PLASTID DYNAMICS DURING SURVIVAL OF *DINOPHYSIS CAUDA DATA* WITHOUT ITS CILIATE PREY\(^1\)

Myung Gil Park\(^2\), Jong Soo Park, Miran Kim

Laboratory of HAB Ecophysiology (LOHABE), Department of Oceanography, Chonnam National University, Gwangju 500 757, Korea

and Wonho Yih

Department of Oceanography, Kunsan National University, Kunsan 573 701, Korea

To survive, the marine dinoflagellate *Dinophysis caudata* Saville-Kent must feed on the plastidic ciliate *Myrionecta rubra* (=*Mesodinium rubrum*), itself a consumer of cryptophytes. Whether *D. caudata* has its own permanent chloroplasts or retains plastids from its ciliate prey, however, remains unresolved. Further, how long *D. caudata* plastids (or kleptoplasts) persist and remain photosynthetically active in the absence of prey remains unknown. We addressed those issues here, using the first established culture of *D. caudata*. Phylogenetic analyses of the plastid 16S rRNA and *psbA* gene sequences directly from the three organisms (*D. caudata*, *M. rubra*, and a cryptophyte) revealed that the sequences of both genes from the three organisms are almost identical to each other, supporting that the plastids of *D. caudata* are kleptoplastids. A 3-month starvation experiment revealed that *D. caudata* can remain photosynthetically active for 2 months when not supplied with prey. *D. caudata* cells starved for more than 2 months continued to keep the plastid 16S rRNA gene but lost the photosynthesis-related genes (i.e., *psaA* and *psbA* genes). When the prey was available again, however, *D. caudata* cells starved for more than 2 months were able to reacquire plastids and slowly resumed photosynthetic activity. Taken all together, the results indicate that the nature of the relationship between *D. caudata* and its plastids is not that of permanent cellular acquisitions. *D. caudata* is an intriguing protist that would represent an interesting evolutionary adaptation with regard to photosynthesis as well as help us to better understand plastid evolution in eukaryotes.

Key index words: dinoflagellate; *Dinophysis*; kleptoplastid; *Myrionecta rubra*; plastid evolution

Abbreviations: BA, Bayesian analysis; DIC, differential interference contrast; *F*\(_{\text{m}}\), maximum fluorescence; *F*\(_{\text{v}}\), variable fluorescence; MCMC, Markov chain Monte Carlo; ME, minimum evolution; ML, maximum likelihood; MP, maximum parsimony

Dinoflagellates are highly intriguing protists in several ways. They are one of the important planktonic members causing harmful algal blooms in many parts of the world (Hallegraeff 1993). Dinoflagellates play diverse roles in aquatic ecosystems, serving as hosts, parasites, and even hyperparasites (i.e., parasites that infect other parasites) (Park et al. 2004). They also display a variety of nutritional modes, ranging from phototrophy to heterotrophy, and even mixotrophy (Schnepf and Elbrächter 1992, Stoecker 1999). Furthermore, dinoflagellates harbor an array of plastid types derived from several algal groups, including haptophytes, diatoms, cryptophytes, and prasinophytes (Schnepf and Elbrächter 1999), each with its own evolutionary history. For this reason dinoflagellates have been considered the "champions" of plastid endosymbiosis among eukaryotes (Hackett et al. 2004).

Species belonging to the genus *Dinophysis* are ideal for investigating plastid evolution, as the genus includes both photosynthetic and heterotrophic species (Hallegraeff and Lucas 1988). Rather than having peridinin-containing plastids as in most dinoflagellates, photosynthetic *Dinophysis* have aberrantly pigmented plastids of a cryptophyte origin (Schnepf and Elbrächter 1988, Lucas and Vesk 1990, Hewes et al. 1998, Takishita et al. 2002, Hackett et al. 2003, Janson and Granéli 2003, Janson 2004). Whether the cryptophyte-type plastids of *Dinophysis* species are permanent or periodically derived kleptoplastids (stolen plastids) remains unresolved. The presence of only two surrounding membranes, absence of plastid endoplasmic reticulum, and lack of a cryptophyte nucleomorph support the argument that *Dinophysis* species have a permanent chloroplast (Lucas and Vesk 1990, Schnepf and Elbrächter 1999). Furthermore, digested or partially digested plastids have never been observed inside food vacuoles of *Dinophysis* species (Lucas and Vesk 1990). By contrast, the remarkable similarity between 16S rRNA and *psbA* (encodes PSII reaction center protein...
D1) genes of plastids in *Dinophysis* and cryptophytes, along with occasional presence of polymorphic plastids in *Dinophysis* species (Hackett et al. 2003, Janson 2004, Minnhagen and Janson 2006), suggests that *Dinophysis* derives its plastids through kleptoplastidy.

Recently, we reported the first successful cultivation of *D. acuminata* and discovered that this species feeds on the ciliate *M. rubra* (Park et al. 2006), itself a consumer of cryptophyte prey (Yih et al. 2004). We have since established *D. caudata* in culture. In this study, we used cultures to compare plastid 16S rRNA and *psbA* genes sequences for *D. caudata*, its prey ciliate *M. rubra*, and the cryptophyte prey of *M. rubra*. The three experimental species belong to different evolutionary lineages. Thus, the plastid encoded 16S rRNA and *psbA* genes would be expected to have diverged if those genes were derived from permanent plastids of three independent species and if those genes in the plastids have coevolved with each genomic DNA. Further, we investigated how long *Dinophysis* plastids (or kleptoplastids) persist and remain photosynthetically active in the absence of prey *M. rubra*. To do this, we examined ecophysiology, morphology, and molecular signature (plastid 16S rRNA, *psbA*, and *psaA* [encoding the PSI P700 apoprotein A1] gene sequences) of *D. caudata* during a 3-month starvation experiment.

**MATERIALS AND METHODS**

**Cultures.** *D. caudata* (strain DC-LOHABE01) was established in culture by isolating single cells from seawater samples collected on 28 August 2006 near Namhae (34°50′10.16″ N, 127°48′48.25″ E) on the south coast of the Republic of Korea. *D. caudata* was grown in 30 psu 1/2-St medium (+5% v/v soil extract) at 20°C on a 14:10 light:dark (L:D) cycle, with cool-white fluorescent lamps providing 60 μmol photons m⁻² s⁻¹. As prey for *D. caudata*, the marine ciliate *M. rubra* (strain MR-MAL01; GenBank accession number EF195734, Park et al. 2007) was added to cultures every 2 to 3 d to give a ratio of *M. rubra* cells per *D. caudata* cells of 5:1.

Cultures of *M. rubra* were grown as described in detail elsewhere (Yih et al. 2004, Park et al. 2007), using a cryptophyte (strain CR-MAL01; GenBank accession number EF195735, Park et al. 2007) as prey. The cryptophyte culture was grown under the same conditions described above for *D. caudata*. *D. caudata* measured 84–93 μm in length and 37–54 μm in width; *M. rubra*, 18–36 μm in length and 14–25 μm in width; and the cryptophyte, 6–8 μm in length and 4–6 μm in width.

**Plastid gene sequences.** To determine if *Dinophysis*, *M. rubra*, and cryptophyte plastids are similar, we sequenced and compared the plastid 16S rRNA and *psbA* genes directly from *D. caudata* and *M. rubra* cells (strain MR-MAL01) and a cryptophyte strain CR-MAL01 in exponential growth phase. Procedures for DNA extraction, PCR amplification, DNA sequencing, and phylogenetic analysis are described in detail below.

**Plastid retention and functionality.** Stock cultures of *D. caudata* in exponential growth were fed *M. rubra* daily for 2 weeks to ensure that the dinoflagellate culture was well fed. The well-fed *D. caudata* cells were washed free of prey using gentle gravity filtration through 20 μm Nitex mesh (Sefar, Rüschlikon, Switzerland), split into two aliquots, and then diluted with fresh medium to prepare duplicate 300 mL flasks at cell densities of ~900 cells·mL⁻¹. The flasks were incubated under standard growth conditions and sampled at 3–7 d intervals over 3 months to assess cell numbers, photosynthetic efficiency, the presence of plastids and their fluorescence intensity, cellular starch content, presence of acid vacuoles, and the status of the plastid 16S rRNA, *psaA*, and *psbA* genes.

Estimates for cell abundance were obtained from acid Lugol’s-fixed (final concentration 2%) samples (3 mL) by enumerating cells present in microscope transects (×100, Olympus BX51 microscope; Olympus, Tokyo, Japan) of triplicate Sedgewick-Rafter chambers (Vision Scientific, Gwangju, Korea). For each chamber, successive transects were examined until 10 transects (~20% of the chamber area) had been scanned. Frequency of paired cells was determined by scoring at least 200 cells per sample as single or paired.

In vivo fluorescence measurements before (*F₀*) and after (*Fₘ*) addition of 3,3’,5’-dichlorophenyl-1,1’-dimethyl urea were made on 4 mL of culture using a Turner Designs (Sunnyvale, CA, USA) model 10-AU fluorometer to derive the maximum photochemical quantum yield of PSII (*Fₘ*/*F₀* where *Fₘ* is variable fluorescence, i.e., *Fₘ* – *F₀*, and *F₀* is maximum fluorescence), as an indicator of photosynthetic efficiency (Parkhill et al. 2001).

In addition to in vivo fluorescence measurements, plastid functionality was assessed at ~7 d intervals by monitoring variation in starch accumulations after shifting *D. caudata* cells to dark conditions for 1, 2, and 3 d and then shifting the 1, 2, and 3 d dark-treated cells back to light conditions for 1 d. Cellular starch accumulations were observed from acid Lugol’s-fixed samples.

The presence of acid vacuoles was examined from live cells stained with neutral red (Sigma-Aldrich, Germany). The neutral red stains any acid-containing vacuole, including autophagic vacuoles, not just food vacuoles, and thus we cannot differentiate acid vacuoles from food vacuoles in this study.

Light and epifluorescence micrographs of live and acid Lugol’s-fixed *D. caudata* cells were taken at 1,000 magnification using a digital camera (PowerShot G5; Canon, Tokyo, Japan) coupled to the Olympus BX51 microscope (Olympus) equipped with differential interference contrast and fluorescence cube (U-MWB2, 450–480 nm excitation, 500 nm emission).

**Recovery potentials of feeding and starch accumulation.** The potential for starving *D. caudata* to recover feeding and starch accumulation was assessed at 22 and 90 d after starvation by providing the saturating concentrations of prey, and the potential was investigated by observing the uptake of new plastids and starch accumulation at 1 and 7 d after addition of prey.

**DNA extraction, PCR amplification, and DNA sequencing.** Prior to extraction and purification of nucleic acids, stock cultures of *D. caudata* (strain DC-LOHABE01) and *M. rubra* (strain MR-MAL01) were washed free of prey with sterile seawater using 20 μm Nitex mesh and 12 μm Isopore membrane filters (Millipore Corp., Carrigtwohill, Ireland), respectively. Each fraction was examined under the light microscope to confirm no contamination with prey organisms. Prey-free *D. caudata* (strain DC-LOHABE01), prey-free *M. rubra* (strain MR-MAL01), and cryptophyte (strain CR-MAL01) used to feed *M. rubra* were harvested from 4 mL samples of the cultures using centrifugation for 3 min at 10,000 g. Nucleic acids were extracted and purified using AccuPrep™ Genomic DNA Extraction Kit (Bioneer, Seoul, Korea) according to the manufacturer’s instructions.
Amplifications of plastid 16S rRNA, psbA, and psaA genes were performed using common primer sets, cyb101 (5′-GARRGRCGTGGCAGTGA-3′) and bac2 (5′-ACCTTGTTACGCACTTACCG3′) for the plastid 16S rRNA gene (Janson 2004), baF3 (5′-ATCTCGCTCACCAGTTAYATHAGG-3′) and baR1 (5′-GTTGTTAGGCGTTACCGTTCGATCAGTATC-3′) for the psbA gene (Zhang et al. 2000), and psaA130F (5′-AACWACG-ACCTGATTTGGAA-3′) and psaAI600R (5′-GGATATGATGATGATGAW-3′) for the psaA gene (Yoon et al. 2002), respectively. The size of the PCR products from plastid 16S RNA, psbA, and psaA genes of each experimental species was ~800, 0.9, and 1.6 kb, respectively, and this was confirmed by agarose gel electrophoresis. The amplified products were purified using a PCR purification kit (Bioneer) according to the manufacturer’s instructions and then ligated into the pGEM-T Easy vector supplied with the pGEM-T Easy Vector System (Promega, Seoul, Korea) according to the manufacturer’s protocols. Plasmid DNA from putative positive colonies was harvested using a Bioneer plasmid purification kit. Typically, five to six positive clones from each strain were partially sequenced using the T7 promoter sequencing primer (i.e., 5′-ATATACGACTCACTATAG-3′) derived from the cloning vector, and subsequently, all partial sequences (~700 bp) were identified by a BLASTN search. Among the positive clones, including the identified partial sequences, one to two positive clones were selected and completely sequenced using the SP6 promotor sequencing primer (i.e., 5′-ATATACGACTCACTATAG-3′). Sequencing was performed with an Applied Biosystems (Seoul, Korea) automated sequencer (ABI 3730xl) at Macrogen Corp. in Seoul, Korea. The plastid 16S rRNA gene sequences from the cultured D. caudata (strain DC-LOHABE01), M. rubra (strain MR-MAL01), and cryptophyte (strain CR-MAL01) have been deposited in the GenBank under the following accession numbers: D. caudata strain DC-LOHABE01 (EU123324), M. rubra strain MR-MAL01 (EU123322), and a cryptophyte strain CR-MAL01 (EU123323). In addition, psbA sequences from the organisms have been deposited in GenBank under the accession number EU123327 for D. caudata (strain DC-LOHABE01), EU123325 for M. rubra (strain MR-MAL01), and EU123326 for a cryptophyte (strain CR-MAL01).

Phylogenetic analysis. The plastid gene sequences from the cultures were compared to the sequences of related taxa obtained from the GenBank database using a BLASTN search. The sequences from the cultures were manually aligned and edited with previously known sequences in the database. A total of 1,220 and 799 of unambiguously aligned sites was retained for phylogenetic analysis of plastid 16S RNA and psbA genes, respectively. Phylogenetic trees were inferred by the maximum likelihood (ML, Felsenstein 1981), maximum evolution (ME), and maximum parsimony (MP) methods using PAUP* 4b10 (Swofford 2002), and by Bayesian analysis (BA) using MrBAYES 3.0 (Hueslenbeck and Ronquist 2001) program. For the analyses except parsimony, HKY + gamma + I (~ln L = 5974.3879) and general-time-reversible (GTR) + gamma + I (~ln L = 4259.2251) models were selected for other phylogenetic analyses of plastid 16S RNA and psbA sequences using Modeltest version 3.04, respectively (Posada and Crandall 1998). For each ML analysis, the best tree was determined using 20 random additions and tree-bisection-reconnection (TBR), and 200 replicates were performed (neighbor-joining starting tree, then TBR). For distance and parsimony analyses, the ME and MP trees were found using 20 random additions and TBR branch swapping, and a bootstrap analysis (Felsenstein 1985) was performed with 10,000 replicates (five random additions and TBR). To estimate posterior probabilities, four simultaneous Markov chain Monte Carlo (MCMC) chains were run for 1,000,000 generations and sampled every 500 generations (burn-in 200,000 generations).

RESULTS

Plastid 16S rRNA and psbA genes. The plastid 16S rRNA gene sequence of D. caudata (DC-LOHABE01) was almost identical (99.9%–100% similarity) to those of M. rubra (MR-MAL01) and cryptophyte strain CR-MAL01. In the phylogenetic tree (Fig. 1a), D. caudata (DC-LOHABE01) formed a clade with M. rubra (MR-MAL01) and the cryptophyte strain (CR-MAL01), with high bootstrap support (ML, 99%; MP, 100%; ME, 100%) and posterior probability of 1. Interestingly, the plastid 16S rRNA genes from all Dinophysis species clustered together with those from the two cryptophytes, strain CR-MAL01 and Teleaulax amphioxia (AY453067), with the exception of A. acuminata (DQ006804), which grouped with Geminigeria cryophila (AB073111) with moderate bootstrap support (ML, 67%; MP, 85%; ME, 67%; Fig. 1a) and posterior probability of 0.63 (data not shown).

The psbA gene sequences from D. caudata (DC-LOHABE01), M. rubra (MR-MAL01), and a cryptophyte strain (CR-MAL01) were almost identical to each other, showing 99.9%–100% similarity in sequences. The psbA sequences from D. caudata (DC-LOHABE01), M. rubra (MR-MAL01), and a cryptophyte strain (CR-MAL01) grouped together within the chlorovelaotes as a monophyletic group with strong bootstrap supports (ML, 100%; MP, 100%; and ME, 100%) or high posterior probability of 1 in all our tree construction methods (Fig. 1b). The psbA gene sequences from our three cultured organisms clustered with the sequences from other Dinophysis species and the cryptophyte T. amphioxia (AY453068), with the cryptophyte G. cryophila as a sister group with high bootstrap support (ML, 99%; MP, 100%; ME, 100%) and posterior probability of 1. Interestingly, the cluster with sequences from Dinophysis species showed almost zero branch length.

Growth of D. caudata in the absence of prey. In the absence of prey, D. caudata cell numbers increased exponentially with a growth rate of 0.10 (±0.016) d⁻¹ over the first 7 d. Thereafter, D. caudata abundance increased slowly (0.02 ± 0.007 d⁻¹) through day 50, reaching a maximum concentration of ~5,000 cells mL⁻¹ and then rapidly declined toward the end of the experiment (Fig. 2a). Frequency of paired D. caudata cells increased to 80% over the first 7 d and thereafter sharply decreased to zero on day 31.

Plastid retention. Light and epifluorescence microscopic observations of live cells revealed that D. caudata plastids persisted throughout the 3-month starvation period (Fig. 3). In the beginning of the starvation experiment, D. caudata cells were characterized by bright yellow-orange fluorescence under blue-light excitation and apparent dark-brownish color under bright-field LM. Thereafter, the color of the cells gradually shifted to green, and
their fluorescence signals slightly diminished with increasing time of starvation. Neutral red staining of live cells revealed that *D. caudata*, which seems to digest the aged chloroplasts, contained several acid vacuoles after 50 d of starvation (Fig. 4). However, PCR products of the plastid 16S rRNA gene amplified from *D. caudata* with the primer set cyb101 and bac2 were detected without a significant decrease in band intensity on agarose gel throughout the 3-month starvation experiment (Fig. 5a).

**Plastid functionality.** The maximum photochemical quantum yield of PSII (i.e., $F_v/F_m$), as an indicator of photosynthetic efficiency, was $\sim 0.6$ over the first 17 d and thereafter gradually decreased to zero by the end of the experiment (Fig. 2b).

The PCR products of both *psbA* and *psaA* genes were obviously detected up to 56 d after starvation (Fig. 5, b and c). After day 56, band intensity of the PCR product of *psbA* gene on agarose gel gradually decreased, whereas the intensity of *psaA* gene quickly declined. Neither *psbA* nor *psaA* genes were detected at the end of the experiment.

Microscopic observations revealed that *D. caudata* cells accumulated starch as the product of photosynthetic activity mainly within their ventral projection and along the periphery of the dorsal side (Fig. 3). By day 7, starving *D. caudata* cells accumulated more starch than was present in well-fed cells on day 0, but starch content then declined with increasing starvation time, with only a few starch granules usually present by day 56. Thereafter, starch accumulations were not detected in *D. caudata*.

When well-fed *D. caudata* cells at day 0 were shifted to dark conditions, starch accumulations gradually began to be reabsorbed and then were exhausted completely within 3 d (Fig. 6). Shifting the 1, 2, and 3 d dark-treated *D. caudata* cells back to light conditions for 1 d resulted in the formation of new starch accumulations. The longer *D. caudata* cells were starved, the faster starch accumulations were exhausted when cells were shifted to dark conditions (not shown).

**Recovery of feeding and starch accumulation after starvation.** When *D. caudata* cells starved for 22 d...
were offered *M. rubra* as prey, they not only began to feed, but also accumulated starch granules (not shown). By contrast, *D. caudata* cells starved for 90 d were capable of feeding on *M. rubra* when reexposed to the prey for 1 d but failed to accumulate starch granules (Fig. 7). After exposure to prey for 7 d, however, they resumed starch accumulation.

**DISCUSSION**

Our results suggest that *D. caudata* can retain and use kleptoplasts up to 2 months after stealing them from its prey. Whether *Dinophysis* plastids are permanent or temporary kleptoplasts has long been a source of controversy (Schnepf and Elbrächter 1999, Takishita et al. 2002, Hackett et al. 2003, Janson 2004, Minnhagen and Janson 2006, Park et al. 2006, Nagai et al. 2008). However, both availability of established cultures of *Dinophysis* species and new recognition of the pathway of the plastid flow among the three cultured protists (Park et al. 2006, this study) now enable us to easily test this long-standing issue. To do this, we sequenced plastid 16S rRNA and *psbA* genes directly from the three cultured predators and prey (*D. caudata, M. rubra*, and a cryptophyte). Given that the *psbA* gene encoding the PSII reaction center protein D1 is considered highly conserved due to a low rate of base substitution (Morden and Sherwood 2002) and that the three experimental species in this study belong to different evolutionary lineages, the plastid 16S rRNA and *psbA* genes would be expected to have diverged if those genes originated from permanent plastids of three independent species and if those genes have coevolved with the genome of each species. By contrast, our cultured *D. caudata* had identical plastid 16S rRNA and *psbA* genes to its prey *M. rubra*, which in turn had identical plastid.

Fig. 2. Time course of (a) *Dinophysis caudata* cell abundance (filled circles) and percent paired cells (bars) and (b) photosynthetic efficiency ($F_v/F_m$) in the absence of prey. Error bars are ±SE.

Fig. 3. Plastid retention and functionality in *Dinophysis caudata*. (a–e) Light and epifluorescence (f–j) images of live *D. caudata* cells. (k–o) Starch accumulation in Lugol’s fixed samples. Scale bar = 20 μm. The scale bar in (a) applies to all panels.
16S rRNA and psbA genes as its cryptophyte prey. These results suggest that the plastids in *D. caudata* are kleptoplastids, rather than permanent plastids.

Our results, however, do not prove that *D. caudata* has kleptoplastids, as our data can also be interpreted as recent incorporation of plastids. Also, it is likely that molecular sequence data alone cannot fully answer the question. For example, previous molecular studies (Janson 2004, Minnhagen and Janson 2006) showing that genes (i.e., 16S rRNA and psbA genes) of *Dinophysis* plastids had the same sequence as those in *T. amphioxeia* suggested that *Dinophysis* ate cryptophytes directly (Nishitani et al. 2005, Carvalho et al. 2008). We now know that this is not the case (Park et al. 2006). To determine whether *D. caudata* has kleptoplasts or permanent plastids, future studies should use feeding experiments encompassing *M. rubra* strains having different plastid genes or using the same *M. rubra* strain grown on different cryptophyte species to follow the fate of prey plastids. TEM data would also be helpful in assessing whether *D. caudata* has its own plastids or is keeping those from *M. rubra* and/or the prey of *M. rubra*. For example, Hansen and Fenchel (2006) recently showed with TEM micrographs that the *Teleaulax* prey and endosymbiont of *M. rubra* have morphologically distinct plastids and reported that their isolate of *M. rubra* from temperate Danish waters feeds *Teleaulax* to obtain growth factors to keep its endosymbiont working properly. Whether the Korean isolate of *M. rubra* used in this study has permanent plastids or kleptoplastids obtained from its cryptophyte prey is uncertain. Further, we cannot completely rule out the possibility that *D. caudata* has some plastids of its own.

While most kleptoplastidic protists retain functional plastids of a few hours to ~14 d (Stoecker and Silver 1990, Fields and Rhodes 1991, Skovgaard 1998, Lewitus et al. 1999), *D. caudata* maintained plastids in the photosynthetically active state for ~2 months. Long-term retention of the plastids in protists can be seen in the ciliate *M. rubra* (Johnson et al. 2007) and a novel Antarctic dinoflagellate with haptophyte-type plastids (Gast et al. 2007). In addition to the plastids, these protists are known to retain the chloroplast-mitochondrial complexes (CMCs) and have the ability to divide and maintain CMCs (in case of *M. rubra*), as well as, more importantly, have the prey’s nucleus and/or the second eukaryotic nucleus (i.e., karyoklepty; Johnson et al. 2007). These characteristics make these protists distinguishable from other kleptoplastidic protists and probably help stabilize the stolen plastids within the new host. However, this is not the case in *D. caudata* as neither an algal nucleus, nucleomorph, nor
CMCs have been observed in electron microscopic studies (Lucas and Vesk 1990).

What sustains the long-term functional activity of the plastids in *D. caudata*? We may get some hints to answer the question from the sea slug (*Elysia chlorotica*)/algal (*Vaucheria litorea*) plastid association, in which the kleptoplastids continue to function for up to 9 months in the cytosol of the sea slug in the absence of an algal nucleus (Mujer et al. 1996, Rumpho et al. 2001). Recently, Worful et al. (2007) demonstrated the presence of algal nuclear genes (e.g., *prk* and *psbO*) in the genomic DNA of the sea slug as a result of horizontal gene transfer. In the case of *D. caudata*, the most plausible explanation is that many of the genes necessary for plastid function exist in its nucleus due to dinoflagellate evolutionary history. Dinoflagellates have acquired and lost plastids many times during their evolution. It is now believed that most dinoflagellates arose from a photosynthetic ancestor with peridinin-type plastids, and that heterotrophic species have lost the plastid and photosynthetic dinoflagellates lacking peridinin have replaced their peridinin containing plastids with a different type of plastid (Schnepf and Elbrächter 1999, Saldarriaga et al. 2001). Recently, Sanchez-Puerta et al. (2007) reported the presence of plastid genes in the heterotrophic dinoflagellate *Cryptophidium cohni*. Interestingly, *Karlodinium micrum*, a dinoflagellate with a tertiary haptophyte-derived plastid, has recently been reported to retain and use genes originating from the secondary endosymbiont (Patron et al. 2006). In this context, it seems likely that *D. caudata* may recycle remnant photosynthetic genes inherited from a photosynthetic ancestor for long-term functional activity of the plastids. To dissect the nature of this exceptionally long-lived plastid function in *D. caudata*, future studies should search for plastid-targeting genes originating from a peridinin-type plastid and encoding within the nuclear genome of the dinoflagellate.

Finally, long-term starvation (>2 months) of *D. caudata* resulted in the loss of photosynthetic activity, as indicated by the absence of starch accumulation and the lack of cellular fluorescence. In addition, unlike the plastid 16S rRNA gene, the photosynthesis-related genes (i.e., *psaA* and *psbA* genes) were no longer present in *D. caudata* starved for >2 months. In addition to DNA data, future studies need to explore the expression of those genes to estimate activity of the plastids, although not addressed in this study. We cannot rule out the possibility that lack of detection of the photosynthesis-related genes in *D. caudata* starved for 2 months may have resulted from differences in gene copy numbers. For example, the plastid genome of the cryptophyte *Guillardia theta* is known to have two copies of the 16S rRNA gene and one copy of the photosynthesis-related genes, such as *psaA*, *psbA*, and *rbcL* genes (Douglas and Penny 1999). Alternatively, the loss of some plastid genes (i.e., *psaA* and *psbA*) but not others (i.e., 16S rRNA) in starved *Dinophysis* cells may have resulted from the difference in location encoding those genes (e.g., the nucleomorph or the inner part of the plastid).

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**Fig. 6.** Variation in starch contents when shifting well-fed *Dinophysis caudata* at day 0 to dark conditions: (a) 1 d after shift to dark conditions; (b) 2 d after shift to dark conditions; (c) 3 d after shift to dark conditions; (d) 1 d after shifting 1 d dark-treated cells back to light conditions; (e) 1 d after shifting 2 d dark-treated cells back to light conditions; (f) 1 d after shifting 3 d dark-treated cells back to light conditions. Scale bar = 20 μm. The scale bar in (a) applies to all panels.
However, we did not obtain any sequence related to the nucleomorph from the whole DNA of *D. caudata* (data not shown). In addition, an ultrastructural study reported that *Dinophysis* lacks the nucleomorph (Lucas and Vesk 1990). Further, previous studies demonstrated that these genes in other organisms are encoded from the plastids, not the nucleomorph (Douglas et al. 2001, Wang et al. 2005, Gilson et al. 2006, Lane et al. 2007). Interestingly, cells starved for >2 months were able to reacquire plastids and slowly resumed starch accumulation when reexposed to prey for 7 d. Taken all together, the results indicate that the nature of the relationship between the *Dinophysis* species and its plastids is not that of permanent cellular acquisitions. The dinoflagellate appears to require photosynthesis to survive but perhaps does not have all the required “machinery” to support and maintain permanent plastids. The plastids in starving *Dinophysis* cells may not be lost completely and rather may exist as nonphotosynthetic “proplastids.” Thus, starving cells may only reacquire parts of plastids or gene products to run them.

Research on *D. caudata* using the established culture is still at its beginning, and the next step in research on *D. caudata* is to determine how its kleptoplastids remain functional for months. Future studies should search for plastid-targeting genes encoded in the nuclear genome of this dinoflagellate. This research would contribute to our understanding of plastid evolution and lateral transfer of genes and organelles in eukaryotes.

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Fig. 7. Recovery of feeding and starch accumulation of 3-months-starved *Dinophysis caudata* cell when fed *Myrionecta rubra* again: (a, d, g) before addition of prey *M. rubra*; (b, e, h) 1 d after addition of the prey; (c, f, i) 7 d after addition of the prey. Note that newly ingested plastids (indicated by arrows) are dark-brownish in color and strongly orange-fluorescent. Scale bar = 20 µm. The scale bar in (a) applies to all panels.
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