Systematics of a Kleptoplastidal Dinoflagellate, 
Gymnodinium eucyaneum Hu (Dinophyceae), and Its Cryptomonad Endosymbiont

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Abstract

New specimens of the kleptoplastidal dinoflagellate Gymnodinium eucyaneum Hu were collected in China. We investigated the systematics of the dinoflagellate and the origin of its endosymbiont based on light morphology and phylogenetic analyses using multiple DNA sequences. Cells were dorsoventrally flattened with a sharply acute hypocone and a hemispherical epicone. The confusion between G. eucyaneum and G. acidotum Nygaard still needs to be resolved. We found that the hypocone was conspicuously larger than the epicone in most G. eucyaneum cells, which differed from G. acidotum, but there were a few cells whose hypocone and epicone were of nearly the same size. In addition, there was only one site difference in the partial nuclear LSU rDNA sequences of a sample from Japan given the name G. acidotum and G. eucyaneum in the present study, which suggest that G. eucyaneum may be a synonym of G. acidotum. Spectroscopic analyses and phylogenetic analyses based on nucleomorph SSU rDNA sequences and chloroplast 23 s rDNA sequences suggested that the endosymbiont of G. eucyaneum was derived from Chromonas (Cryptophyta), and that it was most closely related to C. coerulea Skuja. Moreover, the newly reported kleptoplastidal dinoflagellates G. myriopyrenoides and G. eucyaneum in our study were very similar, and the taxonomy of kleptoplastidal dinoflagellates was discussed.

Introduction

Dinoflagellates are a diverse group of single-celled eukaryotic algae that occur in marine and freshwater all over the world [1]. Some have acquired chloroplasts via endosymbiosis [2]. This phenomenon provides insights into the Seral Endosymbiosis Theory that some algal groups arose via the ingestion and retention of photosynthetic, eukaryotic organisms and the subsequent reduction of their nonphotosynthetic organelles [3,4,5]. The origins and structures of endosymbionts are highly diverse. Karenia Hansen & Moestrup, Karlodinium Larsen, and Takayama de Salas have chloroplasts that originated from haptophyte algae, which are surrounded by three membranes but no other organelles remain from the endosymbiont [6,7]. Dinovibrio Carty & Cox, Kryptoperidinium Lindemann, and Peridinium Ehrenberg contain chloroplasts derived from diatoms [8,9]. The nucleus and mitochondria of the diatom remain Dinophyceae in the host cell where they are surrounded by a single membrane [10,11]. The chloroplasts of Dinophys Ehrenberg originated from a cryptophyte, probably Telonema Hill [12,13,14,15], and they are surrounded by two membranes [16].

The retention time of plastids in dinoflagellates also varies greatly depending on the species involved and the conditions under which they are grown [17]. Some endosymbionts are permanent, whereas others are engulfed and temporarily retained in a functional state for a few weeks. The temporary retention of engulfed chloroplasts is known as “kleptoplastidy” and the endosymbionts (chloroplasts) are referred to as “kleptochloroplasts” [18]. The relationship between the endosymbiont and the host remains obscure. In a recent study of Dinophys acuminata Claparède & Lachmann, it was observed that the kleptoplastids were serviced by nucleus-encoded proteins and horizontal gene transfer from the endosymbiont to the host nucleus was detected [19]. Therefore, studies of kleptoplastidy are very interesting and important for increasing our understanding of endosymbiosis and the evolution of algae.

A relatively small group of dinoflagellates have been described as having a blue-green coloration and researchers are keen to understand the source of their coloration [20]. Studies of Gymnodinium acutatum Nygaard, G. aeruginosum Stein, and G. myriopyrenoides Yamaguchi, Nakayama, Kai et Inouye had indicated that a cryptophycean endosymbiont was housed temporarily within the dinophycean cell, which was the source of the blue-green chloroplasts [20,21,22,23,24]. The discovery of nonphotosynthetic organelles in the endosymbionts in dinophycean cells suggested that these are examples of an early stage in the evolutionary process [23]. Thus, systematic studies of this group may be of great evolutionary interest. However, most previous studies are based on pigmentation and morphological observation,
whereas the phylogenetic relationships among the blue-green group of dinoflagellates and their endosymbionts remain uncertain.

The blue-green freshwater dinoflagellate *Gymnodinium eucyaneum* Hu (Hu et al. 1980, as *G. cyaneum*; Hu 1983) was originally described from China as processing phycobilin like cryptomonads, suggesting that it probably contained a cryptophycean endosymbiont [25,26]. At present, it has only been reported in China. In this study, we collected new specimens of *G. eucyaneum* from China and their cell morphology was observed by light microscopy, while the systematics of the dinoflagellates were investigated via phylogenetic analyses based on partial nuclear LSU rDNA sequences. To identify the origin of the endosymbiont, the nucleomorph SSU rDNA and chloroplast 23S rDNA sequences were determined, and the absorption spectrum of the phycocyanin was measured. The sequences of some cryptomonads were also determined for reference.

**Results**

**Description**

*Gymnodinium eucyaneum* (Hu, Yu et Zhang) Hu 1983, Hu, p.198–199; *Gymnodinium cyaneum* Hu, Yu et Zhang 1980, Hu et al., p. 651–653. Non *Gymnodinium cyaneum* Schiller 1955.

Unicellular, freshwater unarmored dinoflagellate. Cells were obviously dorsoventrally flattened, measuring 29–48 µm in length, 16–22 µm in width, and 12–17 µm in thickness. In most cases, the hypocone was conspicuously larger than the epicone (Figs. 1A–D). The epicone was hemispherical and its length was approximately one-third of the total cell length (Figs. 1A–D). The hypocone was sharply acute (Figs. 1A–E). In a few cells, the hypocone and epicone were more or less the same size (Fig. 1E). The cingulum was wide, deeply excavated, and encircled the middle-upper part of the cell (Figs. 1G, H, J, K). There was no displacement of the cingulum and its ventral ends were at the same level, where both curved posteriorly at the junction with the sulcus (Figs. 1G, H, J, K). The sulcus was wide, expanding into the posterior part (Figs. 1G, H, J, K). Two flagella were inserted on the ventral side of the cingulum (Fig. 1G).

A large spherical nucleus was situated in the anterior part of cell (Figs. 1B–E, G, H). Hundreds of granules of variable size were observed beneath the plasmalemma (Fig. 1). Numerous blue-green chloroplasts were located peripherally in the cell (Figs. 1A–H). Determining the actual number was difficult because they were very dense. The chloroplasts gradually became smaller when cells were retained in the lab (Fig. 1F). Colorless cysts with a brownish accumulation of corpuscle formed after 2–4 weeks culture in filtered local water (Fig. 1).

The voucher specimens examined were: HBI 3586× from Lake Donghu in Wuhan City, Hubei Province, collected by SX on April 18, 2012; HBI 3597× from a fishpond in Wuhan City, Hubei Province, collected by SX on May 3, 2012. The specimens are deposited in the Freshwater Algal Herbarium (HBI), Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei, China.

**Spectroscopy**

The absorption spectrum of the phycocyanin extracted from *G. eucyaneum* in this study is shown in Fig. 2. Two absorption peaks were observed at 641 nm and 585 nm. The peak at 641 nm was slightly higher than the peak at 585 nm.

**Figure 1.** Micrographs of *Gymnodinium eucyaneum*. Figs. A–E. Different cell shapes of the field samples. F Cells kept for 2–4 weeks in the laboratory, showing that the chloroplast became smaller. G Ventral view showing the insertion of the flagella. H, J, K Ventral view showing the detail of the cingulum and sulcus. I Cysts each with a brownish accumulation of corpuscles. E: epicone; H: hypocone; N: nucleus; C: chloroplasts; Cl: cingulum; S: sulcus; AC: accumulation of corpuscle. Scale bars: A–I = 10 µm; J–K = 2 µm. doi:10.1371/journal.pone.0053820.g001

**Figure 2.** Absorption spectrum of phycocyanin extracted from *Gymnodinium eucyaneum* samples and a *Chroomonas* sp. strain. doi:10.1371/journal.pone.0053820.g002

**Phylogenetic Analyses**

**Host phylogeny based on the nuclear LSU rDNA.** The LSU rDNA sequences aligned in this study contained 572 nucleotides for 38 taxa of dinoflagellates and their putative relatives. Of these nucleotides, 389 sites (68.0%) were variable and 309 sites (54.0%) were parsimoniously informative. The base frequencies were found to be homogeneous across taxa. The overall average pairwise distance was 0.253. The phylogenetic trees constructed by the ML and Bayesian analyses produced similar topologies to their composition, although only the Bayesian trees are presented. In the phylogenetic tree, the members of the genus *Gymnodinium* sensu stricto formed a well supported clade (0.96/76 for BA/ML) (Fig. 3). *G. eucyaneum* was...
present in this clade and it formed a robust subclade (1.00/100 for BA/ML) with *G. acidotum*. The interspecific pairwise divergence between them was 0.002 and there was only one site difference. Both clustered with *G. palustre*, *G. myripyrenoidosum*, and *Amphidinium poecilocraum* with high support (1.00/100 for BA/ML). The interspecific pairwise divergence between *G. eucyaneum* and *G. myripyrenoidosum* was 0.108. This group did not show strong affinity to any others.

**Endosymbiont phylogeny based on the nucleomorph SSU rDNA and chloroplast 23S rDNA**

The nucleomorph SSU rDNA sequences aligned in this study contained 1872 nucleotides for 32 taxa of dinoflagellates, cryptomonads, and their putative relatives. Of these nucleotides, 741 sites (39.6%) were variable and 477 sites (25.5%) were parsimoniously informative. The base frequencies were found to be homogeneous across taxa. The overall average pairwise distance was 0.077. Three main clades were distinguished in the
phylogenetic tree, which represented the blue-green, brownish-green, and red cryptomonad species (Fig. 4). The sequence of *G. eucyaneum* was positioned in the clade containing *Chroomonas* sp., *C. mesostigmatica*, *C. placidea*, *C. pauciplastida*, and *C. coerulea* with high support (1.00/86 for BA/ML). The sequence of *G. eucyaneum* was most closely related to two *C. coerulea* sequences. One was from *C. coerulea* strain UTEX 2780 and the other was from *C. coerulea* collected from the lake where *G. eucyaneum* was collected in the present study. Another member of *Chroomonas*, *C. pochmannii*, was distantly related to this group. The *Komma* and *Hemiselmis* species, which are also blue-green in color, formed two robust groups (1.00/100 for BA/ML and 1.00/100 for BA/ML) with relatively distant relationships to *G. eucyaneum*.

The chloroplast 23S rDNA sequences aligned in this study contained 976 nucleotides for 28 taxa of cryptomonads, dinoflagellates, diatoms, and other algae. Of these nucleotides, 292 sites (29.9%) were variable and 523 sites (22.2%) were parsimoniously informative. The base frequencies were found to be homogeneous across taxa. The overall average pairwise distance was 0.090. The algae from different phyla were well separated in the reconstructed phylogenetic tree (Fig. 5). The sequence of *G. eucyaneum* was positioned in the cryptomonad clade with high support (1.00/100 for BA/ML). The sequences of *G. eucyaneum* and *C. coerulea* from the same lake formed a robust lineage (1.00/98 for BA/ML) and the pairwise distance between them was 0.001. Several diatoms and dinoflagellates that contained endosymbiont derived from diatoms formed a well-supported clade (1.00/100 for BA/ML).

Discussion

Previous studies of *Gymnodinium eucyaneum* in China

Several studies have investigated *G. eucyaneum* in China since 1980 [25–29]. However, they are not widely known because they were written in Chinese. Evidence for the presence of phycobilin [25] and the ultrastructure of the chloroplasts [29] suggested that the chloroplasts of *G. eucyaneum* were derived from cryptophytes. Observations of the nucleus and nuclear substance [27] showed that one dinokaryon was present in all cells and the numbers of second eukaryotic nuclei ranged from 0–4 (rarely 7–10). This data may suggest that the second eukaryotic nuclei were temporary and that the chloroplasts of *G. eucyaneum* were “stolen” and could be lost. Similar report on the number of nuclei was made by Field and Rhodes [22].

*Gymnodinium eucyaneum* and *G. acidotum*

Since it was first described in China, *G. eucyaneum* has often been confused with another blue-green unarmored dinoflagellate, *G. acidotum*. Traditionally, unarmored dinoflagellates have been classified based mainly on the relative sizes of the epicone and hypocone [30]. According to their original descriptions, the epicone and the hypocone were nearly equal in *G. acidotum* [31] (Fig. 6A), whereas the hypocone was conspicuously larger than the epicone in *G. eucyaneum* [26,27] (Figs. 6B, 6C). The hypocone was 1.3–1.8 times as long as the epicone in *G. eucyaneum* according to our observations. Schnepf et al. (1989) suspected that the organism studied by Wedemayer (1984) under the name “*Gymnodinium*
acidotum” was identical to *G. aeruginosum* [24] (Figs. 6D–E). After examining the relative sizes of the epicone and hypocone in the images provided in previous studies, we considered that the organisms studied by Farmer and Roberts (1990) [21], Fields and Rhodes (1991) [22], and Barsanti et al. (2009) [32] (Figs. 6F, G, H, I) under the name “*G. acidotum*” were different from the lectotype and *G. acidotum* in other studies (Figs. 2A, J, K), and they may be identical with the *G. eucyaneum* analyzed in our study. However, recent studies indicate that the classification based on the relative sizes of the epicone and hypocone does not reflect their phylogenetic relationships [7,33,34,35]. In a recent study by Yamaguchi et al. (2011) [23], a partial nuclear LSU rDNA sequence of *G. acidotum* were included. There was only one site difference in the nuclear LSU rDNA sequence of *G. acidotum* in that study and *G. eucyaneum* in the present study. An image of *G. acidotum* was not provided in that paper, but the authors gave us the usage of a photo of *G. acidotum* collected from the same sample on their web site. (Fig. 6K) In the photo, the hypocone and the

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**Figure 5. Bayesian phylogenetic tree constructed from the chloroplast 23S rDNA sequences.** The numbers on the nodes represent the posterior probabilities (PP)/bootstrap support values (BP) produce by the Bayesian inference and maximum-likelihood analyses. Values >0.50 for PP and >50 for BP are shown. The sequences obtained in our study are shaded in gray.

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Gymnodinium eucyaneum and other kleptoplastidal dinoflagellates

Recently, a new unarmored blue-green kleptoplastidal dinoflagellate, Gymnodinium myriopyrenoides Yamaguchi, Nakayama, Kai et Inouye was reported from Isonoura Beach in Japan [23]. Although the habitat of G. myriopyrenoides was marine and sand-dwelling, while G. eucyaneum was a freshwater species, we thought G. myriopyrenoides and G. eucyaneum were quite similar: in our phylogenetic analyses, the two were very close to each other; as to morphological characters, both of the two were dorsoventrally flattened and elongate-elliptical in ventral view; their epicone was conspicuously smaller than the hypocone; and they both had a wide, deeply incised cingulum with no displacement; both their endosymbionts came from blue-green cryptomonads. The symbiont of G. myriopyrenoides was found to be derived from Chromononas or Hemiselmis via phylogenetic analyses based on plastid-encoded SSU rDNA, but could not be identified at species level because plastid-encoded SSU rDNA sequences of Chromononas and Hemiselmis were insufficient. In the present research, the symbiont of G. eucyaneum was confirmed to be derived from Chromononas via phylogenetic analyses based on nucleomorph SSU rDNA and spectrophotometric pigment analyses.

G. myriopyrenoides and G. eucyaneum, together with some other species of Gymnodinium and Amphidinium who also harbored blue-green kleptochloroplasts, such as G. aeruginosum, A. poecilochroum, A. latum and A. segense, formed a relatively distinct group in Dinophyceae in view of their kleptoplastidal behavior, morphological characters and close relationships in phylogenetic analyses. As mentioned above, recent ultrastructural and molecular phylogenetic studies revealed that the traditional taxonomy of unarmored dinoflagellates based mainly on the relative sizes of the epicone and hypocone was problematic. As revealed in ultrastructural and molecular phylogenetic studies, the genus Gymnodinium sensu Hansen er Moestrup and Amphidinium were polyphylectic [33,34,35,36]. Yamaguchi et proposed to establish a new genus for these kleptoplastidal dinoflagellates based on morphological and molecular characters, and we considered this proposal was more reasonable than the traditional taxonomy.

Identification of the endosymbiont

Previous studies based on spectrophotometric pigment analyses suggested that the phycocyanins in G. eucyaneum resembled PC 645 and that G. eucyaneum may contain a blue-green cryptomonad endosymbiont [25], although the origin of the endosymbiont remained uncertain. Three cryptomonad genera contain blue-green endosymbionts [37], i.e., Hemiselmis, Komma, and Chromononas, and Komma and Chromononas both contain PC 645 [38]. The absorption spectrum of the phycocyanin extracted from G. eucyaneum in this study was almost the same as that extracted from G. eucyaneum in a previous study [25], which matched the PC 645 extracted from Chromononas sp. strain CCMP 1221 [38]. In the phylogenetic analyses based on the nucleomorph SSU rDNA and chloroplast 23S rDNA in this investigation, the sequences of the endosymbiont were firmly included in the Chromononas clade and they had relatively long distances from Komma and Hemiselmis.

Thus, we suggest that the endosymbiont originated from Chromononas. Furthermore, the sequence of the endosymbiont indicated that it was most closely related to two C. corulea sequences. It is quite remarkable that C. corulea and G. eucyaneum were collected from the same lake in the same month, in the present study. Thus, we suspect that the endosymbiont of G. eucyaneum detected in this study originated from C. corulea. However, the species level identification could be problematic because the taxon sampling and taxonomic studies of Chromononas were inadequate. In addition, DNA changes may have occurred after the endosymbiont was engulfed by the host, which may make the species level identification more complex.

In our study, G. eucyaneum survived for 2–4 weeks without feeding, so the chloroplast was likely to be the nutritional source for the dinoflagellate host, which agreed with previous observations of starch grain accumulation in some kleptoplastidal dinoflagellates [39,40]. The chloroplasts gradually became smaller and fewer, and host cell division was rarely seen, while attempts to establish clonal cultures failed. In a previous Chinese study, it was reported that the location and number of the nuclear substances (probably cryptomonad nuclei and nucleomorphs) varied greatly among the G. eucyaneum cells in the same samples, because the nuclear substances were randomly distributed to two daughter cells when host cell division occurred [27]. These studies suggest that the endosymbiont of G. eucyaneum is neither a food nor a permanent endosymbiont and that G. eucyaneum is a kleptoplastidal dinoflagellate.

Why cryptomonads?

In some dinoflagellates, the symbionts are derived from Chromononas and other cryptomonads, such as Teleaulax spp and Rhodomonas spp. [39,41,42,43]. Thus, these cryptomonads must have unique characteristics that allow them to become symbionts of dinoflagellates. We propose four main reasons for this phenomenon, as follows.

First, cryptomonads are an appropriate size for engulfment by dinoflagellates.

Furthermore, the cell surface of cryptomonads is a delicate proteinaceous periplast rather than a cellulose wall, which is found only in cryptomonads, although the same term is applied to euglenoids but they have a different arrangement [44]. The periplast is vulnerable to rupturing or distortion [45], so it is probably easily disrupted and digested by the hosts after engulfment.

Moreover, the periplastidial compartment (periplastidial complex or chloroplast endoplasmic reticulum) may maintain a relatively appropriate intracellular environment for the endosymbiont in the host cells.

Finally, the chloroplast of cryptomonads may have the ability to adapt to new intracellular environments. It is widely believed that cryptomonads obtain their chloroplasts by ingesting a red algal-like photosynthetic endosymbiont [46,47]. Thus, from an evolutionary viewpoint, the chloroplasts are fairly unique because they possess the remnants of a eukaryotic nucleus, the nucleomorph [48,49,50,51]. Some genes may be encoded in the chloroplast or nucleomorph, which help the endosymbiont to adapt to the intracellular environment of the host. In addition, some alterations may have occurred in the genes or the ultrastructure of the chloroplasts, which help to integrate the newly engulfed organelles into the host cell. The nucleomorph appears to be important for the chloroplasts because it is retained in the host cell in most cases [20,21,22,23,24]. During the secondary endosymbiosis of cryptomonads, the genes encoded in the nucleomorph are highly compacted and most of the genes with metabolic functions are
Figure 6. Drawings and photographs of *Gymnodinium eucyaneum* and *G. acidotum*. The organisms shown in F–I were considered to be the same as *G. eucyaneum* in the present study.
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eliminated, leaving 30 genes for chloroplast-located proteins [52]. Gene transfer and reduction may occur more in the nucleomorph after engulfment by the dinoflagellates. In a recent study of *Dinophysis acuminata*, horizontal gene transfer was detected from the kleptoplastidal chloroplast obtained from a cryptomonad to the host nucleus [21], which supports our hypothesis. In addition, endosymbiont genes were relocated to the nucleus via massive gene transfer in *Kairomia brevis*, although the endosymbiont was a haptophyte instead of a cryptomonad, but the plastid still originated via a red algal secondary endosymbiosis [53]. The cryptomonad nucleus is probably not as important as the nucleomorph for the survival of the symbiont, because it is frequently lost in the host cell [20,21,24]. The lack of a cryptomonad nucleus in some dinoflagellates did not appear to affect the cell’s ability to photosynthesize or move in response to varying levels of illumination [20]. In the present study, the genes encoded by the cryptomonad nucleus could not be amplified, although we tried many times, which suggested that the nucleus was probably lost.

All these unique characteristics might help the chloroplasts to adapt to new intracellular environments, although no clear evidence was available until now. Thus, it is necessary to study kleptoplastidal dinoflagellates and cryptomonads using comparative genomics and biochemistry methods to achieve a better understanding of the evolution of chloroplasts and algae.

**Materials and Methods**

**Sample collection**

Samples of *G. eucyaneum* were collected in April 2012 from Lake Donghu (30°32'55" N, 114°21'15" E) and from a fishpond in Wuhan, Hubei Province, China (30°28'34" N, 114°21'41" E) in May 2012, where it bloomed and accounted for more than 99% of the phytoplankton cell density. Samples of *G. eucyaneum* cells were isolated using a serial dilution pipetting technique [54] under an inverted microscope (CKX41; Olympus, Tokyo, Japan) and cultivated in sterilized lake water added with Bold’s Basal Medium added [55]. The total DNA was extracted using the CTAB method [56].

**DNA extraction, PCR amplification, and DNA sequencing**

Approximately 30 *G. eucyaneum* cells were isolated using a serial dilution pipetting technique [54] under an inverted microscope (CKX41; Olympus, Tokyo, Japan). The cryptomonads were isolated in the same way and cultivated in sterilized lake water with 4% Bold’s Basal (BB) Medium added [55]. The total DNA was extracted using the CTAB method [56].

The partial nuclear LSU rDNA sequence was amplified using the primers described by Scholin et al. [57]. The conditions used for PCR amplification of the partial LSU rDNA sequence and thermal cycling were those described in Hansen et al. [6]. The specific primers for the nucleomorph SSU rDNA (CMsF, 5'-TGGCT CATTCA CAACA GTTAT AG-3'; CMsR, 5'-AGGCA TTCCT CGTTC AAG-3') and chloroplast 23S rDNA (Cr23S1F, 5'-GAATA GATGC CTGTA CCTTA AACC-3'; Cr23S1R, 5'-TGGAC CGAAC TGTCT CACG-3') of the endosymbionts were designed based on the conserved areas of known sequences of cryptomonads. The amplification profile of the nucleomorph SSU rDNA consisted of an initial denaturation step at 94°C for 4 min, followed by 35 cycles of 1 min at 94°C, 1 min at 60°C, and 90 s at 72°C, with 10 min at 72°C for the final extension. The chloroplast 23S rDNA PCR amplification started with 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 75 s at 72°C, ending with a final hold of 10 min at 72°C. All PCR amplicons were cleaned using an E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, Norcross, GA, USA), before some PCR products were cloned into the pMD18-T vector (Takara, Dalian, China). All clones were sequenced using the universal sequencing primer M13 with an ABI 3700 sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were deposited in GenBank under the Accession Nos. JX470945~JX470953, JX545331, and JQ639750.

**Phylogenetic analyses**

The sequences of putative relatives were downloaded from GenBank. Initially, the sequences were aligned using ClustaX 1.83 [58] and refined manually in SEA VIEW [59]. The final alignment of LSU rDNA sequences comprised a matrix of 38 sequences, including *Perkinus marinus* (Perkinsozoa) as an outgroup taxon. A total of 32 cryptomonads, a dinoflagellate, and putative relative taxa were selected for the nucleomorph SSU rDNA sequence analyses. Species of Bangiophyceae were used as outgroup phylogenies because a previous study showed that members of the Bangiophyceae had close relationships with the nucleomorphs in cryptomonad cells [60]. The alignment of chloroplast 23S rDNA sequences comprised a matrix of 28 sequences including three blue-green algae as an outgroup.

Mutational saturation was evaluated in the variable positions of the alignments by plotting the pairwise distances against model-corrected distances for Tamura and Nei (1993) and Kimura (1980) models, which were estimated using MEGA (v.4.0) [61].

The phylogenies were estimated using the maximum-likelihood (ML) method in PAUP 4.0b10 [62] and Bayesian inference (BI) in MrBayes (v. 3.1.2) [63]. For the ML analysis, ModelTest 3.06 [64] was used to select the evolutionarily best fit model given Akaikes Information Criterion (AIC). The best fit model for the LSU rDNA datasets was TrN+H+I. The best fit model for the nucleomorph SSU rDNA and chloroplast 23S rDNA datasets were GTR+I+G. In the ML analysis, a heuristic search option with random added sequences (100 replicates) and the tree bisection and reconnection branch-swapping algorithm were used to find the best tree.

**Spectrophotometry**

Fishpond samples of *G. eucyaneum* and cultivated strain of *C. corulea* were used. A 10 ml aliquot of the samples was settled for 3 min at 10,000 x g and resuspended in phosphate buffer at pH 7.2 on three occasions. The cells were broken up by three cycles of freezing at −80°C and thawing. The cell debris was pelleted at 15,000 x g. The absorption spectra of the supernatant was measured and recorded using a UV-1700 PharmaSpec spectrophotometer (Shimadzu, Kyoto, Japan).
were used for tree search. A bootstrap analysis with 1000 replicates of the ML dataset was performed to estimate the statistical reliability. A Bayesian Markov Chain Monte Carlo analysis was conducted with seven Markov chains (six heated chains and one cold) for 5,000,000 generations, with the trees sampled every 1000 generations. The first 25% of the trees (burn-in samples) were discarded and the remaining samples were used to construct a Bayesian consensus tree and to infer the posterior probability.

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