J Appl Phycol (2014) 26:1773–1782
DOI 10.1007/s10811-013-0216-z

The diversity and phylogeny of the commercially important algal class Eustigmatophyceae, including the new clade *Goniochloridales*

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Received: 24 September 2013 / Revised and accepted: 20 November 2013 / Published online: 12 December 2013

Abstract The Eustigmatophyceae is a class of yellow-green algae allied with the Chrysophyceae and other chlorophyll *c* possessing stramenopile (heterokont) algae. Some members of the class, especially the marine species of the genus *Nannochloropsis*, are under intense investigation for their potential for production of biofuels and beneficial fatty acids. The class has generally been thought to comprise a small number of genera and species, and these organisms were considered rare or infrequently encountered. In this study, we examined the phylogeny and diversity of this class by analysis of nuclear 18S rDNA sequence data. Our analysis included sequences from all the named members of the Eustigmatophyceae held in culture collections as well as a number of strains identified in culture collections as Xanthophyceae, new strains with features characteristic of the Eustigmatophyceae, and published data for uncultured DNA clones. The results of these analyses show that the Eustigmatophyceae is far more diverse than generally recognized. Two major lineages are supported in the class, the previously recognized order Eustigmatales and the new clade, *Goniochloridales*. Additional new lineages were also resolved within each of these major lineages; however, the results of our analyses were considered insufficient for naming these subordinate clades. Several of these lineages comprised only unnamed strains or uncultured DNA clones. Overall, our results indicate that the Eustigmatophyceae is a highly diverse class, with many new species, genera, and families awaiting taxonomic treatment.

Keywords Biofuels · *Eustigmatos* · *Goniochloris* · *Monodopsis* · *Monodus* · *Nannochloropsis* · Fatty acids · *Trachydiscus* · *Vischeria*

Introduction

Species from the algal class Eustigmatophyceae D.J. Hibberd and Leedale are receiving increased attention as potential biofuel organisms and for possible production of fatty acids for nutritional supplements. Most of these studies have focused on marine species of the genus *Nannochloropsis* D.J. Hibberd, a genus of tiny unicellular algae. *Nannochloropsis* species have been used for many years to provide a source of polyunsaturated fatty acids in the diet of aquaculture-raised marine invertebrates (Apt and Behrens 1999). The recent interest in biofuel organisms has prompted a host of genomic studies of *Nannochloropsis* species (Pan et al. 2011; Radakovits et al. 2012; Vieler et al. 2012; Jinkerson et al. 2013; Wei et al. 2013; Carpinelli et al. 2013) as well as developing methods for genetic transformation (Kilian et al. 2011; Radakovits et al. 2012). The combination of high lipid content, ease of growth on a large scale, new genomic information, and genetic transformation techniques has led to the suggestion that *Nannochloropsis* has high potential for

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photons-to-fuel commercial systems (Jinkerson et al. 2013). *Nannochloropsis* is now firmly established as a model organism for the production of lipids.

Other species of the Eustigmatophyceae have not been examined as thoroughly as *Nannochloropsis* species for their production of lipids and other compounds of biotechnology interest. This difference may relate to the marine habitat of most *Nannochloropsis* species, whereas other Eustigmatophyceae are freshwater or soil organisms. The use of *Nannochloropsis* in aquaculture led to the early development of growth systems for this alga, whereas freshwater Eustigmatophyceae had no such importance. However, lipid production has been studied in some species of the genera *Eustigmatos* D.J. Hibberd, *Vischeria* Pascher, and *Monodopsis* D.J. Hibberd, (Volkman et al. 1999a, b; Khozin-Goldberg and Cohen 2006; Wan et al. 2012), but most other genera have not been examined for biotechnology potential. Recently, *Trachydiscus minutus* (Bourr.) H. Ettl has been shown to produce very high concentrations of polyunsaturated fatty acids, which makes this alga a good candidate for commercial production of these lipids (Řezanka et al. 2010). *T. minutus* was originally described as a member of the Xanthophyceae, but it has been transferred to the Eustigmatophyceae on the basis of DNA sequence analysis, pigments, and morphology (Přibyl et al. 2012). The potential of *T. minutus* for biotechnology applications suggests that other Eustigmatophyceae could also prove useful for these applications. In addition, genomic studies of diverse Eustigmatophyceae strains could provide new insights on the genetics of lipid production.

The potential for commercial exploitation of additional Eustigmatophyceae algae has also been limited by a very low known diversity for this class of algae. Only a few genera and species have been described in only five families and one order (Table 1). The Eustigmatophyceae are also thought to be infrequently encountered, rarely reaching high densities in freshwater systems. Biodiversity reviews have suggested that the total number of species in the class, described and undescribed, could be as few as 30 (Adl et al. 2007). This low diversity leaves little room to explore the potential of Eustigmatophyceae algae for possible biotechnological applications. Recent analyses of Eustigmatophyceae diversity have begun to change this view. The only described freshwater species of *Nannochloropsis*, *N. limnetica* Krienitz, Hepperle, Stich & W. Weiler, is actually a complex of genetically distinct organisms (Fawley and Fawley 2007). In addition to *T. minutus*, the former Xanthophyceae alga *Goniochloris sculpta* Geitler is allied with the Eustigmatophyceae (Přibyl et al. 2012). Moreover, partial characterization of some new freshwater strains of Eustigmatophyceae indicated much more diversity in the class than was previously known (Prior et al. 2009). Many of these new strains are morphologically simple and similar to taxa assigned to the Xanthophyceae. Thus, many taxa that have traditionally been placed in the Xanthophyceae may actually be Eustigmatophyceae, a suggestion also made by Hibberd (1990).

Here we present analyses of new nuclear 18S ribosomal RNA gene sequence data from Eustigmatophyceae strains that clearly demonstrate that the Eustigmatophyceae is a diverse class of algae. We analyzed strains that included named and unnamed Eustigmatophyceae from public culture collections and uncharacterized strains from our own collections. Sampling from the genus *Nannochloropsis* was limited to one exemplar from each named species. This genus has been the subject of many sequencing studies and will be the focus of a separate analysis in the future. The new strains of Eustigmatophyceae included in our analyses were collected from many soil and freshwater habitats, including mesotrophic, dystrophic, and eutrophic ponds and lakes. Most of the new strains are not closely allied to any named species of the Eustigmatophyceae. The results of our phylogenetic analyses show two major lineages, the order Eustigmatales and the new ordinal-level clade, *Goniochloridales*. Each of these lineages includes multiple internal clades which likely represent additional new families in the traditional hierarchical taxonomic system. We also present a phylogenetic framework that should facilitate the naming of new species, genera, and families from among this diversity. This framework will allow researchers who are interested in the biotechnology potential, phylogeny, and genomics of the Eustigmatophyceae to select strains that are representative of the diversity across the entire class.

Materials and methods

Origin of algal strains

Fifteen strains of Eustigmatophyceae were acquired from public culture collections (Table 2). Eight of these strains were

Table 1 Current taxonomy of the Eustigmatophyceae

Order	Families	Genera
Eustigmatales	Chlorobotryaceae	*Chlorobotrys*
	Eustigmataceae	*Eustigmatos*
		Vischeria
	Loboceae	*Pseudotetraëdriella*
	Monodopsidaceae	*Monodopsis*
		Nannochloropsis
	Pseudocharaciopsidaceae	*Botryochloropsis*
		Pseudocharaciopsis
	Incertae sedis	*Pseudellipsoidion*
Incertae sedis		*Goniochloris*
		Pseudostaurastrum
		Trachydiscus

Based on Hibberd (1990), Santos (1996), Neustupa and Němcová (2001), Hegewald et al. (2007), and Přibyl et al. (2012)

Table 2 Eustigmatophyceae strains from culture collections used in this study and GenBank accession numbers

Taxon	Strain code	18S rDNA accession
Characiopsis saccata	SAG 15.97	KF848925
Chloridella neglecta	SAG 48.84	KF848924
Chloridella simplex	CCALA 279	KF848923
Chlorobotrys regularis	CCAP 810/1	KF848934
Eustigmatos polyphem	CAUP Q 102	KF848922
Eustigmatos cf. *polyphem*	CAUP H 4302	KF848921
Monodopsis subterranea	CCALA 830	KF848930
Monodus guttula	CCALA 826	KF848927
Monodus guttula	CCALA 828	KF848928
Monodus cf. *guttula*	CCALA 825	KF848929
Monodus sp.	CAUP D 901	KF848926
Pseudellipsoidion edaphicum	CAUP Q 401	KF848933
Pseudocharaciopsis ovalis	CAUP Q 301	KF848931
Pseudocharaciopsis ovalis	CAUP Q 302	KF848932
Vischeria helvetica	CCALA 514	KF848920

CAUP Culture Collection of Algae at Charles University in Prague, *CCALA* Culture Collection of Autotrophic Organisms, Academy of Sciences of the Czech Republic, *SAG* The Culture Collection of Algae at Goettingen University

previously placed in the Xanthophyceae. Twenty-four of our new algal strains (Table 3) were isolated as part of the Itasca Microbial Observatory project in Itasca State Park (ISP), Minnesota, USA. For that project, coccoid algae were isolated from seven sites in the park, initially chosen to represent a diversity of water chemistries and habitats. Both phytoplankton and tychoplankton were sampled for all sites, and samples were collected from multiple depths for larger lakes (Lake Itasca and Mary Lake). A list of the collection sites and a summary of the water chemistry for each site is presented in Fawley et al. (2004).

We have also isolated two Eustigmatophyceae strains from a surface grab sample from eutrophic Lake Chicot in southeast Arkansas, USA (Table 3). New algal strains were isolated from the water samples using a spread-plate method as described in Phillips and Fawley (2000), using the media WH+ (Fawley et al. 1990) and DYIV (Keller and Andersen, in Andersen et al. 1997). Strains were identified as potential Eustigmatophyceae by the presence of an orange-red body in the cytoplasm or by sequence analysis of the 18S rDNA. Cultures were maintained at 17–20 ° C under cool white fluorescent lamps on agar slants.

Light microscopy

Light microscopy of living cells from liquid batch culture of the strains from ISP and Lake Chicot was performed with either a Nikon E-600 or Nikon N*i*-U (Nikon, USA) microscope equipped with differential interference contrast optics using × 60 or × 100 objectives (NA 1.40). Digital images were acquired with a Pixera Pro 150ES camera (Pixera Corporation, USA). Only vegetative morphologies were examined, although autospores and occasionally zoospores were observed.

DNA sequence analysis

DNA was isolated from liquid cultures using the technique of Fawley and Fawley (2004) or from cultures grown on agar slants as described in Přibyl et al. (2012). The nuclear 18S rDNA region was amplified by polymerase chain reaction (PCR) using the primers NS1X and 18LX according to Fawley and Fawley (2004) or using primers F and R (Katana et al. 2001) according to Přibyl et al. (2012). No product useful for sequencing could be obtained with the standard primers for *Chlorobotrys regularis* (W. West) Bohlin CCAP 810/1. Hence, a combination of the F primer (Katana et al. 2001) with a newly designed Eustigmatophyceae-specific primer EustigR1 (5′GTTATA AACTCGTTGAACGCA3′) was employed; the 18S rDNA sequence obtained for *C. regularis* CCAP 810/1 is thus shorter (1,170 bp) than our other sequences. The primers used for PCR and the primer NS5 (White et al. 1990), the new primer 18JX (5′GCATCACAGACCTGTTATTG3′), or the sequencing primers described by Katana et al. (2001) were used for sequencing. Sequencing was performed by the DNA Resource Center at the University of Arkansas at Fayetteville or by the DNA Sequencing Laboratory of the Faculty of Science, Charles University in Prague. Sequences were compiled and edited using the Staden Package v 2.0.0b9 (http://staden.sourceforge.net/) or BioEdit v 7.0.5.3 (Hall 1999) and the CAP3 assembler server (http://pbil.univ-lyon1.fr/cap3.php), and terminal primer sequences were removed. The GenBank accession numbers for newly generated sequences are given in Tables 2 and 3.

An alignment of 18S rDNA sequences from our new sequences, Eustigmatophyceae sequences in GenBank, and outgroup taxa from representative stramenopiles was generated with the Muscle (Edgar 2004) server (http://www.ebi.ac.uk/Tools/msa/muscle/) and visually edited in MacClade 4.08 (Maddison and Maddison 2000). Outgroup taxa were selected as representative of other stramenopile algae. For brevity, only one sequence for each of the six recognized species of *Nannochloropsis* was included in the alignment. Potentially ambiguous regions of the alignment were excluded from further analyses using GBlocks (Castresana 2000) through the GBlocks server version 0.91b (http://molevol.cmima.csic.es/castresana/Gblocks_server.html). Maximum parsimony (MP) analyses were performed with PAUP* 2.0b (Swofford 2002). Maximum likelihood (ML) analysis used Garli 0.96 (Zwickl 2006), and Bayesian inference (BI) was performed using MrBayes 3.2.1 (Ronquist et al. 2012). Both

Table 3 Unidentified strains of Eustigmatophyceae used in this study with sample origin, sample date and GenBank accession number

Strain	Sample origin	Sample date	18S rDNA accession
BogD 9/21 T-2d	Itasca State Park, Minnesota, Bog D	21 Sept 2000	KF757230
Chic 10/23 P-6w	Lake Chicot, Chicot County, Arkansas	23 Oct 2006	KF757231
Chic 10/23 P-37w	Lake Chicot, Chicot County, Arkansas	23 Oct 2006	KF757232
Itas 6/3 T-8w	Itasca State Park, Minnesota, Lake Itasca	03 Jun 2001	KF757233
Itas 8/18 S-5d	Itasca State Park, Minnesota, Lake Itasca	18 Aug 2001	KF757234
Itas 9/21 S-8w	Itasca State Park, Minnesota, Lake Itasca	21 Sept 2000	KF757235
Itas 9/21 S-11w	Itasca State Park, Minnesota, Lake Itasca	21 Sept 2000	KF757236
Mary 6/3 T-1w	Itasca State Park, Minnesota, Mary Lake	03 Jun 2001	KF757240
Mary 8/18 T-2d	Itasca State Park, Minnesota, Mary Lake	18 Aug 2001	KF757237
Mary 8/18 T-3d	Itasca State Park, Minnesota, Mary Lake	18 Aug 2001	KF757238
Mary 8/18 T-4d	Itasca State Park, Minnesota, Mary Lake	18 Aug 2001	KF757239
Mary 8/18 T-4w	Itasca State Park, Minnesota, Mary Lake	18 Aug 2001	KF757241
Pic 8/18 P-2d	Itasca State Park, Minnesota, “Picnic Pond”	18 Aug 2001	KF757242
Pic 8/18 P-13d	Itasca State Park, Minnesota, “Picnic Pond”	18 Aug 2001	KF757243
Pic 8/18 T-15d	Itasca State Park, Minnesota, “Picnic Pond”	18 Aug 2001	KF757244
Pic 8/18 T-19w	Itasca State Park, Minnesota, “Picnic Pond”	18 Aug 2001	KF757245
Pic 9/21 T-1d	Itasca State Park, Minnesota, “Picnic Pond”	21 Sept 2000	KF757246
Tow 2/24 P-2d	Itasca State Park, Minnesota, “Tower Pond”	24 Feb 2001	KF757247
Tow 8/18 T-2d	Itasca State Park, Minnesota, “Tower Pond”	18 Aug 2001	KF757248
Tow 8/18 T-4w	Itasca State Park, Minnesota, “Tower Pond”	18 Aug 2001	KF757251
Tow 8/18 T-6d	Itasca State Park, Minnesota, “Tower Pond”	18 Aug 2001	KF757249
Tow 8/18 T-8w	Itasca State Park, Minnesota, “Tower Pond”	18 Aug 2001	KF757252
Tow 8/18 T-12d	Itasca State Park, Minnesota, “Tower Pond”	18 Aug 2001	KF757250
Tow 9/21 P-2w	Itasca State Park, Minnesota, “Tower Pond”	21 Sept 2000	KF757253
WTwin 8/18 T-5d	Itasca State Park, Minnesota, West Twin Lake	18 Aug 2001	KF757254
WTwin 8/18 T-15d	Itasca State Park, Minnesota, West Twin Lake	18 Aug 2001	KF757255

ML and BI employed the GTR+I+Γ model of DNA substitution (Tavaré 1986), with parameters selected by the programs. The ML analysis was performed with 20 replicates with different random starting trees. Bootstrap analyses employed 1,000 resamplings of the data for the MP analysis, whereas the ML analysis was bootstrapped with 500 resamplings, each with two random starting trees. Bayesian analysis was conducted with two independent runs of four Markov chains for 2.5 million generations, with the first one million generations discarded.

Results

Phylogenetic analyses

Results of the phylogenetic analyses of the 18S rDNA sequence data (Fig. 1) were similar for MP, ML, and BI techniques. The Eustigmatophyceae are a monophyletic lineage with robust support in all analyses. Two major lineages of the Eustigmatophyceae are present, the Eustigmatales and the new lineage, clade *Goniochloridales*. Each of these lineages has several internal clades. Some of the previously described families of the Eustigmatophyceae are either not monophyletic or are immersed in other lineages.

The Eustigmatales comprises the majority of the previously known diversity of the class, including the well-supported family Monodopsidaceae D.J. Hibberd and the lineages with the working names, the Eustigmataceae group and the *Pseudellipsoidion* group, as shown in (Fig. 1). The Eustigmataceae group was monophyletic in our analyses and included the established family Eustigmataceae D.J. Hibberd with the genera *Eustigmatos* and *Vischeria*. *Vischeria*, *Eustigmatos*, and two strains from culture collections identified as *Chloridella* Pascher (presently assigned to the Xanthophyceae) are a monophyletic group with very little variation among the 18S rDNA sequences. *C. regularis*, which had not been previously studied, is allied with the Eustigmataceae and is sister to the other genera of the family, *Vischeria* and *Eustigmatos*. This genus is presently given its own family, Chlorobotryaceae Pascher (alternate name, Chlorobotrydaceae).

Clade Ia, a potential new lineage within the Eustigmatales, is comprised of three strains from Itasca State Park and perhaps the ISP strain BogD 9/21 T-2d. Although this clade is

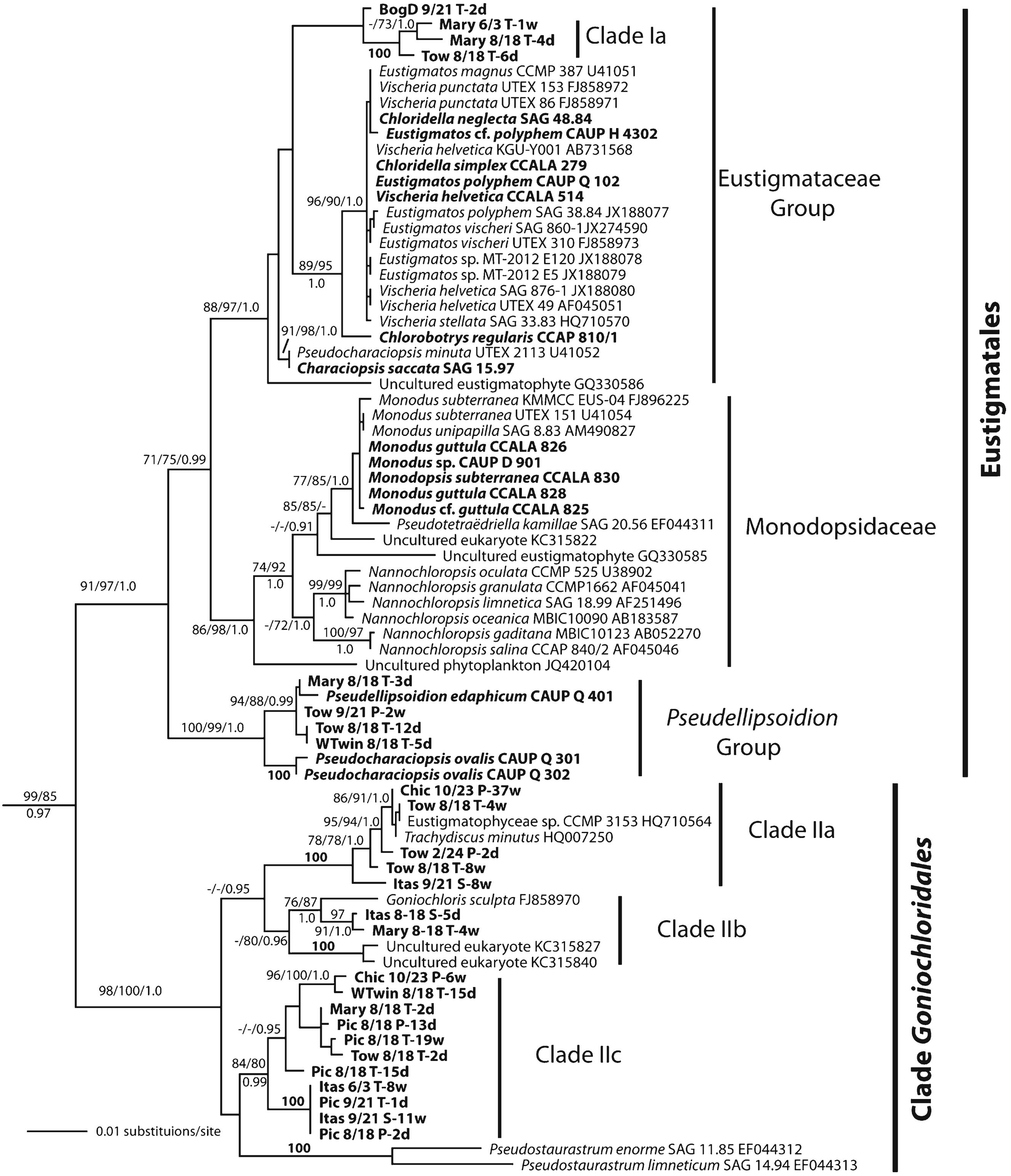

Fig. 1 Results of phylogenetic analysis of the 18S rDNA of Eustigmatophyceae. The phylogram is from a maximum likelihood analysis, with bootstrap support values and posterior probabilities shown for maximum parsimony/maximum likelihood/Bayesian analyses. A single value of 100 in bold face indicates maximum support in all analyses. Only values greater than 70 for bootstraps and 0.9 for posterior probabilities are shown. The tree was rooted using sequences from the stramenopile algae *Aurearena cruciate* (Aurearenophyceae) AB365192, *Chromulina nebulosa* (Chrysophyceae) AF123285, *Pylaiella littoralis* (Phaeophyceae) AY032606, *Synchroma grande* (Synchromophyceae) DQ788730, and *Botrydium stoloniferum* (Xanthophyceae) U41648 as outgroup taxa

clearly allied with the Eustigmataceae group, the relationships of Clade Ia to other lineages of the group remain unclear.

Pseudocharaciopsis minuta (Braun) D.J. Hibberd and a strain identified as *Characiopsis saccata* N. Carter have 18S rDNA sequences that are identical except for one indel. Together, these strains form a separate lineage in the Eustigmataceae group, basal to all other described members. *Pseudocharaciopsis* Lee and Bold has been assigned to its own family, the Pseudocharaciopsidaceae Lee and Bold ex D.J. Hibberd; however, *Pseudocharaciopsis* is polyphyletic in our analysis, with the only other species, *P. ovalis* (Chodat) D.J. Hibberd, positioned in the *Pseudellipsoidion* group (see below). The final sequence assigned to the Eustigmataceae group is an 18S rDNA clone from an uncultured organism. This sequence is not clearly associated with any lineage of the group.

The Monodopsidaceae is a well-supported lineage that comprises the genera *Nannochloropsis*, *Monodopsis*, and *Pseudotetraëdriella* E. Hegewald, Padisak and Friedl as well as three uncultured clones. Several strains of *Monodus* Chodat (assigned to the Xanthophyceae) from culture collections are immersed in *Monodopsis*. The enigmatic genus *Pseudotetraëdriella* (presently given its own family, the Loboceae E. Hegewald) is sister to the genus *Monodopsis*. Although the genus *Monodopsis* (including *Monodus* strains) is monophyletic in all analyses, bootstrap and posterior probability support for the genus is low in the results of our 18S analysis. The genus *Nannochloropsis* is monophyletic, with poor bootstrap support in the MP and ML analyses. However, analyses that do not include the uncultured marine phytoplankton sequence JQ420104 have high bootstrap support for monophyly of *Nannochloropsis* (results not shown).

The final lineage within the Eustigmatales, the *Pseudellipsoidion* group in (Fig. 1), comprises *Pseudellipsoidion edaphicum* Neustupa and Němcová, *Pseudocharaciopsis ovalis*, and four strains from Itasca State Park. This robust lineage is sister to all other Eustigmatales.

The second lineage of the Eustigmatophyceae is comprised mostly of unnamed strains from Itasca State Park and Lake Chicot. Only four named taxa are included, *Pseudostaurastrum enorme* (Ralfs) Chodat, *Pseudostaurastrum limneticum* (Borge) Chodat, *G. sculpta*, and *T. minutus*. Until recently, these species were all considered to have Xanthophyceae affinities. The organism labeled here Eustigmatophyceae sp. CCMP 3153 has

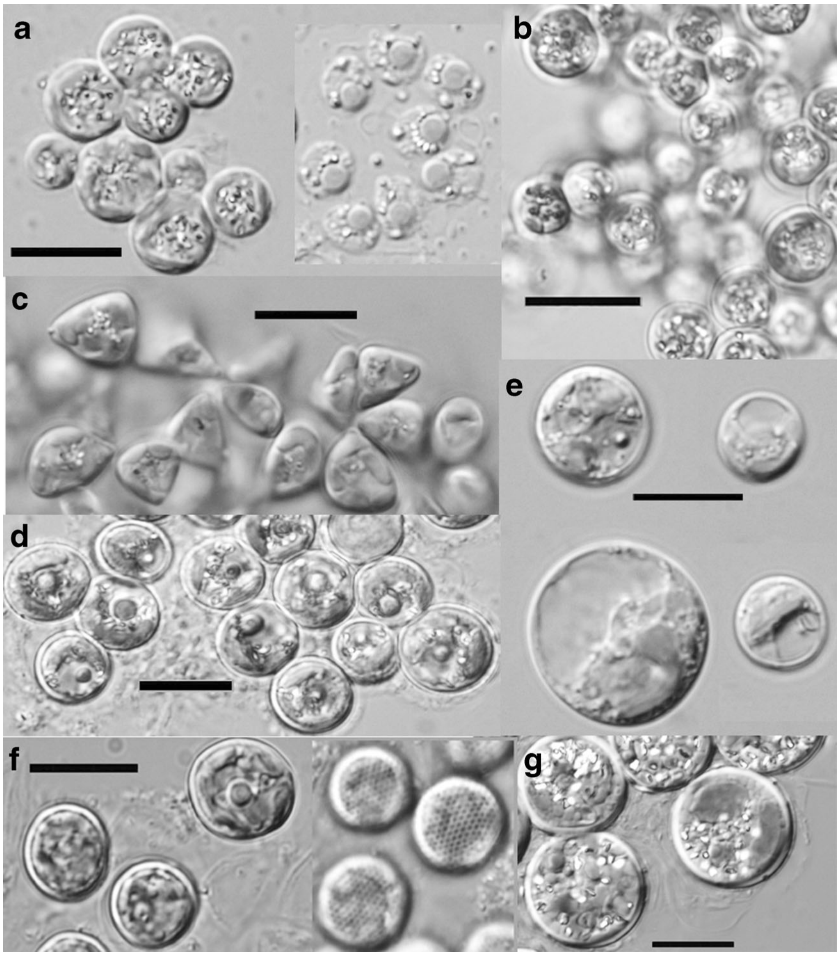

Fig. 2 Light micrographs of select unidentified eustigmatophycean strains. **a**. Strain Mary 8/18 T-4d (Clade Ia) with clumps of vegetative cells with refractive granules (left) and zoospores (right). **b**. Strain Mary 8/18 T-4w (Clade IIb), vegetative cells in clumps. **c**. Itas 8/18 S-5d (Clade IIb), angular vegetative cells similar to *Goniochloris*. **d**. Pic 8/18 T-15d (Clade IIc). **e**. Pic 9/21 T-1d (Clade IIc) vegetative cells. **f**. Chic 10/23 P-37w (Clade IIa) vegetative cells, with cell wall sculpting shown on the right. **g**. WTwin 8/18 T-15d (Clade IIc) vegetative cells with highly refractive granules. Scale bars = 10 μm

18S rDNA sequence identical to that of our strain Tow 8/18 T-4w. CCMP 3153 was referred to as "*Microtalis aquatica* J.C. Bailey" by Yang et al. (2012), but to our knowledge, this name has not been validly published. The results of analysis of 18S rDNA sequence data are robust in placing these species and the unnamed strains as a new lineage of the Eustigmatophyceae. This lineage includes four well-supported clades. Clade IIa includes *T. minutus* and six unnamed strains. Clade IIb includes *G. sculpta* and two unnamed strains. Two uncultured clones, KC315827 and KC315840, are associated with Clade IIb with low bootstrap support. Clade IIc comprises only 11 unnamed strains, and the final clade comprises only the two species of *Pseudostaurastrum*.

New diversity

Our new freshwater strains of Eustigmatophyceae include members of all of the new clades (Clades Ia, *Pseudellipsoidion* Group, IIa, IIb, and IIc), but none of these strains can be identified by 18S rDNA sequence as any known species of the class (Fig. 1). In two cases, strains with identical 18S sequences were isolated from different sites in Itasca State Park. However, the majority of strains from ISP and Lake Chicot (20 of 26 strains) possess unique 18S rDNA genotypes. Moreover, the 18S clones derived from environmental samples are all distinct from the 18S sequence from any cultured strain. Thus, these new strains and clones add greatly to the breadth of the known diversity of the class.

Morphology

The morphologies of the named taxa of Eustigmatophyceae have been previously investigated. However, the unnamed strains from Itasca State Park and Lake Chicot have received only slight attention. Light microscopy of the vegetative cells does reveal some features that may be indicative of the separate lineages within the Eustigmatophyceae. All strains are unicellular, with coccoid, non-motile vegetative cells (Fig 2). Some strains are commonly found as clumps of cells in batch culture (Fig. 2a, b) whereas most strains are typically not clumped, except perhaps in very old cultures or those that are very loosely associated (Fig. 2d). Pyrenoids are found in the plastids of new strains from Clade Ia (Fišerová 2012) but are often difficult to visualize with light microscopy because of abundant highly refractile granules in the cytosol (Fig. 2a). Pyrenoids have not been observed in new strains from other clades. Sculpted cell walls are an interesting feature of all the strains investigated from Clades IIa (Fig. 2f). This feature has not been observed for any member of the Eustigmatales, and sculpted cells are not found in Clade IIc and some of the organisms in Clade IIb of the *Goniochloridales*. However, it is possible that the sculpting on some cells is too small to be seen by light microscopy.

Discussion

The results of our phylogenetic analyses are unambiguous in supporting two major lineages within a monophyletic Eustigmatophyceae. This result has previously been shown for analyses of partial *rbc*L DNA sequence data (Prior et al. 2009) and 18S rDNA data (Přibyl et al. 2012). However, all earlier analyses lacked the breadth of taxon sampling of our analyses. Our analyses included 18S rDNA sequences representing all known genera of the Eustigmatophyceae, except *Botryochloropsis* Preisig and C. Wilhelm. No culture of *Botryochloropsis* is available. Undescribed strains and 18S clones from environmental samples were also included and add considerable detail to the results of our analysis. Although the taxon sampling for the *rbc*L analysis of Prior et al. (2009) does not include as many taxa as the present analysis, both that analysis and our 18S analysis support the same two major lineages within the Eustigmatophyceae. One of these major lineages includes all the taxa previously assigned to the Eustigmatales, except the genus *Pseudostaurastrum*. A second major lineage is sister to the order Eustigmatales, and thus, in a rank-based taxonomic system, this new lineage would be given ordinal status. However, most of the organisms present in this new lineage are of uncertain taxonomy, with no clear characteristics or boundaries to provide definitions for new species, genera, or families. Many of these strains may already be named as members of the Xanthophyceae. As a result, we are unable to use the ICBN to define a new order within the Eustigmatophyceae. Fortunately, the PhyloCode (version 4c, *Cantino and deQueiroz* 2010*)*, which focuses on well-supported clades rather than ranks, explicitly allows the recognition of lineages that possess unresolved internal clades. Here we use the PhyloCode to formally recognize the new clade *Goniochloridales*.

Goniochloridales, K.P. Fawley, M. Eliáš and M.W. Fawley 2013, new clade name

Definition (branch-modified node-based)

The crown clade originating in the most recent common ancestor of *Goniochloris sculpta*, *Pseudostaurastrum enorme* and all extant strains and species that share a more recent common ancestor with *G. sculpta* than with *Eustigmatos vischeri* D. J. Hibberd, *Nannochloropsis oculata* D. J. Hibberd, and *Pseudoellipsoidion edaphicum*.

Reference phylogeny

Figure 1, this study. Inferred composition: *Goniochloris sculpta*, *Pseudostaurastrum enorme*, *P. limneticum*, and *Trachydiscus minutus* as well as unnamed strains.

Comments

Goniochloridales is chosen as the name for this clade based on the genus *Goniochloris* Geitler, which is one of two genera (the other being *Pseudostaurastrum*) that can be referred to this clade at this time. Although *Trachydisus minutus* is included in this clade, the type of species for this genus, *T. lenticularis* H. Ettl presently remains in the Xanthophyceae. Thus, it would be improper to base a higher taxon on *Trachydiscus*. The *Goniochloridales* clade is intended as a clade above the level of genus; therefore, all preexisting genus names within this clade are retained, subject to further taxonomic treatments.

Members of the *Goniochloridales* clade that have been characterized possess several features in common with the Eustigmatales (Přibyl et al. 2012). The pigments of two taxa have been examined. *T. minutus* and *P. limneticum* both have the suite of photosynthetic pigments typical for the Eustigmatophyceae (Schnepf et al. 1996; Přibyl et al. 2012). These taxa also have plastids without girdle lamellae and without a peripheral ring of DNA. The chloroplast endoplasmic reticulum is not continuous with the nuclear envelope, and storage vesicles have a fine, lamellate appearance (Schnepf et al. 1996; Přibyl et al. 2012). All of these features are characteristic of the Eustigmatophyceae and clearly separate these algae from the morphologically similar Xanthophyceae (Hibberd 1990; Santos 1996). However, the zoospores of both *T. minutus* and *P. limneticum* lack the eyespot (Schnepf et al. 1996; Přibyl et al. 2012) that is characteristic of the Eustigmatales and one of the defining features of the Eustigmatophyceae (Hibberd and Leedale 1971; Hibberd 1990; Santos 1996). However, zoospores without eyespots are also known from *Pseudotetraëdriella kamillae* (Hegewald et al. 2007), which is a member of the Eustigmatales. As discussed previously in Přibyl et al. (2012), the absence of a stigma in the zoospores may be an ancestral feature in the Eustigmatophyceae.

Within *Goniochloridales*, the following subclades are hypothesized: Clade IIa, Clade IIb, Clade IIc, and the genus *Pseudostaurastrum* (Fig. 1). At the present time, the taxon sampling and characterization of the strains that comprise these lineages are insufficient to clearly delimit the boundaries and characteristics of these possible clades. Using the PhyloCode, we could recognize some well-supported clades within *Goniochloridales* as well as new clades in the Eustigmatales. However, we prefer to leave these clades undefined until additional sampling and characterization is complete. Light microscopy of our new strains from this lineage show that members of Clade IIa as well as *Goniochloris* of Clade IIb (Přibyl et al. 2012) have distinctive sculpting on the cell wall. However, other members of *Goniochloridales*, such as *Pseudostaurastrum* (Schnepf et al. 1996), do not have this sculpting. When our strains and additional new eustigmatophyceaen strains are characterized by light and electron microscopy, it is likely that wall sculpting and other morphological features will help delimit the lineages within *Goniochloridales*. The results that we provide here provide a framework for that additional morphological, phylogenetic, and taxonomic work which can now focus on individual lineages within *Goniochloridales* and the Eustigmatales.

The formal recognition of *Goniochloridales* also legitimizes the class Eustigmatophyceae. In a rank-less, clade-based taxonomic system, a single clade cannot carry two names. Before the inclusion of the clade *Goniochloridales*, the clade defined as the Eustigmatophyceae was the same clade as the order Eustigmatales. Indeed, a recent rank-less classification scheme of eukaryotes proposed by a wide international consortium of protistologists (Adl et al. 2005, 2012) does not use the name "Eustigmatophyceae" at all and keeps the taxon Eustigmatales as directly subsumed to the higher order taxon Stramenopiles. With the recognition of *Goniochloridales*, the Eustigmatophyceae now comprises the clades Eustigmatales and *Goniochloridales*.

The results of our analyses also show the close relationship of *P. kamillae* to the genus *Monodopsis* and indicate that *Pseudotetraëdriella* is immersed in the Monodopsidaceae. *P. kamillae* had been placed in the new family, Loboceae, by Hegewald et al. (2007). Our results indicate that this new family cannot be accepted if we retain the family Monodopsidaceae. In addition, the Loboceae is an invalid name by the ICBN, which requires that a family name be based on the genus type. We recommend that *P. kamillae* be recognized as a member of the Monodopsidaceae for the time being. *P. kamillae* has several features that are uncharacteristic of the Monodopsidaceae which prompted the erection of the Loboceae (Hegewald et al. 2007). Future studies may provide more information to better understand the phylogeny and evolution of this interesting lineage.

Several strains labeled *Monodus* by culture collections are resolved with the *Monodopsis* strains included in our analyses. *Monodus* is a genus in the Xanthophyceae with simple coccoid cells that are morphologically very similar to *Monodopsis*. One species of *Monodus*, *Monodus unipapilla* Reisigl, is represented in our phylogenetic analysis by the authentic strain. The affinities of *M. unipapilla* to the type species of *Monodopsis*, *Monodopsis subterranea*, were confirmed by ultrastructural studies, which led to the appearance in the literature of the combination *Monodopsis unipapilla* (Santos and Leedale 1995; Santos 1996). However, the binomial *Monodopsis unipapilla* has not been validly published. Two strains that we investigated here have been assigned by the CCALA collection to the species *Monodus guttula* Pascher, and one more strain was provisionally identified as *Monodus* cf. *guttula*. Although all these strains should be transferred to *Monodopsis*, a taxonomic revision of the species involved, *M. unipapilla* and *M. guttula*, should be postponed until the species concept is better established for

Monodopsis. The strains examined in this study are not well resolved using 18S rDNA sequences, and more variable loci must be examined. The specimens assigned to *M. guttula* should also be critically studied to determine that they were correctly identified in the collections.

The Eustigmataceae comprises the genera *Eustigmatos*, *Vischeria*, and *Pseudostaurastrum* in the treatment of the Eustigmatophyceae by Ott and Oldham-Ott (2003). However, the results of our analyses, as well as a previous analysis of 18S rDNA sequence data (Přibyl et al. 2012), clearly indicate that *Pseudostaurastrum* is not allied with the original Eustigmataceae genera *Eustigmatos* and *Vischeria*. We now place *Pseudostaurastrum* in *Goniochloridales*. As already pointed out by Přibyl et al. (2012), there is no apparent reason to retain two separate genera for *Vischeria* and *Eustigmatos* based on analyses of 18S rDNA sequence data. These two genera also were not resolved separately in the analysis of *rbc*L data (Prior et al. 2009). Strains assigned to two species of the Xanthophyceae genus *Chloridella* (*Chloridella neglecta*, the type species of the genus, and *Chloridella simpex*) were also resolved with *Eustigmatos*/*Vischeria*. As mentioned above for *Monodus*, these specimens need to be examined for proper identification before any formal taxonomic changes concerning the genus *Chloridella* can be made. The single specimen of *Chlorobotrys* Bohlin (Chlorobotrydaceae) in our analysis, the type species, *C. regularis*, is allied with the Eustigmataceae. Taxonomic treatments of these genera, the Eustigmataceae, and the Chlorobotrydaceae are not appropriate until more studies are completed using more variable loci.

Finally, two other lineages are resolved in the Eustigmataceae group. Lineage Ia, which is represented by only unnamed strains, cannot be characterized until these strains are critically examined. The other lineage, the *Pseudellipsoidion* group, is very well supported in the results of our analyses and comprises the named species *P. edaphicum* (the type species for the genus) and *P. ovalis*. The unnamed strains included in the lineage, Mary 8/18 T-3d, Tow 8/18 T-12d, Tow 9/21 P-2w, and WTwin 8/18 T-5d, have also been shown to be monophyletic in an analysis of *rbc*L data (Prior et al. 2009). Additional work also needs to be done with this clade, including a revision of the genus *Pseudocharaciopsis*, which is polyphyletic in our analysis. It is likely that a new family will be erected for this lineage.

Conclusions

Our results clearly demonstrate the level of diversity that is present within the class Eustigmatophyceae. The results of our phylogenetic analyses (Fig. 1) can easily be used to select candidate taxa and strains for research on the genetics of lipid production, for example, or as exemplars for genome structure studies or phylogenetics above the class level. In addition, the informal clades and groups suggested by the results of our phylogenetic analysis will serve as guides for future taxonomic work in the class Eustigmatophyceae.

Acknowledgments This work was supported by National Science Foundation grants DEB1248291, DBI 0070387, and MCB0084188 to K.P.F and M.W.F., University of Arkansas at Monticello Faculty Research Grants to K.P.F., grants from the Arkansas Space Grant Consortium to K.P.F., grants from Arkansas INBRE (funded by the National Center for Research Resources (grant number 5P20RR016460-11) and the National Institute of General Medical Sciences (grant number 8P20GM103429-11) of the National Institutes of Health) to M.W.F., the grants number P506/10/0705 and 13-33039S from the Czech Science Foundation to M.E. and by project number CZ.1.05/2.1.00/03.0 M.100 (Operational Program Research and Development for Innovations) to M.E. We thank Dr. Larry Bellot, Nikon Instruments, for the assistance with microscopy. We thank two anonymous reviewers for the helpful comments.

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