Multiple losses of photosynthesis in *Nitzschia* (Bacillariophyceae)

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SUMMARY

In order to obtain insights into the evolution of colorless (apochlorotic) diatoms, we investigated newly established apochlorotic strains of *Nitzschia* spp. using light and electron microscopy and molecular phylogenetic analyses. Fluorescence microscopic observations demonstrated that the apochlorotic diatoms lack chlorophylls. Transmission electron microscopy of two apochlorotic strains also demonstrated that their plastids lacked thylakoids; instead, having four-membrane-bound organelles without thylakoids, similar to nonphotosynthetic plastid remnants. From the apochlorotic strains, we also found plastid small subunit rRNA genes that were unusually long branched in phylogenetic analyses, as observed in other nonphotosynthetic plastids. Molecular phylogenetic analysis of the nucleus-encoded large subunit rRNA genes showed eight distinct lineages for apochlorotic diatoms. The eight apochlorotic lineages were not monophyletic, suggesting that the loss of photosynthesis took place multiple times independently within *Nitzschia*. Several diatoms, including *Nitzschia* spp., are mixotrophic, which is an expected mode of nutrition that would help explain the evolutionary switch from a photosynthetic lifestyle to a heterotrophic lifestyle.

Key words: apochlorotic diatoms, genetic diversity, large subunit rRNA, molecular phylogenetic analysis, nonphotosynthetic plastids, plastid 16S rRNA.

INTRODUCTION

Diatoms comprise the most species-rich group of algae and play important ecological roles as primary producers in aquatic environments. It is reported that diatoms contribute up to 20% of global net primary production (Field et al. 1998; Mann 1999). In addition to this beneficial contribution to ecosystems, some diatoms can also form harmful red tides and disrupt economically important industries such as fisheries (Anderson 1989; Hallegaef 1993).

Most diatoms are photosynthetic, and only seven species are known to be obligate heterotrophs: *Hantzschia achrona* Li and Volcani, *Nitzschia alba* Lewin and Lewin, *N. albicostalis* Li and Volcani, *N. fluorescens* Li and Volcani, *N. leucosigma* Benecke, *N. lucisensibilis* Li and Volcani, and *N. putrida* (Cohn) Benecke (Benecke 1900, Lewin & Lewin 1967; Li & Volcani 1987). These apochlorotic diatoms live on different macroalgae such as *Sargassum* sp., *Fucus* sp., *Pelagophycus* sp., *Hesperophycus harveyanus* (Decaisne) Setchell & N.L.Gardner, *Macroystis pyrifera* (Linnaeus) C.Agardh, *Enteromorpha* sp., *Zostera* sp., *Codium fragile* (Suringar) Hariot, and *Phyllospadix torreyi* S.Watson (e.g., Lewin & Lewin 1967; Li & Volcani 1987). In addition, apochlorotic diatoms have also been found in mangroves and intertidal sand (Blackburn et al. 2009a,b). Apochlorotic diatoms are inferred to have lost photosynthesis secondarily, because most diatoms and their nearest relatives, such as the Bolidophyceae (Daugbjerg & Guillou 2001), are photosynthetic. Lauritis et al. (1968) and Schnepf (1969) observed non-photosynthetic plastids in *Nitzschia alba* using transmission electron microscopy (TEM).

Six out of the seven known species of apochlorotic diatoms belong to *Nitzschia*, which are ubiquitous pennate diatoms including more than 1000 species. Lauritis et al. (1967) and Geitler (1968) reported that *Hantzschia*-like cells are produced by some *Nitzschia* species, which calls into question the traditional separation of these two genera (Mann 1980; Pickett-Heaps 1983). Molecular phylogenetic analyses show that the type species of *Hantzschia*, *H. amphioxys* (Ehrenberg) Grunow, is nested within the *Nitzschia* clade (e.g., Trobajo et al. 2009). Apochlorotic diatoms reported so far are not broadly distributed across the tree of diatoms, but are restricted to only one subgroup consisting of *Nitzschia* and *Hantzschia*. However, because the phylogenetic relationships amongst apochlorotic diatoms remain unclear, we cannot trace the evolutionary history associated with the loss of photosynthesis in diatoms. In this study, we addressed the evolution of apochlorotic diatoms by establishing 22 cultures of apochlorotic *Nitzschia* spp. and inferring their molecular phylogenetic relationships.

MATERIALS AND METHODS

Collection and maintenance of cultures

Water samples and mangroves leaves of *Bruguiera gymnorrhiza* (L.) Lamk. and *Rhizophora mucronata* Lamk.

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were collected from Iriomote Island (123°76′ E, 24°39′ N; 123°78′ E, 24°40′ N; 123°81′ E, 24°40′ N) and Ishigaki Island (124°13′ E, 24°47′ N; 124°21′ E, 24°36′ N; 124°23′ E, 24°48′ N) in Okinawa, Japan, and each of these samples was inoculated in autoclaved seawater and maintained at 20°C under dark conditions. Single cells of colorless Nitzschia spp. were isolated by micro-pipetting and cultured on a seawater-based Glucose Yeast Peptone Agar plate (1 L autoclaved seawater including 15 g agarose, 2 g glucose, 0.5 g Yeast extract, 1 g Peptone, 0.05 g kanamycin, and 0.05 g ampicillin) followed by further incubation under dark conditions. The plating procedures were repeated at least three times for each single colony in order to establish pure colonies. Established culture strains were also maintained at 20°C in Erd-Schreiber Modified medium (Kasai et al. 2009) using a 14 h light:10 h dark cycle. The strains used in this study are summarized in Tables 1 and 2. Strains IriIs01, IriIs03, IriIs04, IriLm01, IriL01, IriSm01, and IriSL01 were deposited to the National Institute for Environmental Study (NIES; Tsukuba, Japan) with no. NIES-3579, NIES-3580, NIES-3581, NIES-3582, NIES-3576, NIES-3577, and NIES-3579, respectively. Voucher slides for strain IriIs04, IriSm01, IriL01, IriSm06, B3, and K1 were deposited as sample nos. TNS-AL-55697, TNS-AL-56998, TNS-AL-55696, TNS-AL-56999, TNS-AL-55698, and TNS-AL-55699, respectively, in TNS (Department of Botany, National Museum of Nature and Science, Tsukuba, Japan).

Table 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Morphotypes†</th>
<th>Phylo-types‡</th>
<th>Origins</th>
<th>Nuclear LSU rRNA gene§</th>
<th>Plastid 16S rRNA gene§</th>
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†Corresponding with Figure 1, ‡Corresponding with Figure 5, §GenBank accession numbers. –, Not amplified by PCR.

Table 2. Morphological characters for apochlorotic diatoms used

<table>
<thead>
<tr>
<th>Morphotypes†</th>
<th>Valve faces</th>
<th>Girdle faces</th>
<th>Keel puncta/10 μm</th>
</tr>
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<td>Lanceolate</td>
<td>Rectangular</td>
<td>15–18</td>
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<tr>
<td>B (K1)</td>
<td>Fusiform</td>
<td>Sigmoid</td>
<td>14–18</td>
</tr>
<tr>
<td>C (IriSm01)</td>
<td>Fusiform with slightly sigmoid end</td>
<td>Sigmoid</td>
<td>12–14</td>
</tr>
<tr>
<td>D (IriSs06)</td>
<td>Wide lanceolate/scalpelliform</td>
<td>Rectangular</td>
<td>12–14</td>
</tr>
<tr>
<td>E (IriSL01)</td>
<td>Linear with slightly sigmoid end</td>
<td>Sigmoid</td>
<td>12–15</td>
</tr>
<tr>
<td>F (B3)</td>
<td>Linear/scalpelliform</td>
<td>Sigmoid</td>
<td>12–14</td>
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†Corresponding with Figure 1. Strains used are shown in parentheses.

Light microscopy

For all but one strain, living cells were observed with a light microscope (Axioskop; Zeiss, Oberkochen, Germany) and the images were captured by a digital camera (DP71; Olympus, Tokyo, Japan). The cleaned frustules were prepared following Nagumo (1995). Otherwise, cells were exposed to low temperature plasma as described in Watanabe et al. (2010). Cleaned specimens were mounted in Pleurax (Wako Pure Chemical Industries, Osaka, Japan) followed by a light microscope (SKE; Nikon, Tokyo, Japan) equipped with a digital camera (Infinity1-5-M; Lumenera, Ottawa, Canada).
Fluorescence and electron microscopy

For fluorescence microscopy, the cells were observed using an epi-fluorescence microscope (BX51N-34-FLD; Olympus) under bright field, UV, and green excitation, after staining with 0.1% 4′, 6-diamidino-2-phenylindole (DAPI) (Porter & Feig 1980) without fixation.

For TEM observations, cell suspensions in media were mixed with an equal volume of fixative [2.5% glutaraldehyde and 0.25 M sucrose in 0.05 M sodium cacodylate buffer (pH 7.2)] at 4°C for 2 h. Cells were then rinsed several times with the same buffer and post-fixed with 1% osmium tetroxide for 30 min. The cells were dehydrated through a graded ethanol series to propylene oxide, and embedded in Spurr’s resin (Spurr 1969). Serial ultra-thin sections, made with EM UC6 ultramicrotome (Leica, Solms, Germany), were double stained with 2% (w/v) uranyl acetate and lead citrate (Reynolds 1963) and observed using H7600 microscope (Hitachi, Tokyo, Japan).

DNA extraction, PCR amplification, cloning and sequencing

Total DNA was extracted using cetyl trimethylammonium bromide buffer following Kamikawa et al. (2009). The nuclear large subunit (LSU) rRNA gene was amplified with forward primer (D1R-F: 5′-ACCCGCTGAATTACGATA-3′; Scholin et al. 1994) and reverse primer (D1B-R: 5′-CTTGGTGCGTGTTCAGA-3′; Nunn et al. 1996) under the polymerase chain reaction (PCR) conditions described in Lundholm et al. (2002). The plastid 16S rRNA gene was amplified by PCR with newly designed forward primer (16SF: 5′-AGAGTGTGGATCTCGGCTGAG-3′) and reverse primer (16SR: 5′-TGATCCAAAGCGACCTTTCCA-3′), under the following PCR conditions: 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min. Both PCR amplifications were performed using ExTaq (Toyake Bio., Ohtsu, Japan) following the manufacturer’s instructions. The amplified products were gel-purified and then cloned into pGEMTEasy vector (Promega, Madison, WI). The clones were sequenced on both strands. The sequences were deposited in DNA Data Bank of Japan (Table 1).

Molecular phylogenetic analyses

Sequences of the nucleus-encoded LSU rRNA gene were sampled from species/strains in the Bacillariaceae, especially from Nitzschia and its closest relatives (Appendix S1 in Supporting Information). We included at least one sequence from each species whose nuclear LSU rRNA gene was available (Appendix S1 in Supporting Information). N. paelea was reported as a species complex (Trojano et al. 2009), so we included the two N. paelea sequences that were most distantly related to each other in a previous study (Trojano et al. 2009). Three Eunotia species were also added as outgroup taxa because previous phylogenetic analyses (Stepanek & Kociolek 2014) suggested a close relationship between Eunotia and the Bacillariaceae. 183 ambiguously aligned nucleotide positions were excluded from the 114-taxon alignment using BioEdit (Hall 1999), leaving 765 unambiguously aligned positions for phylogenetic analyses described below.

Maximum-likelihood (ML) and Bayesian phylogenetic analyses were performed on the LSU rRNA alignment. ML analyses were carried out under the general time reversible (GTR) model incorporating among-site rate variation approximated by a discrete gamma distribution (GTR + I model) as the most suitable model for our dataset determined by the program Phylogenetic analyses described below. Maximum-likelihood (ML) and Bayesian phylogenetic analyses were performed under the CAT-GTR + I model using PhyloBayes 3.3 (Lartillot et al. 2009). Two parallel Markov Chain Monte Carlo runs were run for 39 000 cycles, in which seven million trees and log-likelihoods (lnLs) were sampled. We considered independent runs converged when the maximum discrepancy observed across all bipartitions was 0.05, and effective sample size was >100 (Lartillot et al. 2009). The first 10 000 cycles were discarded as burn-in, and trees were summarized to obtain Bayesian posterior probabilities.

Evaluation of the monophyly of apochlorotic diatoms

In order to evaluate the possible monophyly of the apochlorotic diatoms we used the approximately unbiased (AU) test (Shimodaira 2002). An alternative tree was prepared by constraining the monophyly of all of the apochlorotic diatoms (Table 2; Appendix S3 in Supporting Information). Using the same LSU rRNA alignment, site-wise log-likelihoods (site lnLs) were calculated by RAxML with the GTR + I model for the alternative tree and the ML tree. Resultant site lnLs were subsequently analyzed by Consel version 0.1 (Shimodaira & Hasegawa 2001) with the default settings to perform the AU test.

RESULTS

We successfully established 22 culture strains of Nitzschia collected from mangrove leaves and water samples (Table 1). These strains were distinguished from photosynthetic diatoms by appearing colorless. Microscopic observations of six
different strains representing six distinct phylotypes of apochlorotic diatoms (Lineages 1–6, Table 1) in the LSU rRNA phylogeny are shown in Figure 1. The girdle face was rectangular in IriIs04 and IriSs06, and sigmoidal in IriSm01, IriSL01, K1, and B3 (Fig. 1, Table 2). The valve face was lanceolate, wide lanceolate/scalpelliform, fusiform with slightly sigmoid ends, linear with slightly sigmoid ends, fusiform, and linear/scalpelliform in IriIs04, IriSs06, IriSm01, IriSL01, K1, and B3, respectively (Fig. 1, Table 2). Although observations of the precise valve shape in these diatoms were difficult because of vertical undulations of the valve face, the valves were either scalpelliform or almost fusiform to linear with/without sigmoid ends (Table 2). Striae were not observed in all strains, but the keel puncta (fibulae of canal raphe system) were distinctive. The fibulae density was higher in IriIs04 and K1 than that of IriSm01, IriSs06, IriSL01 and B3 (Table 2). A wide gap between central two fibulae, which often reflects the presence of a central nodule in raphe system, was not observed in any of the strains (Fig. 1). We did not find any pigments in these cells (left column in Fig. 2a–f but not shown for all the other strains), which is strongly suggestive of a non-photosynthetic mode of nutrition. When these strains were observed using fluorescence microscopy, although DAPI signals supposedly derived from organellar DNA were observed in addition to those of nucleus (middle column in Fig. 2a–f), no red chlorophyll fluorescence was detected in the cells (right column in Fig. 2a–f).

In order to investigate plastids in our apochlorotic isolates, we characterized the ultrastructure of two strains, IriIs04 and IriSm01. We did not find plastids with conspicuous thylakoids, but instead we observed plastid-like structures with an electron-dense matrix surrounded by four membranes (Fig. 3). The plastid-like structures also possessed a periplastidal compartment positioned between the outer two and inner two membranes (Fig. 3b,d), which is typically seen in the photosynthetic plastids of diatoms. Some of the plastid-like structures in IriIs04 had a few reduced thylakoid-like structures (Fig. 3c).

In order to gain insights into whether the plastid-like structures have retained a genome, we performed PCR-based surveys for the plastid 16S rRNA gene. We determined the sequences of plastid 16S rRNA genes in almost all of the new apochlorotic strains (GenBank accession nos.
The branch lengths of the 16S rRNA sequences obtained from the apochlorotic diatoms were much longer than those of photosynthetic species representing a broad range of lineages across the tree of eukaryotes (Fig. 4). In these analyses, the long-branched apochlorotic Nitzschia spp. were monophyletic and closely related to the Bacillariaceae (highlighted by stars in Fig. 4). In order to more rigorously evaluate the phylogenetic relationships among apochlorotic diatoms, we analyzed the 114-taxon alignment consisting of LSU rRNA sequences from the Bacillariaceae, including the previously reported apochlorotic diatoms N. alba (GenBank accession nos. HQ396817 and HQ337473) and N. leucosigma (GenBank accession no. HQ396836) (Fig. 5). Our dataset also included the presumed apochlorotic diatom Nitzschia sp. CCMP578 (GenBank accession no. HQ396838, see also https://ncma.bigelow.org/ccmp578#.UzPVxfl_tWg). In the LSU rRNA phylogeny (Fig. 5), the apochlorotic diatoms were divided into lineages 1–8. N. alba and Nitzschia sp. CCMP578 correspond to lineages 7 and 8, respectively. Lineage 6 includes the previously reported sequence of the apochlorotic diatom N. leucosigma. The LSU rRNA phylogeny failed to recover the monophyly of lineages 1–8. Nevertheless, lineages 1 and 2, and lineages 3–6 were coalesced into two separate groups with high statistical support. The paraphyletic relationships were not significantly changed if we used other outgroup taxa, such as Phaeodactylum tricornutum (GenBank EF553458) and Pauliella taeniata (GenBank AF417680) (data not shown).
In order to assess alternative possibilities regarding the phylogenetic relationships amongst apochlorotic diatom groups (lineages 1–8), an ML tree was inferred after constraining the apochlorotic diatoms as monophyletic (Appendix S3 in Supporting Information). The log likelihood of the alternative tree was only slightly smaller than that of the ML tree (ΔlnL = −9.0). The P-value of the alternative tree was 0.313, while that of the ML tree was 0.687. Consequently, the AU test under the 5% P-value criterion failed to reject the alternative tree.

**DISCUSSION**

Light and fluorescence microscopic observations allowed us to conclude that the 22 diatom strains were apochlorotic. Prior to this study, *Nitzschia leucosigma* is the only species that has been formally described as an apochlorotic diatom with a sigmoidal girdle face (Benecke 1900; Lewin & Lewin 1967; Li & Volcani 1987). In this study, we successfully isolated four sigmoidal lineages, and these isolates are distinguishable from *N. leucosigma* in the shape of the valve face: The valve face of *N. leucosigma* is straight (Benecke 1900), while those of morphotypes B, C, E, and F were fusiform, fusiform with a slightly sigmoid end, linear with a slightly sigmoid end, and linear scalpeliform, respectively. The variation in the valve face made it difficult to judge whether sigmoidal apochlorotic isolates in lineages 3, 5, and 6 (corresponding to morphotypes C, E, and F, respectively) are *N. leucosigma*, although they formed a robust clade with *N. leucosigma* in the LSU rRNA phylogeny. On the other hand, sigmoidal apochlorotic isolates I4, K1, and K2 (lineage 2/morphotype B) were most likely different from *N. leucosigma* genetically, as the three isolates and *N. leucosigma* were placed in distant positions in the LSU rRNA phylogeny. More detailed morphological characteristics need to be gleaned from the new apochlorotic isolates for their taxonomic treatments in the future.

We observed plastid-like structures without conspicuous thylakoids in strains IriSm04 and IriSm01 and also obtained plastid 16S rRNA sequences from these diatoms, suggesting that the apochlorotic diatoms studied here contain nonphotosynthetic plastids with genomes. This is analogous to those present in apochlorotic euglenids [e.g., *Euglena longa* (Pringsheim) Marin & Melkonian] and cryptomonads [e.g., *Cryptomonas paramecium* (Ehrenberg) Hoef-Emonds & Melkonian] (Gockel & Hachtel 2000; Donaher et al. 2009). Although metabolic pathway working in nonphotosynthetic plastids of other organisms have been well studied (e.g., apicomplexans, a parasitic green alga, and parasitic land plants; de Koning & Keeling 2004; Wickett et al. 2011; Sheiner et al. 2013), the metabolic functions of these nonphotosynthetic plastids in apochlorotic diatoms remain unknown, urging us to investigate the roles of the organelle more comprehensively in the future.

The plastid 16S rRNA genes from apochlorotic diatoms are rapidly evolving, and therefore their branches were much longer than other branches in the phylogenetic tree (Fig. 4). Despite similar increments of the evolutionary rates being observed in the genomes of nonphotosynthetic plastids (e.g., Vernon et al. 2001), the precise reason for this phenomenon has yet to be elucidated. At least, we conclude that plastid 16S rRNA genes are not ideal to investigate the precise phylogenetic positions of apochlorotic diatoms within the context of diatoms as a whole because of the potential for long-branch attraction (LBA) artefacts (Philippe & Germot 2000; Bergsten 2005). It is known that the impact of LBA artefacts can be reduced when the long branch is ‘split’ by including a sequence (or sequences) closely related to the long-branch sequence of interest (Bergsten 2005). Thus, if one uses plastid 16S rRNA gene sequences to infer the accurate phylogenetic relationships between photosynthetic diatoms and apochlorotic diatoms, it is necessary to acquire more sequence data of other photosynthetic members of the Bacillariaceae, which break the long branches leading to the apochlorotic species. Alternatively, nucleotide substitution...
**Evolution of colorless Nitzschia**

Fig. 4. Maximum-likelihood tree of plastids inferred from plastid 16S rRNA gene sequences. Only bootstrap values over 70% are shown. Nodes supported by Bayesian posterior probabilities over 0.95 are shown by thick branches. Closed stars show taxa that belong to the Bacillariaceae. The labels 1 to 6 for apochlorotic diatom lineages correspond to those in Figure 5 and phylotype 1 to 6 in Table 1.
Fig. 5. Maximum-likelihood tree of *Nitzschia* and its close relatives as inferred from large subunit rRNA gene sequences. Taxa were broadly sampled from the Bacillariaceae. Apochlorotic diatoms are shaded in grey. Other information is the same as Figure 4.

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models, in which tempo and mode of sequence evolution can be varied across subtrees, might help overcome LBA artefacts (e.g., Ishikawa et al. 2012).

In contrast to plastid 16S rRNA genes, nuclear LSU rRNA genes are likely more suitable for phylogenetic analyses to investigate the phylogenetic positions of apochlorotic diatoms because the branch lengths are not significantly longer than other species. The analyses of LSU rRNA sequences showed apochlorotic diatoms divided into eight distinct lineages that did not form a clade. This polyphylectic distribution of apochlorotic diatoms suggests that loss of photosynthetic ability has occurred several times independently within Nitzschia; however, the monophyly of these lineages was not confidently rejected by a statistical test. This possibility can be tested by additional analyses using other phylogenetic markers.

Several lineages of eukaryotes have lost their photosynthetic ability, such as some species of cryptomonads (Sepsenwol 1973; Hoef-Emden 2005), euagles (e.g., Gockel & Hachtel 2000; Triemer & Farmer 2007), green algae (e.g., Menke & Ericke 1962; Leliaert et al. 2012), stramenopiles (e.g., Sekiguchi et al. 2002; Yubuki et al. 2008) and alveolates (e.g., Cavalier-Smith 2002; Fernández Robledo et al. 2011). The independent loss of photosynthesis in distantly related lineages of eukaryotes suggests that this evolutionary pattern has also helped shape the current patchy distribution of photosynthetic species across the tree of eukaryotes. Therefore, it is intriguing to understand the evolutionary force behind the loss of photosynthesis. Nitzschia is one of the most species-rich and successful eukaryotic lineages in both oceanic and freshwater environments. Yet, several Nitzschia lineages likely have experienced secondary loss of photosynthesis. Some species of diatoms including photosynthetic Nitzschia spp. are known to grow under dark conditions by utilizing dissolved organic carbon (Hellebust & Lewin 1977). This suggests that some, if not all, species of diatoms have a mechanism for carbon uptake; however, to our knowledge, genes and proteins associated with this mechanism have not been reported. The origin(s) and subsequent maintenance of mixotrophy may have, at least in part, allowed some Nitzschia species to lose photosynthesis under certain conditions. For instance, it may be possible to gain carbon-sources through interactions with the surfaces of macroalgae and mangrove leaves: these are common associations in the isolates of apochlorotic diatoms that have been described so far (Lewin & Lewin 1967; Li & Volcini 1987; this study). If so, apochlorotic diatom species may not be restricted to Nitzschia and Hantzschia because mixotrophy has been reported from many other genera of diatoms (e.g., Melosira, Navicula, Cylindrotheca, and Cocconeis; Hellebust & Lewin 1977). It is also certainly possible that future discoveries will demonstrate new non-photosynthetic species that are more distantly related to Nitzschia.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Nuclear LSU rRNA gene sequences used in this study.

Appendix S2. Plastid 16S rRNA gene sequences used in this study.

Appendix S3. ML tree inferred after constraining the apochlorotic diatoms as monophyletic.