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DNA Sequence Analysis of Freshwater Eustigmatophyceae, a Potential Source of Essential Fatty Acids

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Abstract

Freshwater Eustigmatophyceae are a group of microalgae that are considered rare and of low diversity, with only a few genera and species in a single order. Some Eustigmatophyceae produce fatty acids that are important nutrients for aquaculture, as well as for human food consumption. In addition, some Eustigmatophyceae produce hydrocarbons that may be useful in biofuel production. In our studies of the diversity of coccoid algae from Itasca State Park, Minnesota, we discovered several isolates that we tentatively identified as Eustigmatophyceae. Preliminary molecular characterization indicated that these isolates were highly diverse and probably represented species new to science. In this study, we examined fifteen of the Eustigmatophyceae isolates from Itasca State Park using DNA sequence analysis of the plastid *rbc*L gene. Phylogenetic analyses of these sequences strongly supported Eustigmatophyceae as a monophyletic group and indicated two distinct lineages among our isolates within Eustigmatophyceae. Our results suggest that many of these isolates represent new genera and species. We can also infer the existence of at least two orders in the Eustigmatophyceae, based on the presence of two distinct lineages in the class. In addition to the taxonomic implications, this study will aid in the selection of isolates for further characterization of fatty acids and hydrocarbons, or as part of a regenerative life support system during extended space missions.

Key words. Algal diversity, Eustigmatophyceae, phylogeny, *rbc*L, sequence analysis

Introduction

The class Eustigmatophyceae is one class of a diverse assemblage of algae in the eukaryotic lineage known as the stramenopiles. This major lineage includes over 10,000 described species of diatoms, oomycetes, kelps, small heterotrophic flagellates and other photosynthetic algae. Stramenopiles are named for the strawlike hairs on the flagellar body (stramen=straw; pila=hairs). The vegetative or reproductive cells typically have two differently structured flagella; a long flagellum with tripartite hairs and a short, naked flagellum (Graham et al. 2006).

All known members of the Eustigmatophyceae are small unicellular coccoid algae with yellow-green plastids. This class consists of 5 families, 10 genera and 35 species in a single order (Guiry and Guiry 2009). They can be distinguished from other green coccoid algae by the presence of a red or orange body within the cytoplasm. The name Eustigmatophyceae refers to the large orange-red eyespot (eustigma) that, when produced, is present in the zoospores. Most stramenopile algae possess both chlorophyll *a* and chlorophyll *c* as major photosynthetic pigments. However, members of the Eustigmatophyceae lack chlorophyll *c*. The presence of violaxanthin as the major accessory pigment is also characteristic of the class. These organisms can be found in a diverse range of habitats, which include marine, freshwater, and terrestrial (soil) environments (Graham and Wilcox 2000). Some organisms within this class are known to produce fatty acids, such as eicosapentaenoic acid (Cohen 1994), which have been demonstrated to have important health benefits for humans (Wen and Chen 2003). In addition, some microalgae have been demonstrated to produce lipids and hydrocarbons that may have uses as biofuels (Hu et al. 2008).

In our early studies of the diversity of coccoid algae from Itasca State Park, Minnesota, several isolates were tentatively identified as Eustigmatophyceae by the presence of the red or orange body in the cytoplasm. Preliminary molecular characterization using 18S rDNA indicated a high level of diversity among these isolates (unpublished observation). However, 18S evolves too slowly to resolve species level diversity in the Eustigmatophyceae (Suda et al. 2002). The plastid *rbc*L gene was chosen for this study because this locus is more informative than 18S and it is easier to

Table 1. Algal isolates examined in this study and their sources. All locations are in Itasca State Park, Minnesota.

Isolate	Source location	Sample Date
BogD 9/21 T-2d	BogD, 47 10.63' N, 95 09.93' W, tychoplankton	21 September, 2000
Itas 9/21 S-8w	Lake Itasca, 47 14.05' N, 95 12.10' W, phytoplankton	21 September, 2000
Mary 6/3 T-1w	Mary Lake, 47 11.25' N, 95 10.05' W, tychoplankton	3 June, 2001
Mary 8/18 T-2d	Mary Lake, 47 11.25' N, 95 10.05' W, tychoplankton	18 August, 2001
NDem 6/3 T-6w	North Deming Pond, 47 10.28' N, 95 09.98' W, tychoplankton	3 June, 2001
NDem 6/3 T-9w	North Deming Pond, 47 10.28' N, 95 09.98' W, tychoplankton	3 June, 2001
NDem 9/21 P-10w	North Deming Pond, 47 10.28' N, 95 09.98' W, phytoplankton	21 September, 2000
NDem 9/21 T-17w	North Deming Pond, 47 10.28' N, 95 09.98' W, tychoplankton	21 September, 2000
Pic 8/18 T-13w	Picnic Pond, 47 14.41' N, 95 12.15' W, tychoplankton	18 August, 2001
Pic 9/21 T-1d	Picnic Pond, 47 14.41' N, 95 12.15' W, tychoplankton	21 September, 2000
Tow 2/24 P-6d	Tower Pond, 47 11.41' N, 95 10.84' W, phytoplankton	24 February, 2001
Tow 8/18 T-4w	Tower Pond, 47 11.41' N, 95 10.84' W, tychoplankton	18 August, 2001
Tow 8/18 T-8w	Tower Pond, 47 11.41' N, 95 10.84' W, tychoplankton	18 August, 2001
WTwin 8/18 T-5d	West Twin Lake, 47 10.52' N, 95 09.99' W, tychoplankton	18 August, 2001
WTwin 8/18 T-6d	West Twin Lake, 47 10.52' N, 95 09.99' W, tychoplankton	18 August, 2001

sequence than some other loci. This combination of features makes *rbc*L useful for the examination of both broad diversity and species level relationships.

Materials and Methods

Algal Cultures. Fifteen cultures from the Itasca State Park, Minnesota, Microbial Observatory collection of algae tentatively identified as Eustigmatophyceae were used in this study (Table 1). These cultures were isolated from phytoplankton and tychoplankton samples from lakes, ponds, and bogs. For descriptions of the collections sites and isolation methods, see Fawley et al. (2004).

Light Microscopy. Isolates were examined using a Nikon E-600 microscope (Nikon, Melville, NY, USA) equipped with differential interference contrast optics. Digital images were acquired with a Pixera 150ES digital camera (Pixera Corporation, Los Gatos, CA, USA).

Molecular Characterization. Sample DNA was isolated from liquid WH+ (Fawley et al. 1990) cultures using the isolation procedure outlined in Fawley and Fawley (2004). The *rbc*L plastid DNA was amplified by PCR using one of four following primer combinations: ND*rbc*L2 and ND*rbc*L8 (Daugbjerg and Andersen 1997), eustig*rbc*LR (5′-TTAAGTAATTGG TGCATTTGT-3′) and eustig-*rbc*LF (5′-GATCCRAT TGAAGCTGC-3′), ND*rbc*L2 and eustig*rbc*LR, and eustig*rbc*LF and ND*rbc*L8. Polymerase chain reaction conditions were as given in Fawley and Fawley (2007). Sequencing was performed by the DNA Resource Center at the University of Arkansas, Fayetteville, using the same primers as those used for PCR. The Staden Package (http://www.sanger.ac.uk/Software/production/staden/) was used to process raw sequence data and sequences were aligned with published sequences from GenBank using GeneDoc V.2.6.02 (Nicholas et al. 1997) and MacClade 4.03 (Maddison and Maddison 2000). Phylogenetic analyses were carried out using PAUP* 4.0b10 (Swofford 2002). GenBank accession numbers for all new sequences and published Eustigmatophyceae sequences used in the alignment and phylogenetic analyses are listed in Table 2. Representatives of the Synchromophyceae, Chrysophyceae, Xanthophyceae, Aurearenophyceae and Phaeophyceae (Table 2) were used as outgroups in this study based on their close phylogenetic relationship to the Eustigmatophyceae (Kai et al. 2008). The alignment included 912 characters with 404 total variable characters; 311 characters were parsimony informative. Maximum parsimony analysis employed a heuristic search with the tree bisection and reconstruction branch-swapping method and 10 repetitions of random taxon addition. Neighbor-joining analysis was performed with the HKY85 model used to generate a distance matrix. PAUP* was used to generate a matrix of total character differences. Maximum parsimony and neighbor-joining analyses were bootstrapped with 1000 replicates.

Table 2. Accession numbers for new Eustigmatophyceae sequences and sequence data retrieved from GenBank that were used in the phylogenetic analysis.

	Accession Number	Class
BogD 9/21 T-2d	GQ405004	Eustigmatophyceae
Itas 9/21 S-8w	GQ405009	Eustigmatophyceae
Mary 6/3 T-1w	GQ405005	Eustigmatophyceae
Mary 8/18 T-2d	GQ405011	Eustigmatophyceae
NDem 6/3 T-6w	GQ405012	Eustigmatophyceae
NDem 6/3 T-9w	GQ405013	Eustigmatophyceae
NDem 9/21 P-10w	GQ405016	Eustigmatophyceae
NDem 9/21 T-17w	GQ405018	Xanthophyceae
Pic 8/18 T-13w	GQ405017	Eustigmatophyceae
Pic 9/21 T-1d	GQ405014	Eustigmatophyceae
Tow 2/24 P-6d	GQ405015	Eustigmatophyceae
Tow 8/18 T-4w	GQ405008	Eustigmatophyceae
Tow 8/18 T-8w	GQ405010	Eustigmatophyceae
WTwin 8/18 T-5d	GQ405007	Eustigmatophyceae
WTwin 8/18 T-6d	GQ405006	Eustigmatophyceae
Nannochloropsis limnetica	EU165325	Eustigmatophyceae
N. oculata	AB052286	Eustigmatophyceae
N. granulata	AB052280	Eustigmatophyceae
N. oceanica	AB052283	Eustigmatophyceae
N. maritima	AY680702	Eustigmatophyceae
N. gaditana	AB052735	Eustigmatophyceae
N. salina	AB052287	Eustigmatophyceae
Eustigmatos magnus	AB280615	Eustigmatophyceae
Synchroma grande	DQ788731	Synchromophyceae
Chromulina nebulosa	AF155876	Chrysophyceae
Botrydium stoloniferum	AF064743	Xanthophyceae
Aurearena cruciata	AB365193	Aurearenophyceae
Pilayella littoralis	X55372	Phaeophyceae

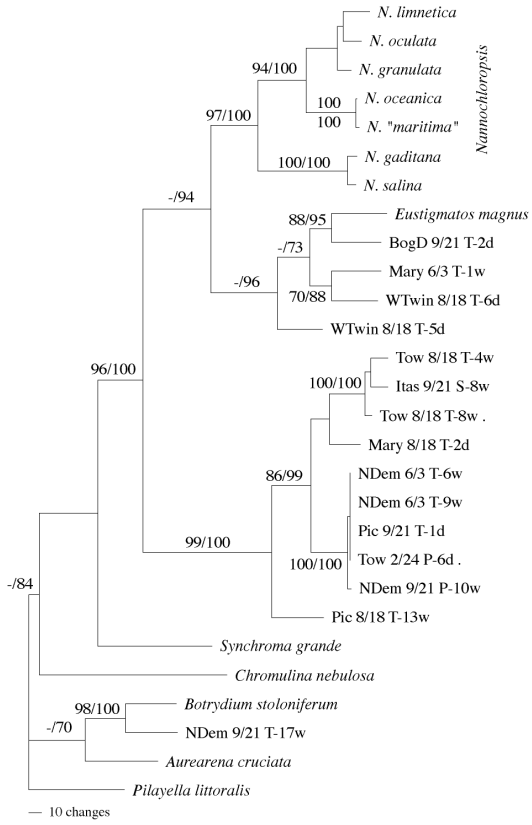

Figure 1. Phylogenetic analysis of *rbc*L sequence data from the Eustigmatophyceae and selected outgroup taxa. The phylogram shows 1 of 4 most parsimonious trees with 1270 steps. Bootstrap percentages (1000 replicates) from Maximum Parsimony analysis are followed by values from the Neighbor-Joining analysis. Only percentages greater than 70 are shown.

Results

Fifteen isolates were used in this study. One of these isolates, NDem 9/21 T-17w, was demonstrated to belong to the Xanthophyceae rather than the Eustigmatophyceae. Sequences from isolates NDem 6/3 T-6w, NDem 6/3 T-9w, Pic 9/21 T-1d, and Tow 2/24 P-6d were found to be identical; another sequence, NDem 9/921 P-10w, was very similar to these four sequences with only five substitutions. The remaining sequences were highly diverse (Table 3).

Phylogenetic analyses support the Eustigmatophyceae as a monophyletic group (Fig. 1). Analyses also show at least two distinct lineages within the Eustigmatophyceae. One lineage is comprised only of our isolates. Our other isolates are allied With *Eustigmatos* and representatives of the genus *Nannochloropsis* in a second lineage, although monophyly of this lineage is only weakly supported. All our isolates are very similar to each other morphologically, with nearly spherical green cells of various sizes (Fig. 2).

Discussion

The *rbc*L sequences of many of our isolates are highly diverse, which indicates that there are probably several new genera and species present among these spherical isolates. For example, the sequence differences among our isolates, except for the nearly identical group NDem 6/3 T-6w, NDem 6/3 T-9w, Pic 9/21 T-1d, Tow 2/24 P-6d and NDem 9/21 P-10w, always exceed 60 substitutions and are often much greater. In contrast, within the fairly species rich genus *Nannochloropsis*, the *rbc*L sequences of many of the

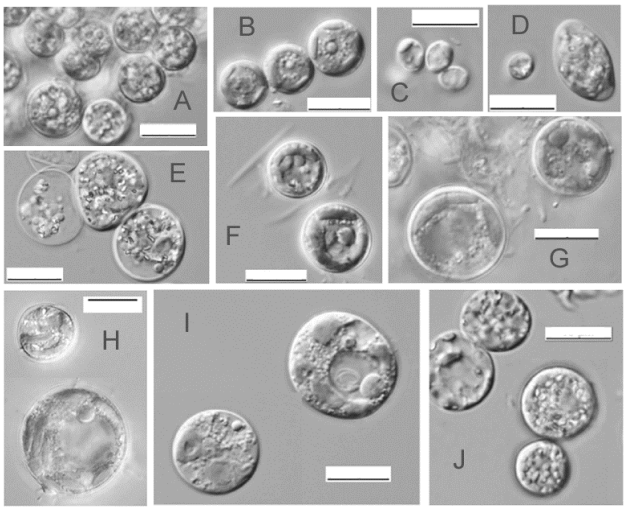

Figure 2. Light micrographs of Eustigmatophyceae isolates; A) BogD 9/21 T-2d, B) Mary 6/3 T-1w, C) WTwin 8/18 T-6d, D) WTwin 8/18 T-5d, E) Mary8/18T-2d; F) Tow8/18T-4w, G) Tow8/18T-8w, H) Itas9/21S-8w, I) NDem6/3 T-9w, J) Pic8/18T-13w. Scale bars represent 10µm.

described species differ by fewer than 60 substitutions. With this knowledge of diversity among these isolates, we will be able to select individual isolates that represent this diversity to screen for the production of useful compounds such as hydrocarbons, lipids, and fatty acids that may have uses in many applications.

Our analysis does not include Eustigmatophyceae other than *Eustigmatos* and *Nannochloropsis* and therefore we may have representatives of other named taxa among our isolates. However, most additional eustigmatophycean taxa that have been named are not spherical (e.g. *Pseudotetraëdriella*, *Pseudocharaciopsis*, *Pseudostaurastrum*), or, if spherical or nearly so, are known from soil rather than phytoplankton or tychoplankton samples (e.g. *Chloridella*, *Ellipsoidion*, *Goniochloris*, *Monodopsis, Vischeria*). These observations support the idea that many of our isolates are new taxa. Future sequencing efforts will include named taxa available from culture collections as well as additional loci that will allow us to describe new taxa from among these isolates.

Phylogenetic analyses of *rbc*L data strongly supported the monophyly of the Eustigmatophyceae. Similar results have been seen using the 18S rDNA sequences from other Eustigmatophyceae (Hegewald et al. 2007). Within the Eustigmatophyceae, two major lineages are present. The magnitude of the sequence variation between the two major lineages (always more than 100 and as many as 148 substitutions) suggests that these lineages may represent different orders within the class. This conclusion is supported by a comparison of *rbc*L sequence variation among different classes of stramenopiles that are closely related to the Eustigmatophyceae. As examples, the sequence of *Aurearea cruciata* (Aurearenophyceae) differs from those of *Botrydium stoloniferum* (Xanthophyceae) and *Synchroma grande* (Synchromophyceae) by 119 and 164 substitutions, respectively, for the studied region of *rbc*L. Thus, the level of sequence variation between the two lineages within the Eustigmatophyceae is similar to that seen in comparisons of different classes of stramenopiles.

This result is significant because, in current literature, Class Eustigmatophyceae contains only one accepted order with five families (Hegewald et al., 2007). However, our results are difficult to put into context with current families, because *rbc*L sequence data are not currently available from representative species. Analysis of sequence data for the 18S rDNA does not suggest two such divergent lineages among the named Eustigmatophyceae that have been examined (Hegewald et al. 2007). However, the genus *Pseudostaurastrum* is highly divergent from other Eustigmatophyceae in 18S analyses. *Pseudostaurastrum* may prove to be a member of our new lineage when the *rbc*L sequences of that genus are analyzed.

Conclusions

The genetic diversity among our collection of simple, spherical Eustigmatophyceae is quite high. The phylogenetic analyses suggest that these isolates represent several new taxa. In addition, our results indicate two possible orders within the Eustigmatophyceae, whereas in the current taxonomy, the class is limited to a single order. Additional studies focused on obtaining *rbc*L from representative species from the accepted families within Eustgmatophyceae, more detailed phylogenetic analyses, and sequence data from other loci are necessary to further clarify the relationships between these isolates and other taxa. Based on results from this study, we will select isolates to examine for the production of interesting fatty acids and hydrocarbons or for their potential use in regenerative systems for extended space missions.

Acknowledgments

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Table 3. Pairwise differences among the partial *rbc*L sequences analyzed in this study.

	Taxon	1	2	3	4	4	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1	*Nannochloropsis limnetica*	-																										
2	*N. oculata*	25	-																									
3	*N. granulata*	19	21	-																								
4	*N. oceanica*	33	26	23	-																							
5	*N. "maritima"*	35	28	25	4	-																						
6	*N. gaditana*	68	66	66	70	74	-																					
7	*N. salina*	65	64	63	66	70	13	-																				
8	*Eustigmatos magna*	120	120	118	122	124	131	130	-																			
9	BogD 9/21 T-2d	111	112	112	117	119	117	119	80	-																		
10	Mary 6/3 T-1w	106	110	111	117	117	131	134	97	95	-																	
11	WTwin 8/18 T-6d	102	102	96	104	106	118	117	97	99	73	-																
12	WTwin 8/18 T-5d	106	104	102	100	102	115	117	98	99	100	91	-															
13	Tow 8/18 T-4w	131	127	127	124	124	133	133	137	143	146	146	131	-														
14	Itas 9/21 S-8w	131	127	128	122	124	134	134	136	140	144	139	136	26	-													
15	Tow 8/18 T-8w	134	130	131	123	123	130	130	137	141	143	141	131	24	24	-												
16	Mary 8/18 T-2d	134	131	130	124	126	140	138	146	145	144	132	130	61	62	57	-											
17	NDem 6/3 T-6w	133	136	132	126	130	144	145	135	146	144	127	132	77	75	72	64	-										
18	NDem 6/3 T-9w	133	136	132	126	130	144	145	135	146	144	127	132	77	75	72	64	0	-									
19	Pic 9/21 T-1d	133	136	132	126	130	144	145	135	146	144	127	132	77	75	72	64	0	0	-								
20	Tow 2/24 P-6d	132	135	131	125	129	143	144	134	145	144	127	131	76	74	71	64	0	0	0	-							
21	NDem 9/21 P-10w	135	136	134	128	132	142	142	134	145	144	127	131	78	76	73	67	5	5	5	5	-						
22	Pic 8/18 T-13w	129	129	127	131	133	136	138	147	148	144	137	133	93	88	89	91	99	99	99	98	99	-					
23	*Synchroma grande*	155	150	158	157	159	156	155	158	170	159	152	154	177	171	171	165	170	170	170	170	171	177	-				
24	*Chromulina nebulosa*	220	214	216	210	212	206	207	212	230	223	209	208	212	209	208	215	211	211	211	211	214	214	178	-			
25	*Botrydium stoloniferum*	168	166	168	171	171	171	171	169	168	164	168	152	169	175	165	165	174	174	174	173	175	163	167	213	-		
26	NDem 9/21 T-17w	172	166	171	171	173	165	165	171	174	166	169	164	155	159	156	166	178	178	178	177	179	154	167	205	77	-	
27	*Aurearena cruciata*	156	151	156	158	158	168	168	175	181	162	157	157	173	167	167	169	168	168	168	167	170	165	164	201	119	118	-
28	*Pilayella littoralis*	186	178	185	184	186	184	182	195	205	184	179	190	197	191	195	199	193	193	193	192	198	187	177	207	158	142	155

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