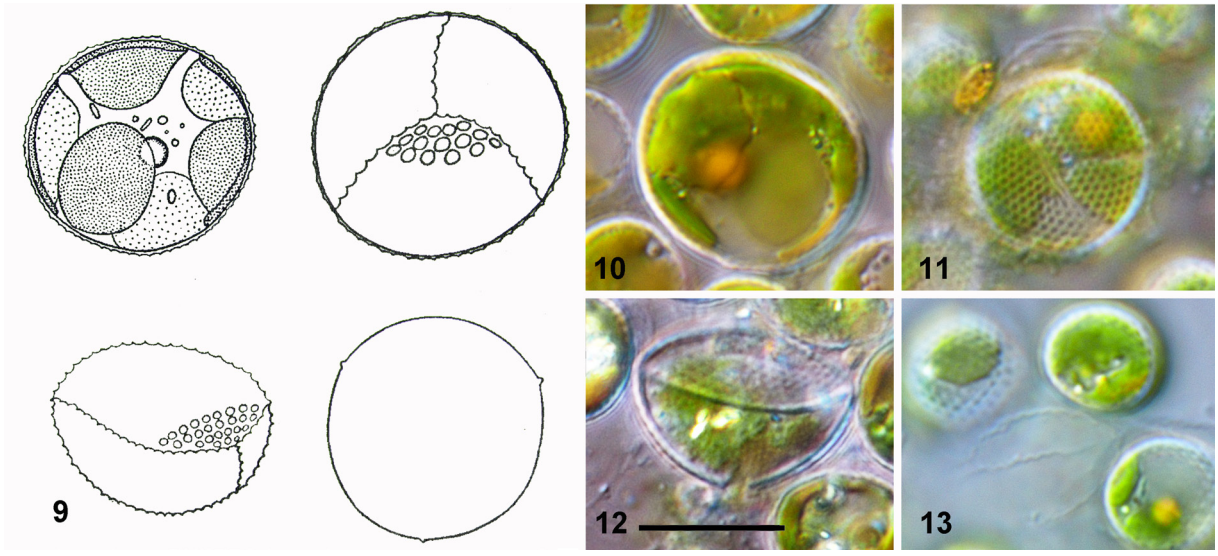
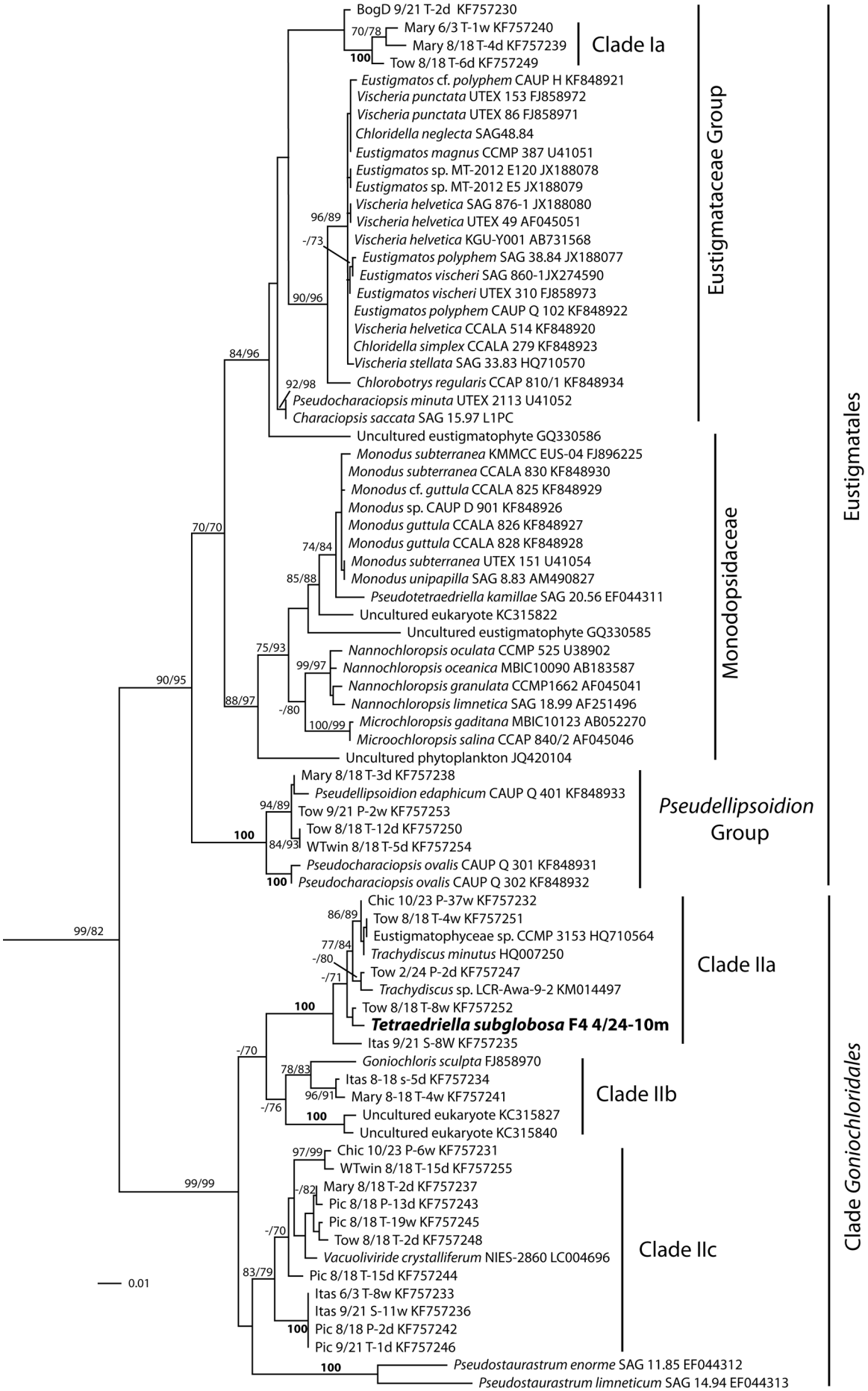


Fig. 9. Original illustrations of *T. subglobosa* from Pascher (1930).

Figs 10-13. Light microscopy showing the features diagnostic for *T. subglobosa* as illustrated in Fig. 9: (10) internal stuctures showing the smooth-edged parietal plastids, large orange lipid body and small granular inclusions; (11) cell surface showing thickened ridge around the cell; (12) cell with flattened, nearly hemispherical shape; (13) old mother cell wall showing longitudinal ridges. Scale bar 10 µm.


BogD 9/21 T-2d KF757230
Mary 6/3 T-1w KF757240
Mary 8/18 T-4d KF757239
Tow 8/18 T-6d KF757249
Clade Ia
Eustigmataceae Group
Eustigmatales
Monodopsidaceae
Pseudellipsoidion Group
Clade Goniochloridales
Clade IIa
Clade IIb
Clade IIc
Eustigmatos cf. polyphem CAUP H KF848921
Vischeria punctata UTEX 153 FJ858972
Vischeria punctata UTEX 86 FJ858971
Chloridella neglecta SAG48.84
Eustigmatos magnus CCMP 387 U41051
Eustigmatos sp. MT-2012 E120 JX188078
Eustigmatos sp. MT-2012 E5 JX188079
Vischeria helvetica SAG 876-1 JX188080
Vischeria helvetica UTEX 49 AF045051
Vischeria helvetica KGU-Y001 AB731568
Eustigmatos polyphem SAG 38.84 JX188077
Eustigmatos vischeri SAG 860-1JX274590
Eustigmatos vischeri UTEX 310 FJ858973
Eustigmatos polyphem CAUP Q 102 KF848922
Vischeria helvetica CCALA 514 KF848920
Chloridella simplex CCALA 279 KF848923
Vischeria stellata SAG 33.83 HQ710570
Chlorobotrys regularis CCAP 810/1 KF848934
Pseudocharaciopsis minuta UTEX 2113 U41052
Characiopsis saccata SAG 15.97 L1PC
Uncultured eustigmatophyte GQ330586
Monodus subterranea KMMCC EUS-04 FJ896225
Monodus subterranea CCALA 830 KF848930
Monodus cf. guttula CCALA 825 KF848929
Monodus sp. CAUP D 901 KF848926
Monodus guttula CCALA 826 KF848927
Monodus guttula CCALA 828 KF848928
Monodus subterranea UTEX 151 U41054
Monodus unipapilla SAG 8.83 AM490827
Pseudotetraedriella kamillae SAG 20.56 EF044311
Uncultured eukaryote KC315822
Uncultured eustigmatophyte GQ330585
Nannochloropsis oculata CCMP 525 U38902
Nannochloropsis oceanica MBIC10090 AB183587
Nannochloropsis granulata CCMP1662 AF045041
Nannochloropsis limnetica SAG 18.99 AF251496
Microchloropsis gaditana MBIC10123 AB052270
Microchloropsis salina CCAP 840/2 AF045046
Uncultured phytoplankton JQ420104
Mary 8/18 T-3d KF757238
Pseudellipsoidion edaphicum CAUP Q 401 KF848933
Tow 9/21 P-2w KF757253
Tow 8/18 T-12d KF757250
WTwin 8/18 T-5d KF757254
Pseudocharaciopsis ovalis CAUP Q 301 KF848931
Pseudocharaciopsis ovalis CAUP Q 302 KF848932
Chic 10/23 P-37w KF757232
Tow 8/18 T-4w KF757251
Eustigmatophyceae sp. CCMP 3153 HQ710564
Trachydiscus minutus HQ007250
Tow 2/24 P-2d KF757247
Trachydiscus sp. LCR-Awa-9-2 KM014497
Tow 8/18 T-8w KF757252
Tetraedriella subglobosa F4 4/24-10m
Itas 9/21 S-8W KF757235
Goniochloris sculpta FJ858970
Itas 8-18 s-5d KF757234
Mary 8-18 T-4w KF757241
Uncultured eukaryote KC315827
Uncultured eukaryote KC315840
Chic 10/23 P-6w KF757231
WTwin 8/18 T-15d KF757255
Mary 8/18 T-2d KF757237
Pic 8/18 P-13d KF757243
Pic 8/18 T-19w KF757245
Tow 8/18 T-2d KF757248
Vacuoliviride crystalliferum NIES-2860 LC004696
Pic 8/18 T-15d KF757244
Itas 6/3 T-8w KF757233
Itas 9/21 S-11w KF757236
Pic 8/18 P-2d KF757242
Pic 9/21 T-1d KF757246
Pseudostaurastrum enorme SAG 11.85 EF044312
Pseudostaurastrum limneticum SAG 14.94 EF044313
0.01

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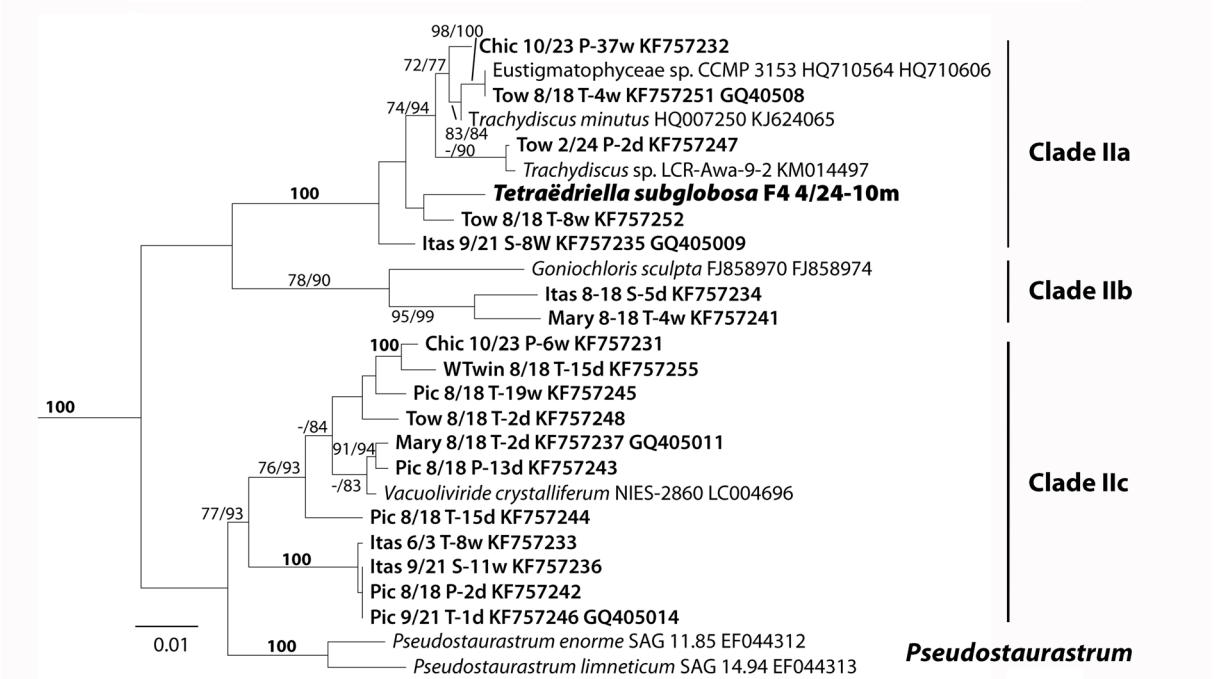

Fig. 15. Phylogenetic analysis of combined nuclear 18S rDNA and plastic *rbc*L DNA sequence data from the Eustigmatophyceae. Because of low taxon sampling for *rbc*L sequence data from the Eustigmatales only the Goniochloridales portion of this ML phylogram is shown. New *rbc*L data (completely new sequences or amended GenBank sequences) were generated for strains shown in bold face. Bootstrap values (70 or higher shown) are for Maximum Parsimony (1000 replicates using the fast stepwise-addition setting in PAUP*) and Maximum Likelihood (200 replicates in GARLI, with 2 random starting trees for each replicate).

Fig. 14. Phylogenetic analysis of nuclear 18S rDNA sequence data from the Eustigmatophyceae showing *T. subglobosa* allied with Clade IIa. The phylogram is from Maximum Likelihood analysis with the outgroup taxa not shown. Bootstrap values (70 or higher shown) are for Maximum Parsimony (1000 replicates using the fast stepwise-addition setting in PAUP*) and Maximum Likelihood (200 replicates in GARLI, with 2 random starting trees for each replicate).

of *T. subglobosa* (Fig. 9) and strain F4 4/24–10m (Figs 10–13). The internal morphology of the cells (Fig. 10), the presence of ridges on the cell wall (Fig. 11), the occasional cell with a flattened face (Fig. 12) and the ridges present on old mother cell walls (Fig. 13) of strain F4 4/24–10m are all identical to Pascher's drawings. In Pascher's illustration, the sculpting was shown as rounded, pore–like indentations, whereas our micrographs resolve the hexagonal sculpting. This difference is likely a result of the increased resolving power of modern microscopes with differential interference contrast.

Phylogenetic analysis of the 18S rDNA sequence data (Fig. 14) confirmed the placement of strain F4 4/24–10m in the Eustigmatophyceae as a likely member of the unnamed lineage, Clade IIa in the clade Goniochloridales (Fawley et al. 2014). The results of analysis of concatenated 18S and *rbc*L sequences from a smaller set of taxa (Fig. 15) provided strong bootstrap support for this alliance, with F4 4/24–10m and the unidentified strain Tow 8/18 T–8w likely comprising a basal lineage of Clade IIa. Although the diversity and overall characteristics of Clade IIa are yet to be determined, strains from this clade all possess some type of cell wall sculpting. The only other named taxon included in Clade IIa is *Trachydiscus minutus* (Bourrelly) Ettl, which has a sculpted cell wall and reproduces by autospores and zoospores. We were unable to stimulate zoospore production in *Tetraëdriella subglobosa*, but we have not attempted the more complex procedure used by Přibyl et al. (2012) to stimulate zoospores in *Tr. minutus*. Pascher (1930) also did not observe zoospore production for *T. subglobosa*.

Although *T. subglobosa* was one of two species originally described for the genus *Tetraëdriella* in the Xanthophyceae (Pascher 1930), the other species, *T. acuta* Pascher, was designated the type species for the genus (Kováčik & Komárek 1976). *Tetraëdriella acuta* possesses cell wall sculpting and an orange lipid body (Pascher 1930), the latter diagnostic of the Eustigmatophyceae (Fawley et al. 2014). In *T. acuta*, the cells typically possess flattened faces and appear triangular in cross–section, with ridges at the angles of the cell and slight points at each apex (Pasher 1930; Ettl 1978). The genus *Tetraëdriella* includes several other species with the basic tetrahedral form of *T. acuta* and varying development of the points at cell apexes Ettl 1978): *T. polychloris* Skuja, without noticeable ridges at the angles of the cells; *T. impressa* Pascher and *T. limbata* Pascher, with more pyramidal shapes (somewhat rounded in *T. limbata*) than *T. acuta*; and *T. spinigera* Skuja, with the points at the apexes extended

forming long spine–like structures. The species *T. jovetii* (Bourelly) Bourelly and *T. regularis* (Kützing) Fott have rounded arms; *T. jovetii* has no projections or ridges and *T. regularis* has short spine–like projections. Although all of the described species of *Tetraëdriella* are considered candidate Eustigmatophyceae (Ott et al. 2015), until the type species *T. acuta* can be isolated and evaluated, we can only speculate that the genus *Tetraëdriella* should be transferred from the Xanthophyceae to the Eustigmatophyceae. Even if *T. acuta* and other species of the genus were to be shown to be in the Eustigmatophyceae they may not be allied with *T. subglobosa*. There is a report of unpublished 18S rDNA sequence data that place two species of *Tetraëdriella* in the Eustigmatophyceae (Ott et al. 2015) and those results should help clarify these issues. Several species of *Tetraëdriella* have an orange lipid body (Ettl 1978) similar to those found in the Eustigmatophyceae, but until these species are isolated and characterized the relationships among these species are open to question.

Tetraëdriella subglobosa might be considered rare based on the paucity of sightings. However, this species and other species of the Eustigmatophyceae are probably frequently overlooked. Most Eustigmatophyceae are spherical or nearly so and quite small, often less than 10 μm in diameter. The yellow, orange or red lipid body is a simple way to recognize Eustigmatophyceae (Fawley et al. 2014), but it can be difficult to see without very good optics. As phycologists become more aware of this interesting and diverse class of algae, it is likely that they will be found more frequently.

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