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Kleptoplasty in an Antarctic dinoflagellate: caught in evolutionary transition?

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Summary

Photosynthetic dinoflagellates contain a diverse collection of plastid types, a situation believed to have arisen from multiple endosymbiotic events. In addition, a number of heterotrophic (phagotrophic) dinoflagellates possess the ability to acquire chloroplasts temporarily by engulfing algae and retaining their chloroplasts in a functional state. These latter relationships typically last from a few days to weeks, at which point the chloroplasts lose function, are digested and replaced with newly acquired plastids. A novel and abundant dinoflagellate related to the icthyotoxic genera *Karenia* and *Karlodinium* was recently discovered by us in the Ross Sea, Antarctica. Sequencing of its plastid small subunit ribosomal gene indicated that it did not share evolutionary history with the plastids of *Karenia* or *Karlodinium*, but was closely related to the free-living haptophyte *Phaeocystis antarctica*, a species that often dominates phytoplankton blooms in the Ross Sea. Chloroplast uptake was observed to occur rapidly (within 2 days), with retention in cultures being long-lived (several months) but not permanent. The dinoflagellate was also incapable of growing indefinitely in continuous darkness with algae as prey. Our findings may indicate an emerging endosymbiotic event yielding a dinoflagellate that is presently neither purely phototrophic nor purely heterotrophic, but occupies a niche juxtaposed between these contrasting nutritional modes.

Introduction

Dinoflagellates are a group of unicellular eukaryotic organisms (protists) that include species capable of photosynthesis, heterotrophy (phagocytosis) and mixotrophy (combined photosynthetic and heterotrophic ability). The photosynthetic dinoflagellates actually represent an evolutionarily diverse collection of different chloroplast types (Delwiche and Palmer, 1997). Dinoflagellate chloroplasts have been divided into five main groups based upon their general pigment types (Dodge, 1989). These include fucoxanthin-type plastids (diatom origin; Chesnick *et al*., 1997), peridinin-type plastids (red algal origin; Takishita and Uchida, 1999; Zhang *et al*., 2000), 19′hexanoyloxyfucoxanthin-type plastids (haptophyte origin; Tengs *et al*., 2000), phycobilin-type plastids (potential cryptophyte origin; Takishita *et al*., 2002; Hackett *et al*., 2003) and chlorophyll-b type plastids (potential prasinophyte origin; Watanabe *et al*., 1987). The ancestral state has been argued to be either the peridinin-type (Tengs *et al*., 2000) or the fucoxanthin-type (Yoon *et al*., 2002), but what is clear is that dinoflagellates are promiscuous with respect to their chloroplasts, and that plastids have been acquired multiple times during their evolutionary history.

Kleptoplasty, the retention of functional chloroplasts from algal prey, has been previously described for a number of heterotrophic dinoflagellates (Fields and Rhodes, 1991; Schnepf and Elbrächter, 1992; 1999; Skovgaard, 1998; Stoecker, 1999). The acquired chloroplasts remain functional for a limited period of time, usually not more than several days, and are eventually digested and replaced with chloroplasts from newly acquired prey. Kleptoplasty is a fascinating behaviour that could represent either an early evolutionary stage in the permanent acquisition of chloroplasts, or a mechanism that permits functional flexibility in the dinoflagellates.

We noted the prevalence of a single dinoflagellate sequence type in an analysis of full-length nuclear small subunit ribosomal gene (srDNA) clone libraries from seawater and slush samples (snow melt-seawater mix from annual pack ice) collected during austral summer of 1999 from the Ross Sea, Antarctica. The dinoflagellate itself was successfully cultured (Fig. 1) from enrichments of seawater samples collected on two separate cruises to the Ross Sea, 2 years apart, yielding two isolates (RS24 and W5-1) whose 18S ribosomal gene sequences were 99.94% similar to each other (Gast *et al*., 2006). Phylogenetic analysis of the sequences indicated that they belonged to a novel dinoflagellate genus that was a sister

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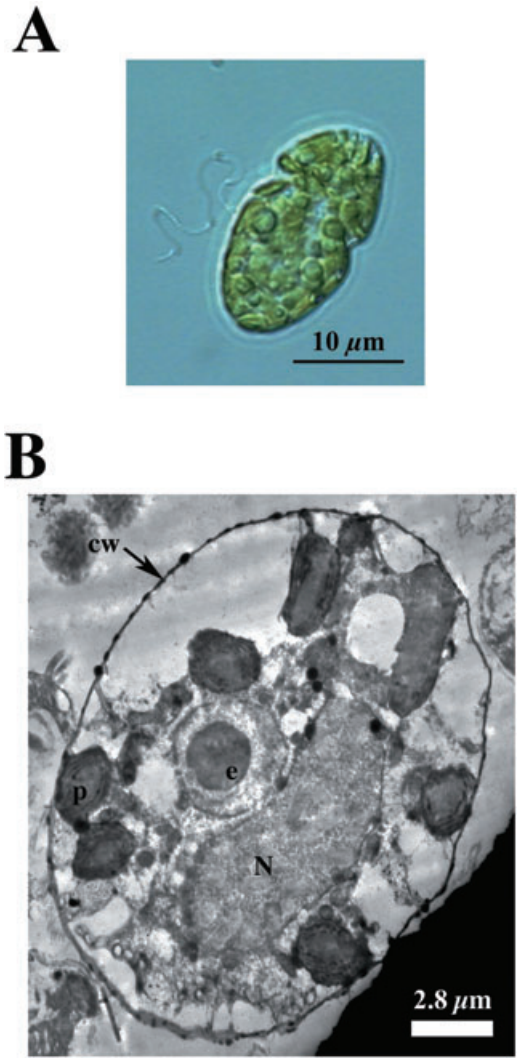

Fig. 1. A. Phase contrast microscope image of kleptoplastidic dinoflagellate cells in the vegetative state.
B. Transmission electron micrograph of dinoflagellate.
N = dinoflagellate nucleus; e = eukaryote-like nucleus; p = plastid (6 are actually visible in this image but only one is labelled); cw = cell wall.

taxon to the dinoflagellate genera *Karenia* and *Karlodinium* (Gast *et al*., 2006). The organism is prevalent in the Antarctic marine environment, with natural abundances as high as 3×10^4 cells l^{-1} of water and 9×10^5 cells l^{-1} of slush determined using quantitative polymerase chain reaction analysis (Gast *et al*., 2006).

Karenia brevis and *Karlodinium micrum* have been shown to carry similar but distinct haptophyte-like plastids (Tengs *et al*., 2000). In this study we set out to clarify the phylogenetic relationship between our dinoflagellate and its sister taxa by conducting a comparison of the plastid sequences. We determined that the plastids from our dinoflagellate were essentially identical to those from the haptophyte *Phaeocystis antarctica*. This organism co-occurred in our original dinoflagellate cultures, and the dinoflagellate can only grow as a clonal culture for a limited amount of time without *Phaeocystis*. We investigated and confirmed the kleptoplastidic nature of this relationship, and discuss the evolutionary implications of the unusual stability of the relationship.

Results

Plastid sequence phylogeny

A portion of the plastid small subunit ribosomal gene from the novel Antarctic dinoflagellate strains RS24 and W5-1 were amplified, cloned and sequenced. Alignment of our dinoflagellate plastid sequences with available haptophyte and bacillariophyte plastid sequences from GenBank (Bilofsky and Burks, 1988) indicated that ours had a haptophyte origin, but were distinct from the plastid sequences of either *Karenia* or *Karlodinium*. Partial 16S sequences were also recovered from several of our *P. antarctica* cultures, and from a culture of pennate diatoms that also co-occur with the dinoflagellate. Inclusion of these sequences in the alignment revealed that our dinoflagellate plastid sequences were most similar to those from *Phaeocystis* (0–2.88% different; Table 1; Fig. 2).

Table 1. Per cent sequence differences between plastid srDNAs from the novel dinoflagellate, three related dinoflagellates, *Phaeocystis antarctica*, and the pennate diatom culture.

Organism	1	2	3	4	5	6	7	8
1. RS 24 dinoflagellate 12 clones[a]	0–1.55							
2. W5-1 dinoflagellate 16 clones[a]	0.44–2.00	0–1.33						
3. W5-1 *Phaeocystis* 14 clones[a]	0.22–2.88	0–2.44	0–2.88					
4. W6-4 *Phaeocystis* 11 clones[a]	0.44–1.77	0–1.33	0–2.44	0–1.11				
5. RS 24 *Phaeocystis* 28 clones[a]	0–1.55	0.44–2.00	0.22–2.00	0.44–1.77	0–1.97			
6. *Gymnodinium breve* CCMP 718[b]	21.73–22.62	21.29–21.73	21.29–22.84	21.29–21.73	21.51–22.62	–		
7. *Gymnodinium galatheanum* KT-77B[b]	21.43–22.32	20.98–21.65	20.76–22.32	20.98–21.43	21.21–22.32	15.11	–	
8. *Gyrodinium aureolum* KT-77D[b]	11.41–12.30	10.96–11.41	10.96–12.30	10.96–11.41	11.19–12.30	22.39	23.06	–
9. W5-1 pennate diatom	14.51–15.62	14.29–14.96	14.29–14.96	14.29–14.73	14.73–16.62	24.12	25.00	19.51

a. Multiple clones were sequenced for the plastid srDNA fragment amplified from these cultures, and the per cent difference values represent the range of differences between the clones.
b. These dinoflagellate plastid srDNA sequences were recovered from GenBank.

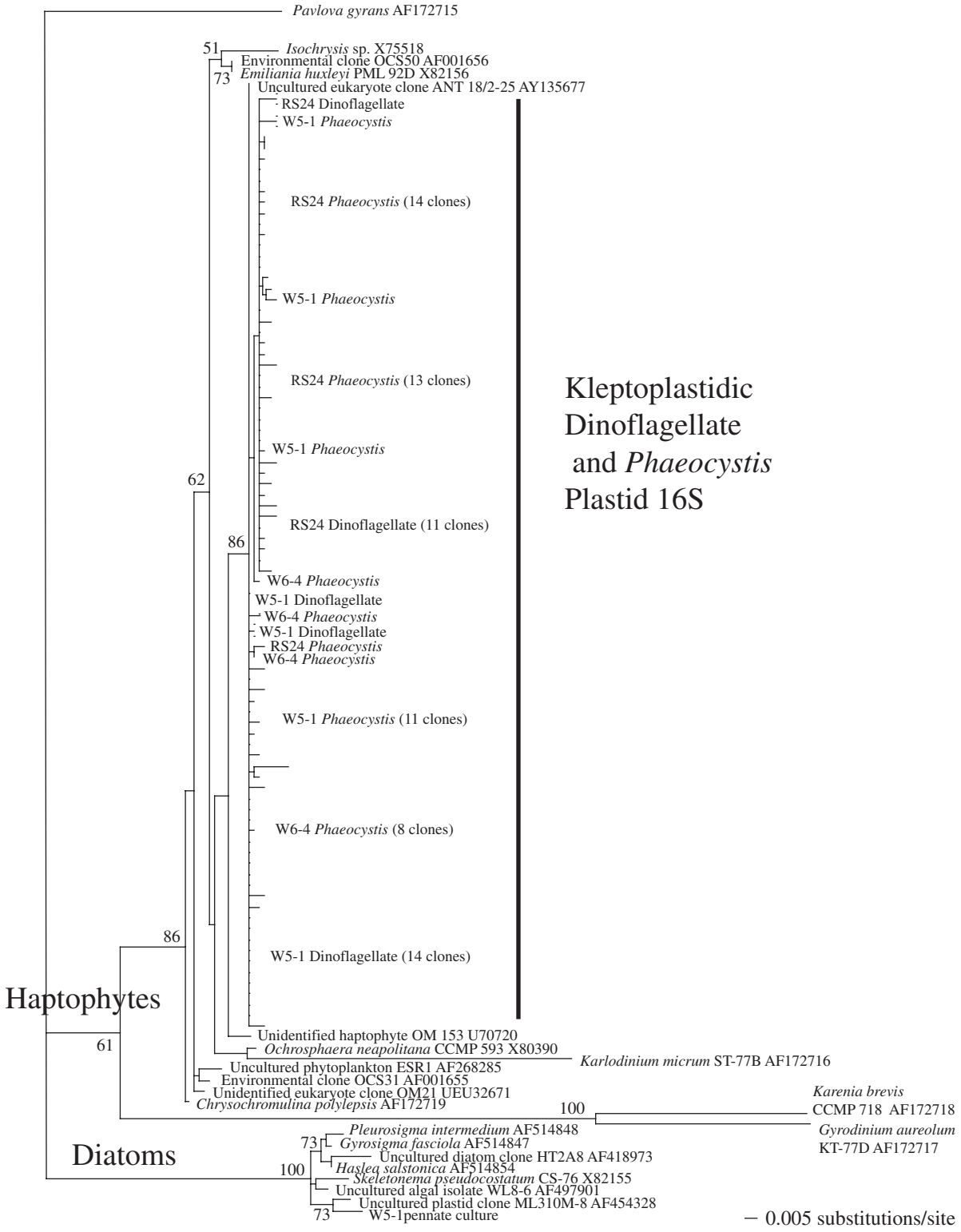

Fig. 2. Phylogenetic reconstruction (maximum likelihood, unrooted) of plastid 16S partial gene sequences (449 characters). Bootstrap values from 100 maximum parsimony replicates are given at the nodes of the tree.

The variability shown by the plastid sequences was within the range that we have observed among individuals of the same species or genus (< 3% different, R.J. Gast *et al*., pers. obs.; Table 1, Fig. 2). Eleven to 14 clones were sequenced from each of our cultures (see *Experimental procedures*) for a total of 81 plastid sequences. Twenty of the 41 sequences from the cultures W5-1 dinoflagellate, W5-1 *Phaeocystis* and W6-4 *Phaeocystis* were identical. Another 14 of the 40 sequences from RS 24 dinoflagellate and RS 24 *Phaeocystis* were also identical to each other, but distinct from the others. The remaining 47 sequences ranged from 0.22 to 2.88% different from each other, and came from all five of our cultures. The within individual variation was not assessed for these data as none of these cultures was clonal. These data suggest that a mixture of strains of *P. antarctica* were likely present in the cultures, and that the dinoflagellates acquire multiple plastids from the culture/environment rather than through replication of a single acquired plastid.

Observation of chloroplast uptake

Our sequence data clearly indicate that the haptophyte *Phaeocystis* was the origin of the novel dinoflagellate plastid. We also noted that we were unable to sustain growth of the novel dinoflagellate as clonal cultures of the organism, and that the haptophyte *P. antarctica* consistently co-occurred in mixed enrichments that yielded the dinoflagellate. These facts suggested a kleptoplastidic relationship between the dinoflagellate and the haptophyte. A feeding experiment was designed and conducted to examine the uptake of the chloroplasts and to determine whether their function was necessary for growth of the dinoflagellate. Monocultures of the dinoflagellate were established, and during a 2-month period they were grown without *Phaeocystis*. The number of chloroplasts per dinoflagellate cell decreased to an average of three, through either dilution during cell division, digestion, or a combination of both. Dinoflagellates were then incubated with (fed) or without (unfed) *Phaeocystis*, in a 14:10 h photoperiod and in complete darkness. Within 2 days of adding algae to the cultures, the number of plastids increased to greater than 20 per cell in the fed dinoflagellate culture with light (Fig. 3A). The fed culture kept in the dark initially took up plastids rapidly, but only reached an average of 7 per cell (Fig. 3A). Unfed cultures of the dinoflagellate continued to gradually lose plastids throughout the experiment.

A second fed plus dark experiment was conducted to assess whether the lower number of plastids per dinoflagellate cell was likely due to the decline of *Phaeocystis* in the culture, and whether the dinoflagellate required functional plastids to grow. *Phaeocystis* were added to the culture every 2 days to keep the level high, and a maximum of 13 plastids per cell were accumulated (Fig. 3A). This indicated that the lower number present in the first experiment was likely due to the decrease in *Phaeocystis* numbers in the dark.

Figure 3B documents the growth of the dinoflagellate and *Phaeocystis* under the experimental conditions of light and dark, fed and unfed. Although plastid uptake was about the same in the light and the dark when given additional algae, the dinoflagellate did not grow during the 40-day experiment while kept in the dark, even with algae continually available as prey (Fig. 3B). In contrast, the fed dinoflagellate in the light doubled several times during the experiment (Fig. 3B). This suggested that the dinoflagellate required a functional plastid to grow, and although plastids might be eaten to maintain the status quo of the culture in the dark, this would not support growth.

Discussion

The nature of the relationship between the dinoflagellate and its plastids appears to be more than kleptoplasty, yet not an endosymbiosis. We have evidence that supports both an incorporation of the plastids, as well as the transient nature of the relationship. Incorporation is suggested by a highly evolved association between the haptophyte chloroplasts and their dinoflagellate host, including dormant stages (cysts) of the dinoflagellate that contain high numbers of healthy-looking plastids, the inability of the dinoflagellate to grow in the dark even when prey are present, and the general stability/longevity of the interaction (up to 8 months). We have also not observed evidence of chloroplast digestion by transmission electron microscopy, or plastids within food vacuoles of the dinoflagellate (Fig. 1B). The presence of a eukaryotic nucleus in close proximity to the dinoflagellate nucleus was noted, but its origin and function are unknown at this time.

Despite the information that indicates the plastid–dinoflagellate association is highly structured, other evidence indicates that the relationship is not permanent. Our starvation/uptake experiments confirm that the dinoflagellate must acquire these plastids from the free-living haptophyte. The novel dinoflagellate does not grow indefinitely in clonal (uniprotistan) culture and can only be maintained as a mixed culture with the bloom-forming haptophyte, *P. antarctica*. Dinoflagellate cultures separated from *P. antarctica* gradually lose chloroplasts over a period of approximately 5–8 months (Fig. 3A). Other kleptoplastidic dinoflagellates generally need to reacquire new plastids within 1 month, presumably because the acquired chloroplasts do not maintain their function for prolonged periods (Fields and Rhodes, 1991; Skovgaard, 1998; Lewitus *et al*., 1999).

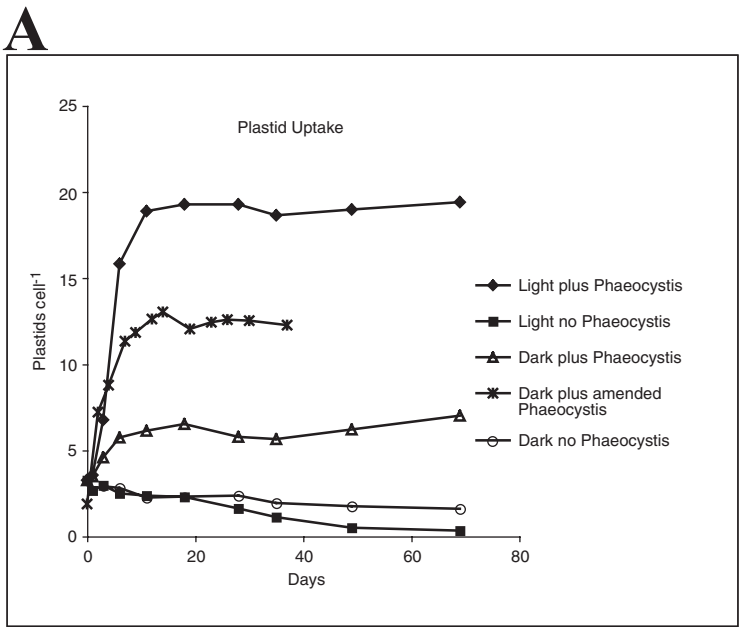

Fig. 3. Plastid uptake by starved cells.
A. *Phaeocystis* plastid uptake. The number of days are given on the *x*-axis, and the average number of plastids per cell are given on the *y*-axis.
B. Dinoflagellate and *Phaeocystis* growth in light and dark. Again, the number of days are given on the *x*-axis, and the natural logarithm of cells per millilitre of culture is given on the *y*-axis.

The kleptoplasty we have observed is potentially one of the most evolved relationships between a heterotrophic dinoflagellate and its 'stolen chloroplasts'. The only other kleptoplasty currently reported to exist for a similar amount of time is that of the marine sea slug, *Elysia chlorotica* (Rumpho *et al*., 2001). The stolen plastids have been shown to continue to function, which is startling considering that many of the genes required for plastid activity are present in the algal nucleus, which is not present in the sea slug. With regard to our novel dinoflagellate, we present two possible scenarios to explain the longevity of the interaction. One could speculate that the second eukaryotic nucleus found next to the dinoflagellate nucleus is from an ingested *Phaeocystis* cell, and that its presence helps stabilize the plastids. The presence of an additional nucleus has been observed in other cells (Dodge, 1971; Farmer and Roberts, 1990; Schnepf and Elbrächter, 1999), appears to be the result of engulfment of a single phytoplankter, and may result in an endosymbiotic association. As each *Phaeocystis* cell has two plastids, multiple cells must be 'eaten' to obtain the large number of plastids observed in our Antarctic

dinoflagellate. The recognition and maintenance of a single *Phaeocystis* nucleus from that process would be remarkable.

Alternatively, the dinoflagellate may have acquired many of the genes necessary to stabilize the function and persistence of haptophyte plastids through its evolutionary history, and the additional nucleus may not be from *Phaeocystis* at all. Based upon identification of the novel dinoflagellate as a sister taxon to the haptophyte plastid lineages *Karenia* and *Karlodinium* (Gast *et al*., 2006), it seems possible that our dinoflagellate may have had a haptophyte-like plastid previously. This could enable the dinoflagellate to acquire and utilize new photosynthetic machinery already well adapted to the extreme cold and extreme light conditions of the Antarctic marine environment during the summer months, and could allow the organism to maintain the plastids throughout the winter for use prior to the availability of *Phaeocystis* in the spring bloom. In either situation, this novel dinoflagellate is a cell chimaera with the potential to provide significant evolutionary information regarding the regulation of plastids in dinoflagellates.

Experimental procedures

Isolation of cells

Two dinoflagellate cultures (W5-1 dinoflagellate, RS 24 dinoflagellate), three *P. antarctica* cultures (W5-1 *Phaeocystis*, W6-4 *Phaeocystis*, RS 24 *Phaeocystis*) and one diatom culture (W5-1 pennate) were used in this study. Dinoflagellates were sorted out of mixed cultures using a Beckman FACSCaliber flowcytometer (W5-1 dinoflagellate) or picked as individual cells by micropipetting (RS 24 dinoflagellate; two picked cells). Mono-algal cultures of diatoms and *Phaeocystis* were established by serial dilution of mixed cultures (W5-1 pennate, W6-4 *Phaeocystis*), micropipetting individual colonies (RS 24 *Phaeocystis*) or using a flowcytometer (W5-1 *Phaeocystis*).

Extraction, amplification and sequencing

DNA was extracted from cells collected onto 25 mm polycarbonate filters following the hot detergent, bead beating method described in Gast and colleagues (2004). The number of cells collected varied, depending upon the type of alga and the growth stage. Between 10 000 and 100 000 cells were collected for the pennate diatom and the *Phaeocystis* cultures, and between 1000 and 10 000 cells for the dinoflagellate cultures. Amplification of approximately 450 bp of the plastid srDNA was accomplished using the plastid-specific primer combination of 394PlaF and 855PlaR (Tengs *et al*., 2000). Polymerase chain reaction products were cloned using pGEM®-T Easy Vector System following instructions (Promega, Madison, WI) and 14 colonies from each ligation were picked for miniprep screening (Wizard® MiniPrep Kit, Promega). Clones were sequenced in a total volume of 10 μl consisting of 1–2 μl of plasmid, 2 μl of ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA), 1 μl of M13F or M13R primer (100 ng μl^{-1} and dH_2O. Completed reactions were run on an ABI 377 automated sequencer (Applied Biosystems). All sequences were assembled and edited using the Sequencher™ 4.1 editing program (Gene Codes Corporation, Ann Arbor, MI). Sequences representative of ones used in the phylogenetic analysis have been submitted to GenBank under the Accession numbers DQ442650–DQ442655.

Phylogenetic analysis

All 81 plastid sequences recovered in this project were aligned with other plastid 16S ribosomal genes from GenBank (Bilofsky and Burks, 1988) using the GCG Wisconsin Package™ SeqLab® v.10 (Genetics Computer Group). MODELTEST (Posada and Crandall, 1998) was used to establish maximum likelihood parameters for analysis of the data set. The maximum likelihood algorithm in PAUP 4.0b10 (Swofford, 1999) was used to reconstruct the plastid phylogeny. Maximum likelihood analysis was run with the following HKY-G parameters: base frequencies A = 0.2936, C = 0.1882, G = 0.2549; a transition/transversion ratio of 2.2462; a gamma distribution of 0.4365; the proportion of invariant sites was zero. Heuristic searches were conducted using starting trees obtained by random stepwise addition. Numbers shown at nodes indicate bootstrap support for those branch points based upon 100 bootstrap replicates using parsimony.

Chloroplast uptake

Approximately 350 dinoflagellates were picked by micropipetting from the mixed dinoflagellate/*Phaeocystis* W5-1 culture. The dinoflagellates were grown for 2 months in the absence of *Phaeocystis* (starved) at 1°C in Sargasso seawater F/2 + Si at 1°C on a 14:10 photoperiod. All wells containing dinoflagellates were confirmed to be unialgal (no *Phaeocystis* contamination) by visual observation, and dinoflagellates were gradually combined over a 2-week period into a 250-ml Costar tissue culture flask. Assuming that each of the original dinoflagellates had at least 20 plastids per cell, they would need to go through six doublings to reach three plastids per cell, giving a total of about 22 400 dinoflagellates.

Four experimental treatments with triplicates were generated by aliquoting 15 ml of unialgal dinoflagellate culture at a density of 10^2 cells per ml into 25-ml culture tissue flasks (a total of 18 000 dinoflagellate cells). *Phaeocystis* was washed twice by centrifuging at 1900 *g* for 20 min at 1°C and resuspended in chilled sterile Sargasso seawater. Half a millilitre of washed *Phaeocystis* at a density of 10^5 cells per ml was added to two treatments (one in normal photoperiod, one in constant dark). Two other treatments had no *Phaeocystis* added (one at normal photoperiod, one in constant dark). All incubations were carried out at 1°C. Aliquots of 100 μl were removed every 2 days for the first 2 weeks, and then every 14 days for the remainder of the experiment, and dinoflagellate and *Phaeocystis* densities were counted using a Palmer Maloney slide. The number of plastids were counted by fluorescence using a FITC long pass filter set for 50 random

dinoflagellates in each sample. It was not possible to reliably count greater than 20 plastids per cell, so this value was designated as the maximum. The average number of plastids per cell for each triplicate set was determined.

To establish that the dinoflagellate could not exponentially grow by ingesting *Phaeocystis* in the dark, a separate amended version of the plus *Phaeocystis* constant darkness treatment was established. Dinoflagellate cells were starved and aliquoted into triplicates as described previously. *Phaeocystis* at a density of 5×10^4 cells ml^{-1} was added as needed to maintain densities high enough to allow maximum ingestion of plastids and/or whole cells. Cells and plastids were counted as above. The dilution factor from adding *Phaeocystis* was taken into account for final dinoflagellate counts.

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