The marine photosynthetic dinoflagellates Dinophysis Ehrenb. species are obligate mixotrophs that require both light and the ciliate prey Myrionecta rubra (= Mesodinium rubrum) for long-term survival. Despite rapid progress on the study of Dinophysis using laboratory cultures, however, whether it has its own permanent plastids or kleptoplastids (i.e., stolen plastids from its ciliate prey) is not fully resolved. Here, we addressed this issue using established cultures of D. caudata Saville-Kent strain DC-LOHABE01 and cross-feeding/starvation experiments encompassing the prey M. rubra strain MR-MAL01 cultures grown on two different cryptophytes (strains CR-MAL01 and CR-MAL11). To follow the fate of prey plastids, psbA gene as a tracer was amplified from individually isolated D. caudata cells, and the PCR products were digested with a restriction enzyme, SfaNI. The RFLP pattern of the PCR products digested by SfaNI revealed that D. caudata continued to keep CR-MAL01-type plastids, while it lost CR-MAL11-type plastids with increasing starvation time. Our results suggest that Dinophysis treats in different ways plastids taken up from different cryptophytes via its ciliate prey M. rubra. Alternatively, D. caudata may already have its own CR-MAL01-type permanent plastid, with two types of plastids (CR-MAL01 and CR-MAL11) obtained from M. rubra being lost within 1 month. This result highlights the need to identify more accurately the origin of plastids in newly isolated photosynthetic Dinophysis species to resolve the issue of plastid permanence.

Key index words: Dinophysis; kleptoplastids; permanent plastids; plastid preference; restriction fragment length polymorphism

Abbreviations: BA, Bayesian analysis; ML, maximum likelihood

Since Park et al. (2006) reported the first successful establishment of Dinophysis acuminata in culture, studies on ecophysiological and biological aspects of Dinophysis species have been greatly accelerated. For example, several Dinophysis species (D. acuminata, D. caudata, D. fortii, and D. infundibululus) subsequently came to be established in laboratory cultures (Nagai et al. 2008, Nishitani et al. 2008a,b, Park et al. 2008, Kamiyama and Suzuki 2009), based on the biological interactions among three organisms: cryptophytes, Myrionecta rubra, and Dinophysis spp. (Park et al. 2006). Diarrhetic-shellfish-poisoning (DSP) toxin analysis of cultured D. acuminata has also been carried out (Kamiyama and Suzuki 2009). Kim et al. (2008) and Riisgaard and Hansen (2009) investigated the ecophysiological responses of D. acuminata, including species-specific growth and feeding characteristics and photosynthesis, and determined that it requires both light and the prey M. rubra for long-term survival (i.e., an obligate mixotroph). Despite recent and rapid progress on Dinophysis research, the important issue of plastid permanence in these photosynthetic dinoflagellates remains unresolved.

While photosynthetic Dinophysis species are well known to have 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 plastids of the photosynthetic species are permanent or periodically derived kleptoplastids (temporarily stolen plastids) has been controversial over the last decade. Ultrastructural studies support the argument that Dinophysis species have a permanent chloroplast, based on the presence of only two surrounding membranes, absence of plastid endoplasmic reticulum, and lack of a cryptophyte nucleomorph, along with no observations of digested or partially digested plastids inside their food vacuoles (Lucas and Vesk 1990, Schnepf and Elbrächter 1999). In contrast, the remarkable similarity between 16S rRNA and psbA (encodes PSII reaction center protein D1) genes of plastids in Dinophysis and cryptophytes (Janson 2004, Minnhagen and Janson 2006), along with occasional presence of morphic plastids in Dinophysis species (Hackett et al. 2003, Minnhagen and Janson 2006), suggests that Dinophysis acquires plastids through kleptoplastidy. Furthermore, Minnhagen et al. (2008) recently analyzed the plastid DNA content of Dinophysis norvegica.
cells in different stages of its cell cycle and found that there was no significant difference in plastid DNA content between the G1 and G2 phases, suggesting that this is consistent with kleptoplastidy.

Recently, we sequenced plastid 16S rRNA and psbA genes from *D. caudata*, its ciliate prey *M. rubra*, and the cryptophyte prey of *M. rubra* and showed that the sequences of both genes from the three organisms are almost identical to each other (Park et al. 2008). This finding suggested that the plastids in *D. caudata* may be kleptoplastids. This result, however, does not prove that *D. caudata* has kleptoplasts, as the data can also be interpreted as the result of recent incorporation of plastids. Thus, to distinguish unambiguously kleptoplastids from fully incorporated plastids, cross-feeding/starvation experiments encompassing either *M. rubra* strains with a different type of plastid gene or using *M. rubra* grown on cryptophyte species having different types of plastids is a prerequisite. If the plastids in *Dinophysis* are kleptoplastids, then the existing plastids would be replaced with newly acquired plastids from the prey. In case of permanent plastids or kleptoplastids, a cross-feeding experiment was performed, as shown in Figure 1. *D. caudata* (DC-LOHABE01) was fed two different cultures of *M. rubra* (strain MR-MAL01) grown on two different cryptophyte strains (CR-MAL01 and CR-MAL11). First, well-fed *M. rubra* (MR-MAL01) grown on cryptophyte strain CR-MAL01 was fully starved (i.e., kept without cryptophyte prey) for 4 months prior to being fed and grown on a new cryptophyte strain, CR-MAL11, for 10 months. Hereafter, the *M. rubra* strain fed strain CR-MAL11 will be for convenience referred to as *M. rubra* (MR-MAL01fed11). Then, 4-months-starved *D. caudata* (DC-LOHABE01) cells were individually isolated and transferred into six-well plates containing *M. rubra* (MR-MAL01fed11) and fresh new 1/2-Si medium using a micropipette and then were allowed to feed on the prey for 1 week. After this, *D. caudata* cells fed on *M. rubra* (MR-MAL01fed11), hereafter referred to as DC-LOHABE01fed11, were transferred into new six-well plates containing 1/2-Si medium only and starved again for 1 month (i.e., incubated without ciliate prey). During this experiment, individual *Dinophysis* cells for psbA gene amplification and its RFLP pattern analysis were picked under the microscope (Olympus, model number SZX7, Tokyo, Japan) from five different conditions: (i) well-fed *D. caudata* with *M. rubra* (MR-MAL01), (ii) 4-months-starved *D. caudata*, (iii) *D. caudata* fed *M. rubra* (MR-MAL01fed11) for 1 week, and (iv and v) 2-weeks- and 1-month-starved *D. caudata* after feeding on *M. rubra* (MR-MAL01fed11).

**DNA extraction.** While cryptophyte strains CR-MAL01 and CR-MAL11 were harvested from 4 mL samples of the cultures
using centrifugation (Vision Scientific, Gwangju, Korea; model number VS-15000 CEN II) for 10 min at 13,000g. Individual cells were used in the case of *M. rubra* and *D. caudata*. Nucleic acids were extracted and purified using AccuPrep® Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea) according to the manufacturer’s instructions.

**PCR amplification and RFLP analysis.** The *psbA* gene was amplified from cryptophytes, *M. rubra*, and *D. caudata* in the first PCR round using the primer set *pbAf3* (5'-AGCGTTACGTTCAGTGCATNACYTC-3') and *pbAr1* (5'-GAGCGTAGTTCTTGACATGATCAGTC-3') (Zhang et al. 2000). The PCR reactions were performed using MyGenie 96 Gradient Thermal Block (Bioneer). The PCR was run as follows: 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, 2 min at 72°C, and a final incubation for 10 min at 72°C. The size of the PCR products from amplified *psbA* gene fragments of each experimental species was 858 bp when analyzed by electrophoresis in a 1% agarose gel stained with ethidium bromide. When amplifying a small amount of DNA from single cells or long-term starved cells, it is likely that a biased template may not be detected. We ran nested-PCR reactions to avoid this. In nested-PCR reactions, 3 μL of the product from the first PCR round was used as a template in the second-round reaction. The second pair of primers was designed by shifting five bases toward the 3' end of the first primers' binding loci: *pbAf3* (5'-CGCTCCAGGTGTAAYTHGAYGTTGCATGCG-3') and *pbAr1* (5'-GAGCCTAGTTCTTGACATGATCAGTC-3'). The second PCR round was run as above, except that 20 instead of 35 cycles of 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C were used. RFLP analysis of the PCR products obtained from the second round was performed by restriction digestion with a SfaNI restriction enzyme (New England Biolabs, Beverly, MA, USA) for 4 h at 37°C. The digested products were then analyzed by electrophoresis in 1% agarose gels and visualized by ethidium-bromide staining and UV transillumination.

**DNA sequencing.** 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, Madison, WI, USA) according to the manufacturer’s protocols. Plasmid DNA from putative positive colonies was harvested using a PCR purification kit (Bioneer). Typically, 17–19 positive clones from each strain were partially sequenced using the T7 promoter sequencing primer (i.e., 5'-ATTAGGTCACATGATC-3') derived from the cloning vector, and subsequently, all partial sequences (5'-700 bp) were identified by a BLAST search. Among the positive clones, including the identified partial sequences, one positive clone was selected and completely sequenced using the SP6 promoter sequencing primer (i.e., 5'-ATTAGGTCACATGATC-3'). Sequencing was performed using an Applied Biosystems automated sequencer (ABI 3730xl) at Macrogen Corp. in Korea. The sequences from the cultures were manually aligned and edited with previously known sequences in the database using a MacGDE version 2.2 (http://www.msu.edu/~lintone/macgde/). A total of 1,568 (22 taxa) and 800 (20 taxa) unambiguously aligned sites were retained for phylogenetic analysis of 18S rRNA and *psbA* genes, respectively. Phylogenetic trees were inferred by the maximum-likelihood (ML) method (Felsenstein 1981) using PAUP* 4.0b10 for Macintosh and UNIX (Swofford 2002) and by Bayesian analysis (BA) using MrBayes 3.1.2 version (Huelsenbeck and Ronquist 2001). (TrN + 1 + G (lnL = 4690.0786) and GTR + I + G (lnL = 4308.1978) models were selected by hierarchical likelihood ratio test (hLRTs) for phylogenetic analyses of 18S rRNA and *psbA* sequences using ModelTest version 3.7, respectively (Posada and Crandall 1998). ML analysis was performed using the RAxML 7.0.4 program (Stamatakis 2006) with the GTR + G model. We used 200 independent tree inferences using # option of the program to identify the best tree. Bootstrap values were calculated using 1,000 replicates with the same substitution model. BA was performed with a GTR + I + G model. The Markov chain Monte Carlo (MCMC) process was set to four chains, and 2,000,000 generations were conducted. The sampling frequency was assigned as every 1,000 generations, and the first 800 trees were deleted to ensure that the likelihood had reached convergence.

**results**

18S rRNA and *psbA* genes of cryptophytes. Two different cryptophyte strains were used as potential plastid donors in this study. Phylogenetic analysis of 18S rRNA gene sequences revealed that the cryptophyte strain CR-MAL01 was closely related to *Teleaulax amphioxeia* (AJ007287) with moderate bootstrap support of 88% and high posterior probability of 1 (Fig. 2A). The 18S rRNA gene sequence of the cryptophyte strain CR-MAL11 was identical to that of *T. acuta* (AF508275) (similarity of 99.88%, data not shown), and phylogenetic analysis also showed that they formed a clade with moderate bootstrap support (ML, 90%) and high posterior probability of 1.

The *psbA* gene sequences from *D. caudata* (DC-LOHABE01fed11), *M. rubra* (MR-MAL01fed11), and the cryptophyte strain CR-MAL11 were almost identical to one another, showing 98.11%–99.65% similarity in sequences. The *psbA* sequences from *D. caudata* (DC-LOHABE01fed11), *M. rubra* (MR-MAL01fed11), and the cryptophyte strain CR-MAL11 clustered together as a monophyletic group with strong bootstrap supports (ML, 97%) or high posterior probability of 0.96 in our all tree construction methods and formed a sister group to other Dinophysis species and the cryptophyte *T. amphioxeia* (AY453068), with a moderate bootstrap support (ML, 80%) and posterior probability of 1 (Fig. 2B).

**Fig. 2.** Phylogenetic trees of 18S rRNA and *psbA* genes. (A) An 18S rRNA gene tree showing the phylogenetic position of cryptophyte strains CR-MAL01 and CR-MAL11 used in this study. (B) A *psbA* gene tree showing the phylogenetic position of *Dinophysis caudata* (DC-LOHABE01fed11), *Myriionecta rubra* (MR-MAL01fed11), and cryptophyte strain CR-MAL11. Bootstrap values (>50%) from maximum likelihood (ML; 1,000 replicates) and a Bayesian posterior probability of 0.5 or greater are indicated at nodes (presented in the order ML/PP). Accession numbers of each taxon are shown in parentheses. *, Bootstrap values of <50%.
RFLP patterns of psbA genes from cryptophytes, M. rubra, and D. caudata. SfaNI digestion of the PCR products amplified on psbA genes from two cryptophyte strains produced distinct RFLP patterns (Fig. 3). While the product from cryptophyte strain CR-MAL01 yielded an RFLP pattern consisting of two major fragments of 712 and 112 bp and one indiscernible small fragment of 34 bp, that from cryptophyte strain CR-MAL11 yielded an RFLP pattern (three discernible fragments of 545, 201, and 112 bp) that was distinct from that of the cryptophyte strain CR-MAL01.

SfaNI digestion of the product amplified on psbA gene from M. rubra strain MR-MAL01 produced the same RFLP pattern as that from cryptophyte strain CR-MAL01 (Fig. 3). Interestingly, the PCR product from M. rubra strain MR-MAL01fed11 displayed a different pattern from that for M. rubra strain MR-MAL01 or the cryptophyte strain CR-MAL11. SfaNI digestion of the product amplified on psbA gene from M. rubra strain MR-MAL01fed11 produced an RFLP pattern that was a kind of hybrid between the two cryptophyte strains CR-MAL01 and CR-MAL11.

SfaNI digestion of the products amplified on psbA genes from both well-fed D. caudata strain DC-LOHABE01 with M. rubra strain MR-MAL01 and 4-months-starved D. caudata strain DC-LOHABE01 produced the same RFLP patterns as those for cryptophyte strain CR-MAL01, as well as M. rubra strain MR-MAL01 (Fig. 3). When D. caudata cells were fed M. rubra strain MR-MAL01fed11 and starved for 2 weeks, they displayed RFLP patterns that were somewhat of a hybrid between those produced from the two cryptophyte strains CR-MAL01 and CR-MAL11. However, D. caudata starved for 1 month after exposure to M. rubra strain MR-MAL01fed11 displayed an RFLP pattern that was the same as those from cryptophyte strain CR-MAL01 and M. rubra strain MR-MAL01.

**Fig. 3.** RFLP patterns produced from SfaNI digestion of the psbA gene amplified from cryptophytes, *Myrionecta rubra*, and *Dinophysis caudata*. Lane 1: size marker; lane 2: cryptophyte strain CR-MAL01; lane 3: cryptophyte strain CR-MAL11; lane 4: M. rubra strain MR-MAL01; lane 5: M. rubra (MR-MAL01fed11) fed cryptophyte strain CR-MAL11; lane 6: well-fed *D. caudata* strain DC-LOHABE01 with M. rubra strain MR-MAL01; lane 7: 4-months-starved *D. caudata* strain DC-LOHABE01; lane 8: *D. caudata* (DC-LOHABE01fed11) fed M. rubra (MR-MAL01fed11) for 1 week; and lines 9 and 10: 2-weeks- and 1-month-starved *D. caudata* (DC-LOHABE01fed11) after feeding on M. rubra (MR-MAL01fed11).

**Fig. 4.** Possible scenarios related to the fate of plastids taken up by *Dinophysis caudata* from different cryptophytes via the ciliate prey *Myrionecta rubra*. (A) *D. caudata* without its own permanent plastid. (B) *D. caudata* with its own permanent plastid. Filled circles and squares represent the plastids having different molecular signatures (e.g., plastid 16S rRNA and *psa* A genes). X inside the plastids of *Dinophysis* indicates digestion. Note that possible scenarios within *M. rubra* were omitted here for simplicity. *T., Teleaulax.*

**DISCUSSION**

The presence of polymorphic plastids in *Dinophysis* cells collected from field samples is not unusual. For example, Hackett et al. (2003) reported that *Dinophysis* spp. have polymorphic plastid sequences of cryptophyte origin as well as of florideophyte origin from samples collected in Rhode Island and interpreted that it may result from the presence of florideophyte DNA within the food vacuoles of *Dinophysis* feeding on red algae. Koike et al. (2005) reported the presence of a haptophyte-type plastid in the heterotrophic dinoflagellate *Dinophysis mitra*. More recently, Minnha gen and Janson (2006) also reported the occurrence of polymorphic plastids in *Dinophysis* spp. from the Greenland Sea, where *Dinophysis* cells contained either the common *Teleaulax amphioxea*-type plastid or the less common *Geminigera cryophila*-type plastid, or both in the same cell. They interpreted the occurrence of polymorphic plastids as a strong indication that *Dinophysis* refills with cryptophyte plastids from the environment. Despite accumulating information about the occurrence of polymorphic plastids, however, little is known about the fate of plastids inside *Dinophysis* cells.

Analysis of RFLP patterns of the *psa* A gene from our study revealed that *D. caudata* can also have polymorphic plastids, as shown in previous studies. Interestingly, while *D. caudata* lost one (*Teleaulax acuta*-type plastid; CR-MAL11) of the two types of plastids with increasing starvation time, it continued to keep one plastid type (*Teleaulax amphioxea*-type plastid; CR-MAL01). However, our results should be interpreted with caution because we do not know the ori-
gin of plastids that persisted throughout 4 months of starvation. It seems that Dinophysis could acquire and temporarily retain a variety of plastid types through an intermediate carrier (i.e., the ciliate M. rubra) from more than one species of cryptophyte. However, it seems likely that Dinophysis does not treat all plastids obtained from M. rubra the same. That is, cryptophyte plastids of type 11 may be handled differently than cryptophyte plastids of type 1. Type 1, if obtained from M. rubra, was retained for 5 months or more, while type 11 was quickly lost within 1 month (Fig. 4A). Perhaps, if newly retained plastids are not fitted to or not similar to the existing “old original” plastids, Dinophysis appears to ultimately digest or eliminate them through cell division without keeping them intact for a longer time. However, it seems unlikely that Dinophysis eliminates plastids through cell division. This situation arises not only because cellular division would not entirely make plastids disappear (i.e., only reduce the number of plastids per cell), but also because it was observed only twice during the starvation experiment. This may in part explain why trials to establish Dinophysis species in culture are often unsuccessful even when fed the ciliate prey M. rubra. For example, Nagai et al. (2008) reported 13.5% and 80.0% of isolation success in D. fortii sampled from the Okhotsk Sea and Hiroshima Bay, respectively.

Alternatively, D. caudata may already have a CR-MAL01-type permanent plastid, with two types of plastids (CR-MAL01 and CR-MAL11) obtained from M. rubra being lost within 1 month (i.e., Fig. 4B). If this is the case, D. caudata could just as easily be holding on to dysfunctional aging plastids until it can feed and replenish them. Park et al. (2008) recently reported through light and epifluorescence microscopic observations that D. caudata plastids persisted throughout the 3-month-starvation period and that long-term-starved (about 2 months) D. caudata lost photosynthetic activity, although it continued to keep the plastid 16S rRNA gene. Whether Dinophysis only manages to use kleptoplastids acquired from prey with a similar type of plastid (e.g., species belonging to Tetraulax amphioxea-type in Fig. 2), however, remains an open question. Given that M. rubra grew well when provided with various cryptophyte prey (i.e., strains CR-MAL01, CR-MAL02, and CR-MAL05), which possessed different 18S rRNA genes (Park et al. 2007), but a similar type of plastid (Fig. 2 in this study), it seems reasonable to postulate that Dinophysis would also grow well when fed with a similar type of plastid.

In summary, this is the first study to show preference for retention of specific cryptophyte plastids in Dinophysis, when feeding on M. rubra prey with multiple plastid types. Our results also indicate the need to address the origin of plastids in newly isolated Dinophysis to resolve the issue of plastid permanence in the photosynthetic Dinophysis species in future study. Future research to address this question would enhance our understanding of plastid evolution and establishment in Dinophysis species.

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