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SEQUENCE ANALYSIS CONFIRMS A NEW ALGAL CLASS

L. K. MEDLIN¹, Y. DESDEVISES²

¹ Marine Biological Association of the UK, The Citadel, Plymouth PL1 2PB, UK

² Sorbonne Université, CNRS, UMR 7232, Biologie Intégrative des Organismes Marins, BIOM, Observatoire Océanologique, 66650 Banyuls/Mer, France

MICROALGAL CLASS
CHLOROMORUM
PHYLOGENETICS

ABSTRACT. – As judged by its position in an SSU rRNA tree, a new algal class in the heterokonts has been recovered from a Bayesian phylogenetic analysis of 12 heterokont algal classes. The sequences were deposited in Genbank as raphidophytes, possibly because cells of these species resemble raphidophytes. A constrained tree placing them in the raphidophytes yielded a significantly worse tree as determined by the Shimodaira-Hasegawa test ($P = 0.0001$). New efforts should be made to describe this class formally because cultures do exist for these microalgae.

INTRODUCTION

In an earlier search for the appropriate number and type of outgroups to recover monophyletic classes in the diatoms (Medlin 2014), there were a group of sequences in the Silva database (SSUREF_96 and SSUREF_199_128) that did not fall within the Raphidophyceae, although they were labeled in Genbank as being new species of Raphidophyceae (Suppl Table I). A revised taxon sampling of the heterokonts with a reduced number of diatoms for the 18S SSU rRNA gene showed that this group of sequences consistently fell into a well supported clade separated from the raphidophytes and thus would appear to be a new microalgal class in the pigmented heterokonts.

MATERIALS AND METHODS

rRNA sequences from the *Chloromorom* spp. in Suppl Table I were uploaded from Genbank and aligned to the SILVA SSU rRNA sequence alignment in the ARB program Version 5.5 using maximum primary and secondary structural similarity (Ludwig *et al.* 2004). No filter was applied to the alignment and full length SSU sequences (2029 bases) from 59 taxa were exported for further analysis. Phylogenetic analyses were performed on the SSU alignment with RAxML (Randomized Axelerated Maximum Likelihood, Stamatakis *et al.* 2014) and a GTR + G model from the CIPRES online analysis portal (<https://www.phylo.org/>, Miller *et al.* 2010). Tree robustness was assessed with a bootstrap procedure with 1000 replicates. This tree was compared with a constrained phylogeny built with all putative raphidophytes forced as a monophyletic group. The comparison was performed with a SH-test (Shimodaira & Hasegawa 1999) using PAUP* (Swofford 2003) with the *FulIOpt* setting and 1000 bootstrap replicates in the testing procedure. The phylogenetic tree was also reconstructed by Bayesian inference with MrBayes 3.2.6 (Ronquist *et al.* 2012) using 4 chains of $2 \cdot 10^6$ generations, trees sampled every 1000 genera-

tions, and burnin value set to 20 % of the sampled trees (400). We checked that standard deviation of the split frequencies fell below 0.01 to ensure convergence in tree search.

RESULTS AND DISCUSSION

The *Chloromorom* sequences fell into a well-supported clade (bootstrap support in % (bt) = 100, posterior probabilities pp = 1) that was sister with no support to a moderately supported larger clade (bt = 61) containing the Rhaphidophyceae, Chrysomerophyceae, Xanthophyceae, Phaeophyceae (Figs 1, 2), basically the SI clade of the pigmented heterokonts of Yang *et al.* (2012). This “SI” clade was collapsed into a polytomy with a posterior probability of 1 with the BI analysis (Fig. 2) and all other sister relationships were the same as the RAxML tree. Shimodaira-Hasegawa tests using a constrained tree (Fig. 3) to place these new sequences inside the raphidophytes yielded a significantly worse tree ($P = 0.0001$). Based on these preliminary analyses, it is concluded that this group of sequences is not raphidophytes but belongs to a new class of microalgae with at least three species based on SSU analysis. There are twelve strains in culture at the MarbioNC Living Algal Resource Collection (Larc) at the University of North Carolina, Wilmington, which should be examined in more detail and with other genes sequenced to ascertain conclusively that they represent a new algae class with their own set of distinct morphological characters separating them from the other pigmented heterokont microalgae and how many new species are present.

This is not the first time that a new algal class has been identified first with sequence data and to be later defined taxonomically with morphological/physiological features from cultured cells. In 1995, Cavalier-Smith *et al.* revised the Ochristan (pigmented heterokont) algae and defined several new classes based on sequence analysis alone, such as the Chrysomerophyceae. Before that, the Pelago-

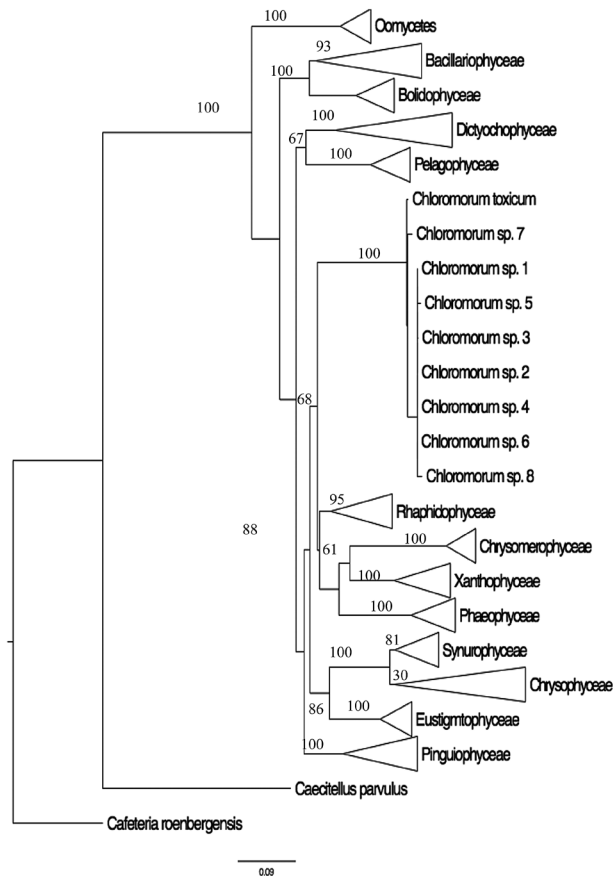


Fig. 1. – Phylogenetic analysis of the *Chloromorom* sequences using a RAxML analysis of selected members of each algal class in the Heterokonta to place them in a phylogenetic context. Bootstrap support values > 50 % are placed at each node.

phyceae were described from sequence data and little supportive morphological data (Andersen *et al.* 1993, Saunders *et al.* 1997), and after that several other microalgal classes were described, all with sequence data providing the strongest and usually the initial support for the new class: Bolidophyceae (Guillou *et al.* 1999), Pinguicophyceae (Kawachi *et al.* 2002), and Phaeothamniophyceae (Bailey *et al.* 1998). The picobiliphytes were recognized first as a new class with molecular data (Not *et al.* 2007) and it was several years and with much effort before cultures could be established to show conclusively that this was not a new algal class as originally predicted but a new heterotrophic protist phylum (Seenivasan *et al.* 2013).

Nor is it the first time that algal species first believed to be raphidophytes have been shown with molecular data to be more closely related to other groups (Bowers *et al.* 2006). In the Bowers *et al.* study, *Chattonella verreculosa* was found to be a dictyochophyte and later moved into a new genus *Pseudochattonella* (Edvardsen *et al.* 2007, Hosoi-Tanabe *et al.* 2007).

The sterols of fatty acids of *Chloromorom toxicum* have already been examined (Giner *et al.* 2008) referring to its original identification as a species of *Chattonella*, a valid member of the Raphidophyceae and compared to

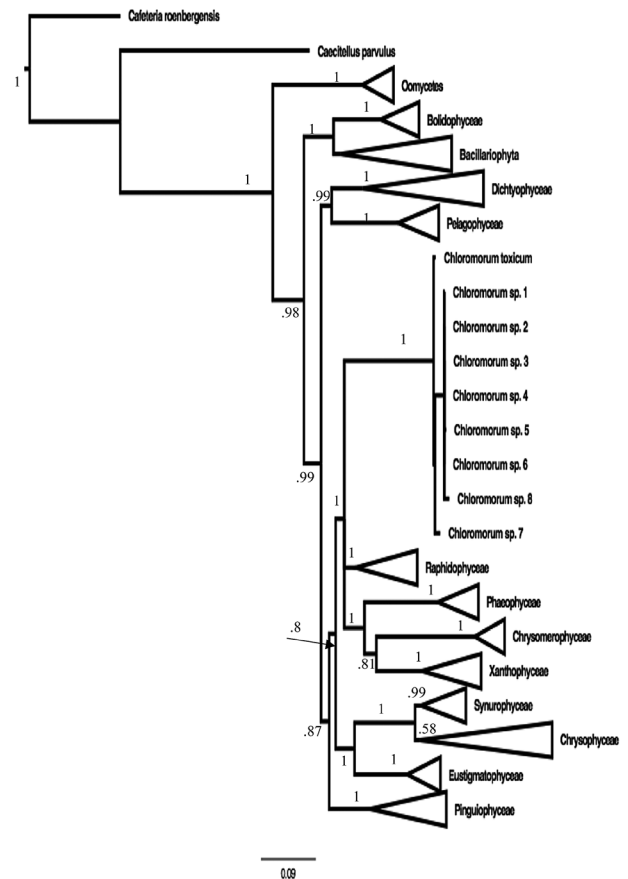


Fig. 2. – Phylogenetic analysis of the *Chloromorom* sequences using a Bayesian analysis of selected members of each algal class in the Heterokonta to place them in a phylogenetic context.

two other microalgae that had originally been described as *Chattonella* spp. Its sterols were significantly different from the other two algae and from those found in another study by Marshall *et al.* (2002) who suggested that sterols and fatty acids were conserved within a genus and thus more informative phylogenetically than pigments, which have been traditionally used to define groups of raphidophytes.

Yang *et al.* (2012) studied the evolution of the pigmented heterokonts and recovered basically three major clades in their analyses, which could primarily be defined by carotenoid pigments. Taxa in the SIII clade have only the diatoxanthin-diadinoxanthin cycle (D-D cycle) carotenoid cycle, as do some taxa in the SI clade, whereas the SII clade is defined by taxa with the violaxanthin-antheraxanthin cycle (V-A) cycle). Based on our phylogenetic analysis, it could be hypothesized that this new algal class contains diatoxanthin-diadinoxanthin cycle (D-D cycle), something that could easily be tested to support their placement in this major clade of the pigmented heterokonts.

The one sequence that has a name, *Chloromorom toxicum* Tomas, has been used without being validly published (Giner *et al.* 2008), having no Latin description or

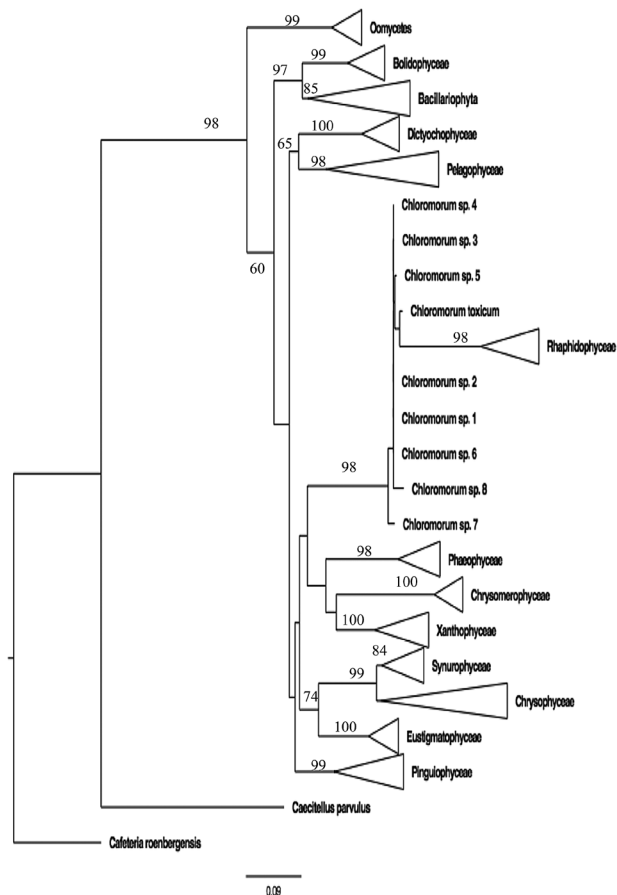


Fig. 3. – Phylogenetic analysis forcing the *Chloromorom* sequences into the Rhabdophyta for testing the validity of these sequences as members of that microalgal class. Bootstrap support values > 50 % are placed at each node.

designation of type material, and thus is a *nomen nudum*, which would make also a class name of Chloromorophyceae also illegal. Thus this species will have to be renamed and another class name linked to the new genus name for a valid description of all taxa.

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Suppl Table I. – Summary of taxa used in this analysis.

Accession Number	Taxon
Bicoecea unpigmented heterokonts	
AF174364	<i>Cafeteria roenbergensis</i> Fench., Pat.
AY642126	<i>Caecitellus parvulus</i> (Greiss) Pat, Nyg. Steinb, Turl.
Oomycetes water molds	
M32705	<i>Achlya bisexualis</i> Cok., Couch
X54266	<i>Lagenidium giganteum</i> Couch
New Algal Class	
EU038275	<i>Chloromorom toxicum</i> Tomas
EU038278	<i>Chloromorom</i> sp. 1
EU038280	<i>Chloromorom</i> sp. 2
EU038274	<i>Chloromorom</i> sp. 3
EU038277	<i>Chloromorom</i> sp. 4
EU038279	<i>Chloromorom</i> sp. 5
EU038276	<i>Chloromorom</i> sp. 6
AY788946	<i>Chloromorom</i> sp. 7
EU038273	<i>Chloromorom</i> sp. 8
Bacillariophyceae	
M87326	<i>Cylindrotheca closterium</i> (Ehr.) Reim., Lewin
M87329	<i>Rhizosolenia setigera</i> Brightw.
M87334	<i>Tryblionella apiculata</i> Greg.
Bolidophyceae	
AF123596	<i>Triparma mediterranea</i> (Guil., Chret.-Din.) Ich., Santos
AF123595	<i>Triparma pacifica</i> (Guil., Chret.-Din.) Ich., Santos
Chrysophyceae	
AF123282	<i>Chromophyton rosanoffii</i> Woronin
AF123291	<i>Dinobryon sociale</i> var. <i>americanum</i> (Brun.) Bach.
AF123293	<i>Ochromonas tuberculata</i> Hibb.
Chrysomerophyceae	
AJ295822	<i>Antarctosaccion applanatum</i> (Gain) Del.
U78034	<i>Giraudyopsis stellifera</i> Dang.
Dictyochophyceae	
U14384	<i>Apedinella radians</i> (Lohm.) Campb.
U14385	<i>Dictyocha speculum</i> Ehr.
AY254857	<i>Florenciella parvula</i> Eik.
U14387	<i>Pseudopedinella elastica</i> Skuj.
Eustigmatophyceae	
U41051	<i>Eustigmatos magus</i> (Pet.) Hibb.
U41054	<i>Monodus subterranea</i> (Pet.) Hibb.

Suppl Table I. – Continued.

Accession Number	Taxon
U41092	<i>Nannochloropsis granulata</i> Karl, Pot.
U41052	<i>Pseudocharaciopsis minuta</i> (Braun) Hibb.
AF045051	<i>Vischeria helvetica</i> (Vis., Pas.) Hibb.
Pelagophyceae	
U40257	<i>Aureococcus anophagefferens</i> Harg., Sieb.
U14386	<i>Pelagococcus subviridis</i> Norr.
U78033	<i>Sarcinochrysis marina</i> Geit.
Pinguiophyceae	
AF123284	<i>Chrysochaete britannica</i> (God.) Ros.
AF438325	<i>Glossomastix chrysoplasta</i> O'Kel.
AF438324	<i>Pinguicoccus pyrenoidosus</i> And, Pot, J. Bail.
AF438322	<i>Polypodochrysis teissieri</i> Magne
Phaeophyceae	
X53229	<i>Costaria costata</i> (C. Ag.) D. Saunders
L43062	<i>Ectocarpus siliculosus</i> (Dill.) Lyng.
AB011423	<i>Fucus distichus</i> L.
L43066	<i>Scytosiphon lomentaria</i> (Lyng.) Link
Rhaphidophyceae	
AY788922	<i>Chattonella antiqua</i> (Hada) Ono
AY788944	<i>Chattonella subsalsa</i> Bieche.
U41649	<i>Chattonella subsalsa</i>
AY788931	<i>Fibrocapsa japonica</i> Tor., Tak.
U41650	<i>Heterosigma akashiwo</i> (Hada) Hada
AB217629	<i>Pseudochattonella verruculosa</i> (Har., Chi.) Tan, Hon, Fuk, In., Sako
U41651	<i>Vacuolaria virescens</i> Cienk.
Synurophyceae	
U73228	<i>Mallomonas caudata</i> (Iw.) Iv.
U73220	<i>Synura mammillosa</i> Tak.
U73219	<i>Tessellaria volvocina</i> (Play.) Play.
Xanthophyceae	
AF083398	<i>Bumilleriopsis filiformis</i> Vis.
U43277	<i>Heterothrix debilis</i> Vis.
AF083399	<i>Heterococcus caespitosus</i> Vis.
AF083400	<i>Mischococcus sphaerocephalus</i> Vis.
U73219	<i>Tessellaria volvocina</i> (Play.) Play.
M55286	<i>Tribonema aequale</i> Pas.
AF083397	<i>Tribonema intermixtum</i> Geit.