Phylogeny and characterization of *Paraeustigmatos columelliferus*, gen. et sp. nov., a member of the Eustigmatophyceae that may represent a basal group within the Eustigmatales

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Abstract: The new taxon, *Paraeustigmatos columelliferus,* was isolated from a mat of the filamentous alga *Zygnema* on the shore of Lake Monticello in Arkansas, USA. The results of the phylogenetic analysis of 18S rDNA and rbcL sequence data suggest that this alga is likely allied with the Eustigmatales; alternately, it may represent a new third lineage of the class. Light and electron microscopy reveal cellular features similar to other members of the Eustigmatophyceae; however, the cell wall bears unusual structures that resemble the columellae of the walls of pollen grains.

Key words: Columelliferus; Eustigmatales; Eustigmatophyceae; *Paraeustigmatos*; Phylogeny

Introduction

Our understanding of the Stramenopile algal class Eustigmatophyceae has advanced over the past two decades from a small group of rarely seen organisms comprising the single order Eustigmatales to a diverse lineage comprising two major clades (Fawley et al 2014; Eliáš et al 2017). Although traditionally thought to primarily inhabit bogs, dystrophic pools and similar freshwater environments, Eustigmatophyceae inhabit environments ranging from oceanic plankton to eutrophic lakes and soil biotic crusts. Eustigmatophyceae have adapted to extreme conditions such as acidic geothermal sites (Hsieh et al. 2018) and permanently ice–covered lakes in Antarctica (Bielewicz et al. 2011).

The taxonomy of the Eustigmatophyceae has not progressed at a rate to compare with the discovery of new diversity in the class. This is partly because of the rather simple morphology of these organisms, which are all coccoid, either solitary or in unorganized clumps. Some eustigmatophytes initially were placed in genera within the Xanthophyceae or even the Chlorophyta. It can be difficult to untangle the taxonomy of these organisms that typically are not represented by a type culture. The extensive cryptic diversity of many coccoid algae makes it very difficult to determine if a new culture is actually the same as a previously described species that looks very similar, or even if they are in the same class.

Our approach to the taxonomy of the Eustigmatophyceae has been to generate a culture collection of these organisms that is large enough to define individual lineages within the class. This approach permits us to understand the features of clades so that we can describe new genera based on shared features and then delimit species within each genus. This approach has been applied to the taxonomy of *Nannochloropsis*, resulting in the identification of new taxa based primarily on DNA sequence and plastid genome analyses (Fawley & Fawley 2007; Fawley et al. 2015).

Here we take a different approach with a strain that stands out from other members of the Eustigmatophyceae. Strain Mont 10/10–1w was isolated from a mat of *Zygnema* sp. on the shore of Lake Monticello, Arkansas, USA. Preliminary DNA sequence analysis suggested that this strain is either basal in the Eustigmatales or represents a possible new third lineage of the class. Here we present phylogenetic and morphological analyses of this organism and describe the new genus, *Paraeustigmatos*.

Materials and Methods

Strain isolation. A mat of *Zygnema* sp. was collected from

the shore of Lake Monticello, Drew County, Arkansas, USA (approximately 33°42'09"N, 91°49'40"W). We isolated the *Zygnema* sp. by washing the filaments in sterile WH+ medium (Fawley et al. 2013) and placing them in a 125 mL Erlenmeyer flask with approximately 70 ml of WH+ medium. After several days' growth, we observed a eustigmatophyte growing in the flask with the *Zygnema*. This eustigmatophyte was isolated from a sample of the *Zygnema* culture, spread on an agar plate of WH+ medium and incubated at room temperature under continuous cool–white fluorescent light at about 50 µm.m². sec⁻¹ illumination. The eustigmatophyte strain, designated Mont 10/10–1w, was maintained on WH+ agar. This strain is now also held by the Culture Collection of Algae at Charles University in Prague as CAUP Q701.

Light Microscopy. For differential interference contrast microscopy we used a Nikon NiU microscope (Nikon, Melville, New York, USA) with a Plan Apochromat 100x objective (numerical aperture 1.45). Digital images were captured with a Nikon DS–Fi2 camera and Nikon Elements BR software. Strain Mont 10/10–1w was grown in WH+ liquid medium at 20 °C with illumination of about 20 µM.m⁻².sec⁻¹ and a 14:10 light:dark cycle and examined within 10 days of inoculation.

Confocal laser scanning microscopy. Cell walls of native cells were stained with 1% Calcofluor white (Sigma–Aldrich, St. Louis, MO, USA) for 10 min. Samples were investigated using Carl Zeiss LSM 880 NLO (Carl Zeiss Microscopy GmbH, München, Germany) multi–functional fluorescence inverted confocal microscope. The samples were acquired using 405 nm laser excitation and collecting emission spectra between 410 and 552 nm. 3D reconstructions were produced using ImageJ version 1.52 with the Fiji image processing package (Schindelin et al. 2012).

Electron Microscopy. For observations in a transmission electron microscope (TEM), samples were fixed for 5 hours at 5 °C in a 2% solution of glutaraldehyde in 0.05 mol.l⁻¹ phosphate buffer, post–fixed for 2 hours at 5 °C in 1% osmium tetroxide in 0.05 mol.l⁻¹ phosphate buffer and at 5 °C in 1% uranyl acetate in methanol for 1 hour. After dehydration through an ethanol series (70%, 96%, 100%), cells were embedded in Spurr's medium (Spurr 1969) via isobutanol. Ultrathin sections, cut with a diamond knife on an Ultracut E (Reichert–Jung, Wien, Austria), were post–stained with lead citrate and examined using a JEOL 1011 TEM (JEOL Ltd., Tokyo, Japan). Photomicrographs were obtained using a Veleta CCD camera equipped with image analysis software (Olympus Soft Imaging Solution GmbH).

Zoospore production. 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 Mont 10/10–1w was grown in liquid WH+ medium as above. Cells were collected by centrifugation and DNA was isolated according to Fawley & Fawley (2004). Conditions for polymerase chain reaction amplification were as described in Fawley & Fawley (2004) for the nuclear 18S rDNA region and Fawley et al. (2015) for the plastid rbcL

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 (Bonfield et al. 1995). New nuclear 18S rDNA (GenBank MK295224) and plastid rbcL (GenBank MK295223) sequences from Mont 10/10–1w were added to the alignments of Fawley & Fawley (2017), and aligned by eye in MacClade 4.08 (Maddison & Maddison 2000).

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 (Taveré 1986) with parameters selected by GARLI. The ML analysis of the concatenated data set was performed with partitions for 18S rDNA and each codon position of the rbcL 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.

Results

Light and electron microscopy of strain Mont 10/10–1w revealed features typical for the Eustigmatophyceae. Cells were spherical or nearly so, with thick walls (Figs 1–9) and were joined in firm clumps without any form. Cells ranged from about 3.7 µm for autospores to 7.8 µm for large vegetative cells. One or more reddish globules were present, which were usually rounded but occasionally they were irregular in outline. Smaller reddish globules appeared to fuse into a larger, rounded body as the cells matured. Plastids were parietal sheets without lobes. Although difficult to discern, by light microscopy there appeared to be multiple plastids per cell, with 2 plastids in small cells and 4 in larger cells (Figs 3–8). The plastids nearly covered the periphery of the cell making it difficult to see internal features clearly. No pyrenoids were observed. Numerous small granules or vesicles were present in the cells, including a few highly refractive bodies. Reproduction was by the production of 2 or 4 autospores (not shown); no zoospores or other flagellate cells were observed.

Cell walls were positively stained with Calcofluor white that binds strongly to structures containing polysaccharides, showing that cell wall is composed of polysaccharide (cellulose?) (Fig. 9). 3D reconstruction of the cell wall revealed a lightly ridged surface (Fig. 9). The cell wall comprised two layers, probably a cellulosic inner wall (50–100 nm wide) succeeded by a layer of trilaminar structure, a narrow dark–light–dark domain (TLS; c.a. 8 nm). Extensions of unknown composition protrude from the outer surface of TLS layer (Fig.10, enlarged upper–left figure; c.a. 50 nm). The most interesting features of the cell wall were the columellae connected to the TLS layer by a widened base. Columellae were of average length 480 nm (n=14), and were regularly distributed over the cell surface. At least in some cases they seemed to connect TLS layers of neighbouring cells (Fig. 11).

However, most columellae simply protruded out of the cell. The columellae probably were not composed of polysaccharide, as they were not stained by Calcofluor white and were not visible even on optical sections through the cell wall (Fig. 9). However, columellae dimensions were near limits of optical resolution of the confocal microscope.

Transmission electron microscopy supports the presence of multiple plastids that lack pyrenoids (Figs 12–15). Smaller and/or younger reddish globules were homogenous (Figs 13, 15) on TEM images, whereas larger bodies were composed of a number of smaller droplets (Figs 12, 14). Ultrastructure confirmed no girdle lamella within the chloroplast and plastoglobuli were sometimes visible. On TEM images lamellated vesicles, highly characteristic structures of eustigmatophytes, were visible freely in the cytoplasm (Fig. 14). Numerous mitochondria contained tubular cristae (Figs 12, 13). There was more than one nucleus in several cells. We observed some nuclei in the stage where the nucleolus was formed (Fig. 15).

Phylogenetic analysis of the 18S rDNA sequence data (Fig. 16) confirmed the placement of strain Mont 10/10–1w in the Eustigmatophyceae. Moreover, Mont 10/10–1w is the only member of a new lineage that is basal within the Eustigmatales, albeit with weak bootstrap support. The results of the analysis of concatenated 18S and rbcL sequences from a smaller set of taxa (Fig. 17) produced identical results for the placement of Mont 10/10–1w, still with poor bootstrap support for the alliance with the Eustigmatales.

Our phylogenetic and morphological results indicate the need for a new genus and species for strain Mont 10/10–1w.

Descriptions of new taxa

***Paraeustigmatos* gen. nov.**
Description: Spherical or nearly spherical cells with smooth, thick walls. Multiple parietal plastids without pyrenoids. The cell wall is composed of two structurally different layers. Reproduction by autospore production.

Paraeustigmatos is also recognized as distinct from other genera based on differences in the plastid rbcL and nuclear 18S rDNA sequences.

Type species: *Paraeustigmatos columelliferus*, sp. nov. Etymology: *Paraeustigmatos* refers to the results of phylogenetic analyses that place the new taxon sister to (Greek *para*, beside) the core Eustigmatales, which includes the genus *Eustigmatos* D.J. Hibberd.

***Paraeustigmatos columelliferus* sp. nov. (Figs 1–15)**
Description: Spherical or nearly spherical cells 3.7 to 7.8 µm in diameter. Forming tight aggregates without specific form. Plastids 2–4, parietal, almost completely covering the surface of mature cells. Pyrenoids lacking. One or more reddish globules present in the cytoplasm along with lamellate vesicles. Refractile granules sometimes present. Reproduction by 2–4 autospores. The cell wall composed of two layers: thicker inner layer and trilaminar outer layer bearing fibrous extensions. Columellae of average size 480 nm in length regularly distributed over the cell surface.

Phylogenetic analyses of nuclear 18S rDNA and plastic rbcL sequence data place *P. columelliferus* in a basal lineage within the Eustigmatales, although with weak bootstrap support.

Type specimen: Strain Mont 10/10–1w, held in liquid nitrogen as CAUP Q701 at the Charles University in Prague.
Type location: A mat of *Zygnema* sp. on the shore of Lake Monticello, Arkansas, USA, (approximately 33°42'09"N, 91°49'40"W) collected 10 October 2011.
**Etymology:** The specific epithet *columelliferus* refers to the presence in this species of cell wall columellae analogous to those found in the walls of pollen grains.

DISCUSSION

Light and electron microscopy showed the presence of reddish globules and lamellate vesicles in the cytoplasm
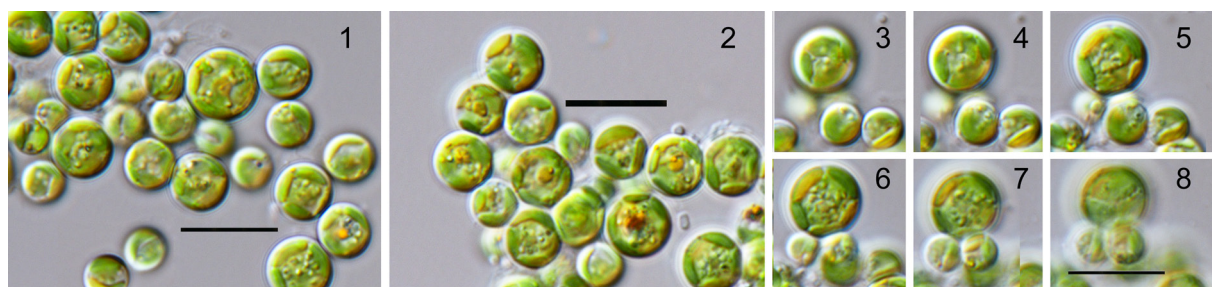
Figs 1–8. Light micrographs of *Paraeustigmatos columelliferus*, gen. et sp. nov.: (1,2) groups of cells showing the nearly spherical form of the cells and the reddish globules; (3) uppermost image of a Z–stack series of vegetative cells showing the mostly smooth cell surface without cell wall sculpting; (4–7) optical sections progressing through the cells that show the multiple plastids and other cellular inclusions; (8) lower surface of the cells. Scale bars 10 µm.
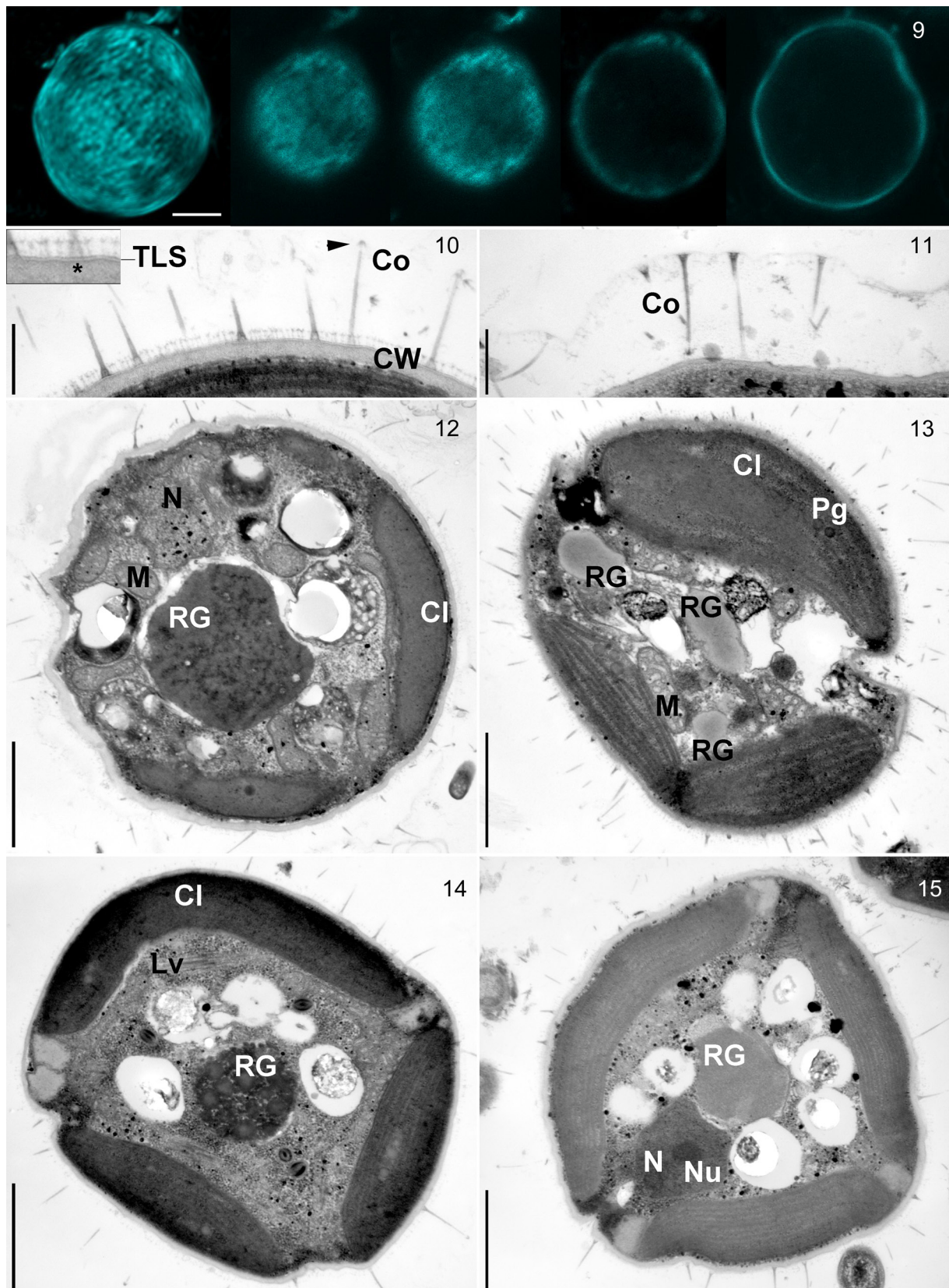
Figs 9–15. Confocal laser scanning and TEM microphotographs of *Paraeustigmatos columelliferus*, gen et sp. nov.: (9) 3D reconstruction of the cell wall stained by Calcofluor white (left) and four optical sections through the cell wall stained by Calcofluor white; (10, 11) cell wall and columellae, (10) cell wall composed of polysaccharide inner wall succeeded by layer of trilaminar structure (TLS), columella (Co) is connected with TLS, the opposite end of the columella is terminated by arrow–like top (arrowhead). Upper–left box shows the detail of fibrous extensions protruding from trilaminar layer and columella's widened base (above asterisk), (11) Columella (Co) seems to connect TLS layer of the neighbouring cell; (12–15) ultrastucture of vegetative cells (12) the cell with a large reddish globule (RG), numerous parietal chloroplasts (Cl) , nucleus (N) and mitochondria with tubular cristae (M), (13) the younger cell with several small homogenous reddish globules (RG), chloroplast (Cl) bearing plastoglobuli (Pg), (14) the cell with pronounced reddish globule (RG) composed of a number of a smaller droplets, lamellated vesicles (Lv), (15) the cell with still homogenous reddish globule (RG) and nucleus (N) bearing visible nucleolus (Nu), Scale bar 2 µm (Fig. 9); 0.3 µm (Figs 10, 11) and 1 µm (Figs 12–15).
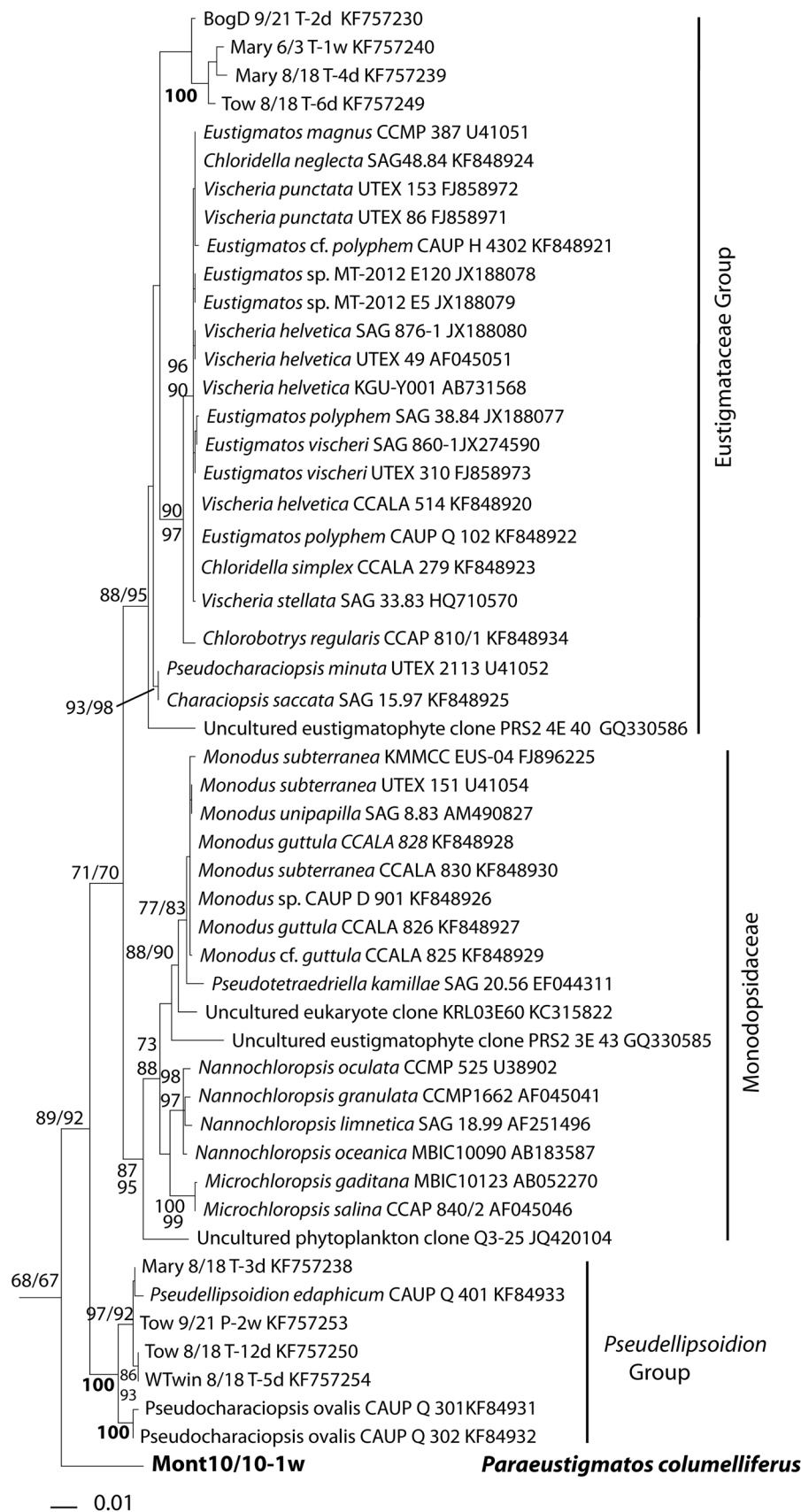
Fig. 16. Results of phylogenetic analysis of nuclear 18S rDNA sequence data from the Eustigmatophyceae showing *P. columelliferus* allied with the Eustigmatales. The full phylogram of the Eustigmatophyceae is shown in Supplemental Fig. S1. The phylogram is from Maximum Likelihood analysis. Bootstrap 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). Bootstrap values for some terminal branches are not shown.
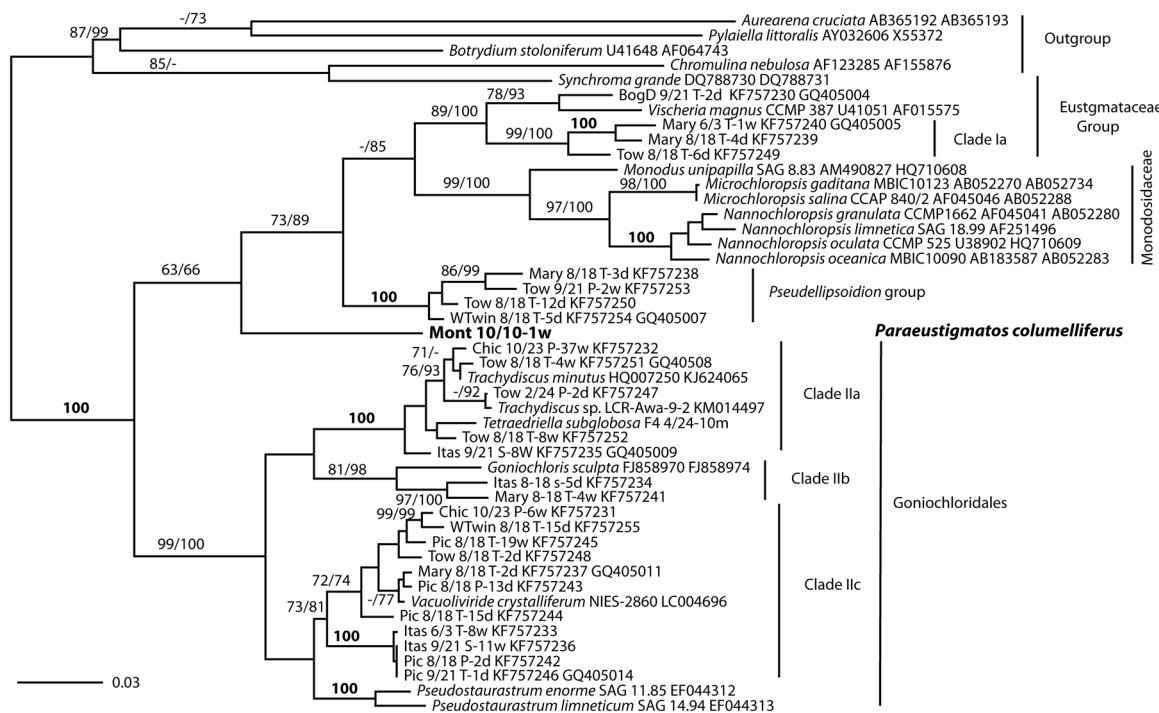
Fig. 17. Results of phylogenetic analysis of combined nuclear 18S rDNA and plastic *rbc*L DNA sequence data from the Eustigmatophyceae. Maximum Likelihood phylogram shown. Bootstrap values as in Fig. 16.

of *Paraeustigmatos columelliferus*, which are key features of the Eustigmatophyceae (ELIÁŠ et al 2017). However, there are few distinguishing features of *P. columelliferus* that can be used to place this organism in any of the existing lineages of the class. The new species is unique in the combination of multiple plastids that lack pyrenoids, the lightly ridged or smooth, thick walls, reproduction only by autospore production and the formation of firm clumps of cells. For the Eustigmatales, to which *P. columelliferus* is likely allied, a single parietal plastid with a polyhedral pyrenoid separated from the plastid by a narrow stalk is typical (HIBBERD 1974; SANTOS 1996; NEUSTUPA & NEMCOVA 2001). The cell wall architecture of *P. columelliferus* is relatively similar to the cell wall of *Microchloropsis gaditana* (formerly *Nannochloropsis gaditana*) that was recently studied in great detail (SCHOLZ et al. 2014). *M. gaditana* possesses a bilayer cell wall consisting of a cellulosic inner wall protected by an outer hydrophobic trilaminar algaenan layer. Also, fibrous extensions (50–100 nm) protruding from the trilaminar layer were observed in both *M. gaditana* (SCHOLZ et al. 2014) and *P. columelliferus*. These fibrous extensions expand the cell surface considerably and may serve for example, to retain humidity during temporal exposure to dry environments or to help form aggregates of cells. In *M. gaditana* the chemical composition of both cell wall layers was confirmed by Fourier transform infrared spectroscopy and carbohydrate analyses. However, in *P. columelliferus* we can only speculate that the inner cell wall layer is composed of cellulose, as Calcofluor white binds strongly to structures containing a wide

spectrum of polysaccharides, and not only cellulose. *Microchloropsis* algaenan was determined to comprise long, straight–chain, saturated aliphatics with ether cross–links (GELIN et al. 1997; SCHOLZ et al. 2014). In thin sections of osmicated, dehydrated specimens, the presence of algaenan may correlate with the presence of a trilaminar structure (TLS), a narrow dark–light–dark domain at the wall surface (ALLARD et. al. 2002). However, the chemical structure of *P. columelliferus* TLS still waits to be elucidated. Cell walls composed of TLS underlain by a thicker (probably pectic) layer was also described in the genus *Chlorobotrys* (HIBBERD 1974). A unique character of *P. columelliferus* was the columella connected with the TLS layer by the widened base; the opposite end of the columella was terminated by an arrow–like top. We can only speculate about the function of the columellae and we also do not know in which phase of the life cycle columellae are produced. They may serve to push the neighbouring cells (e.g. autospores within a mother cell wall) apart from each other to expand the intercellular space. It is possible that the arrow–like top is able to adhere to a neighbouring cell wall, in which case columellae may help to keep cells together. This idea is supported by observation of firm clumps of *P. columelliferus* cells within the liquid culture.

Phylogenetic analyses of nuclear 18S rDNA and plastid rbcL sequence data show that this strain is a member of the Eustigmatophyceae and probably allied with the Eustigmatales. Both Maximum Likelihood and Maximum Parsimony analyses place *P. columelliferus* as basal within

the Eustigmatales, but the bootstrap support for this alliance is quite low; below our normal accepted level of 70%. This weak relationship to the Eustigmatales was found with both 18S rDNA analysis alone and combined with *rbc*L with a smaller taxon set. There is the possibility that *P. columelliferus* actually represents a new third lineage within the Eustigmatophyceae. Thus, the correct phylogeny for *P. columelliferus* is still uncertain. Additional research on the phylogeny of *P. columelliferus* including analyses of organellar genomes is underway and may provide new insights.

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Supplementary material

the following supplementary material is available for this article:

Fig. S1. Results of phylogenetic analysis of nuclear 18S rDNA sequence data from the Eustigmatophyceae.

This material is available as part of the online article (http://fottea.czechphycology.cz/contents)