

## DOES *DINOPHYSIS CAUDATA* (DINOPHYCEAE) HAVE PERMANENT PLASTIDS?<sup>1</sup>

Myung Gil Park, Miran Kim

LOHABE, Department of Oceanography, Chonnam National University, Gwangju 500-757, Korea

Sunju Kim<sup>2,3</sup> and Wonho Yih

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

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. infundibulus*) 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.

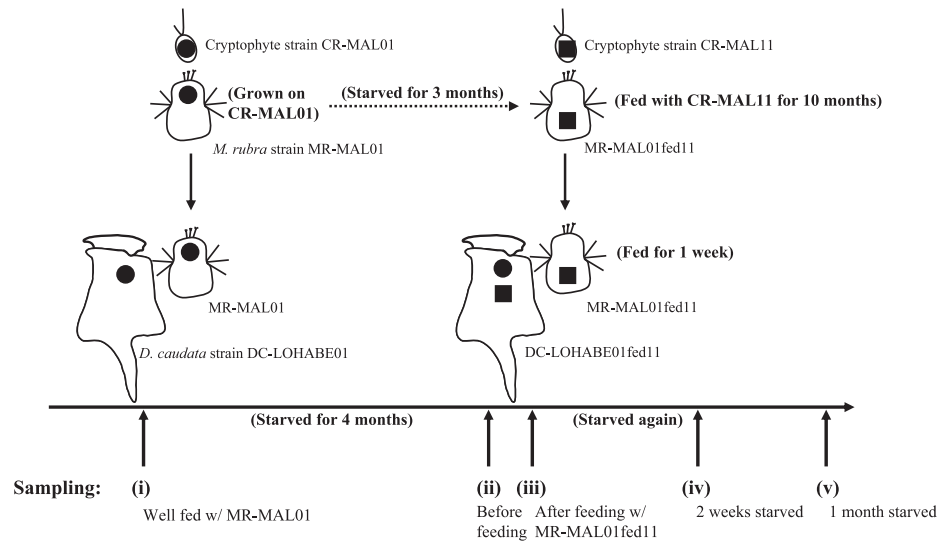
While photosynthetic *Dinophysis* species are well known to have plastids of a cryptophyte origin (Schnepf and Elbrächter 1988, Lucas and Vesik 1990, Hewes et al. 1998, Takishita et al. 2002, Hackett et al. 2003, Janson and Granéli 2003, Janson 2004), whether the plastids of the photosynthetic species 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 Vesik 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 polymorphic 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*

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<sup>2</sup>Author for correspondence: e-mail kimsu@si.edu.

<sup>3</sup>Present address: Smithsonian Environmental Research Center, P. O. Box 28, 647 Contees Wharf Rd., Edgewater, Maryland 21037, USA.

FIG. 1. Schematic representation of the experimental design used for a cross-feeding/starvation experiment in this study. Filled circles and squares represent the plastids having different molecular signatures (e.g., plastid 16S rRNA and *psbA* genes). *Myrionecta rubra* (MR-MAL01fed11) represents *M. rubra* (MR-MAL01) culture fed cryptophyte strain CR-MAL11. *Dinophysis caudata* (DC-LOHABE01fed11) represents *D. caudata* (DC-LOHABE01) culture fed *M. rubra* (MR-MAL01fed11).



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 kleptoplastids, 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 are 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, *Dinophysis* would continue to keep its original plastids even if it ingested the prey. Here, we followed the fate of prey plastids using established cultures of *D. caudata* strain DC-LOHABE01, *M. rubra* strain MR-MAL01, and two different cryptophytes (strains CR-MAL01 and CR-MAL11). We used the molecular signature of *psbA* gene as a tracer and analyzed its RFLP patterns.

#### MATERIALS AND METHODS

**Cultures.** Culture conditions of organisms used in this study were as described in Park et al. (2006, 2008). Briefly, stock cultures of *D. caudata* strain DC-LOHABE01 were grown using the marine ciliate *M. rubra* strain MR-MAL01 as prey in 30 psu f/2-Si medium (+ 5% v/v soil extract) at 20°C on a 14:10 light:dark (L:D) cycle, with cool-white fluorescent lamps provid-

ing  $50 \mu\text{mol} \cdot \text{photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ . Stock cultures of *M. rubra* were grown using the cryptophyte strain CR-MAL01 as prey (Yih et al. 2004, Park et al. 2007). The cryptophyte culture was grown under the same conditions described above for *D. caudata*. In addition, a second cryptophyte strain, CR-MAL11, was grown under the same conditions as strain CR-MAL01 for use as prey for *M. rubra* strain MR-MAL01 in a cross-feeding/starvation experiment (see below). The cell size of cryptophyte strain CR-MAL01 was  $4.2 \pm 0.12 \mu\text{m}$  in length and  $4.2 \pm 0.08 \mu\text{m}$  in width, and cell volume was  $60.8 \pm 3.63 \mu\text{m}^3$  (mean  $\pm$  SE;  $n = 30$ ). The cell size of cryptophyte strain CR-MAL11 was  $14.2 \pm 0.29 \mu\text{m}$  in length and  $7.2 \pm 0.11 \mu\text{m}$  in width (mean  $\pm$  SE;  $n = 30$ ). Its cell volume was  $196.1 \pm 8.80 \mu\text{m}^3$  (mean  $\pm$  SE;  $n = 30$ ), which was 3.2 times greater than that of cryptophyte strain CR-MAL01.

**Cross-feeding/starvation experiment.** To investigate if *Dinophysis* has permanent plastids or kleptoplastids, a cross-feeding/starvation 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 f/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 f/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 CFN 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<sup>®</sup> 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'-ATCTTCGC-TCCACCAGTTGAYATHGAYGG-3') and pbAr1 (5'-GTTGTG-AGCGTTACGTTTCRTGCATNACYTC-3') (Zhang et al. 2000). The PCR reactions were performed using MyGenie<sup>™</sup> 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' direction in the first primers' binding loci: pbAf3-2nd (5'-CGCTCCACCAGT-TGAYATHGAYGGTATCCG-3') and pbAr1-2nd (5'-GAGCGT-TACGTTTCRTGCATNACYTCATACCT-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<sup>®</sup>-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'-AATACGACTCACTATAG-3') derived from the cloning vector, and subsequently, all partial sequences (~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'-ATTTAGGTGACACTATAG-3'). Sequencing was performed using an Applied Biosystems automated sequencer (ABI 3730xl) at Macrogen Corp. in Korea. The sequences of 18S rRNA and *psbA* genes from cryptophyte strain CR-MAL11 have been deposited in GenBank under the accession numbers FJ884690 and FJ884691, respectively.

**Phylogenetic analysis.** The plastid gene sequences from the cultures were compared to the sequences of related taxa obtained from the GenBank database using a BLAST search. 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 + I + 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.

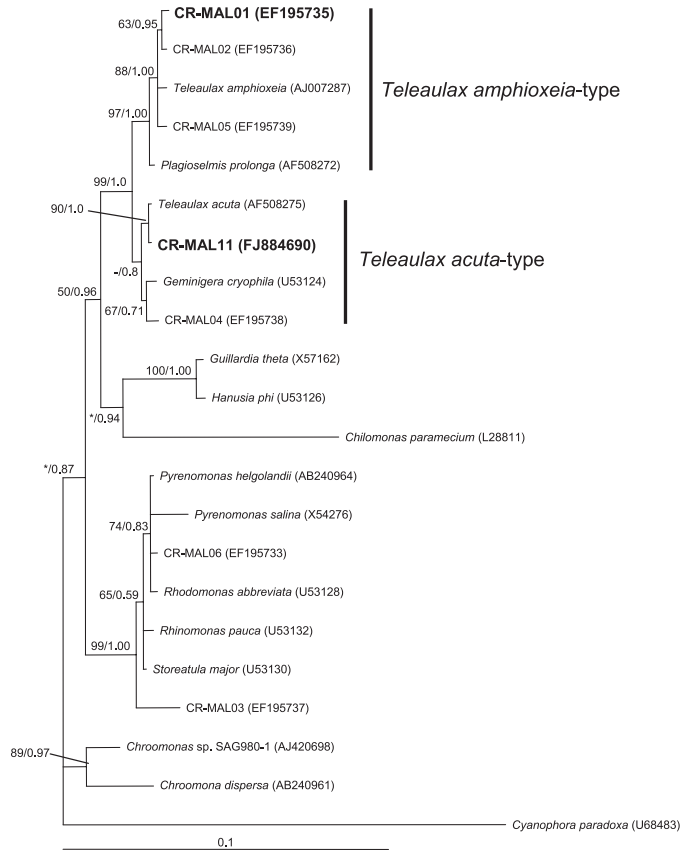
## RESULTS

**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 amphioxieia* (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.

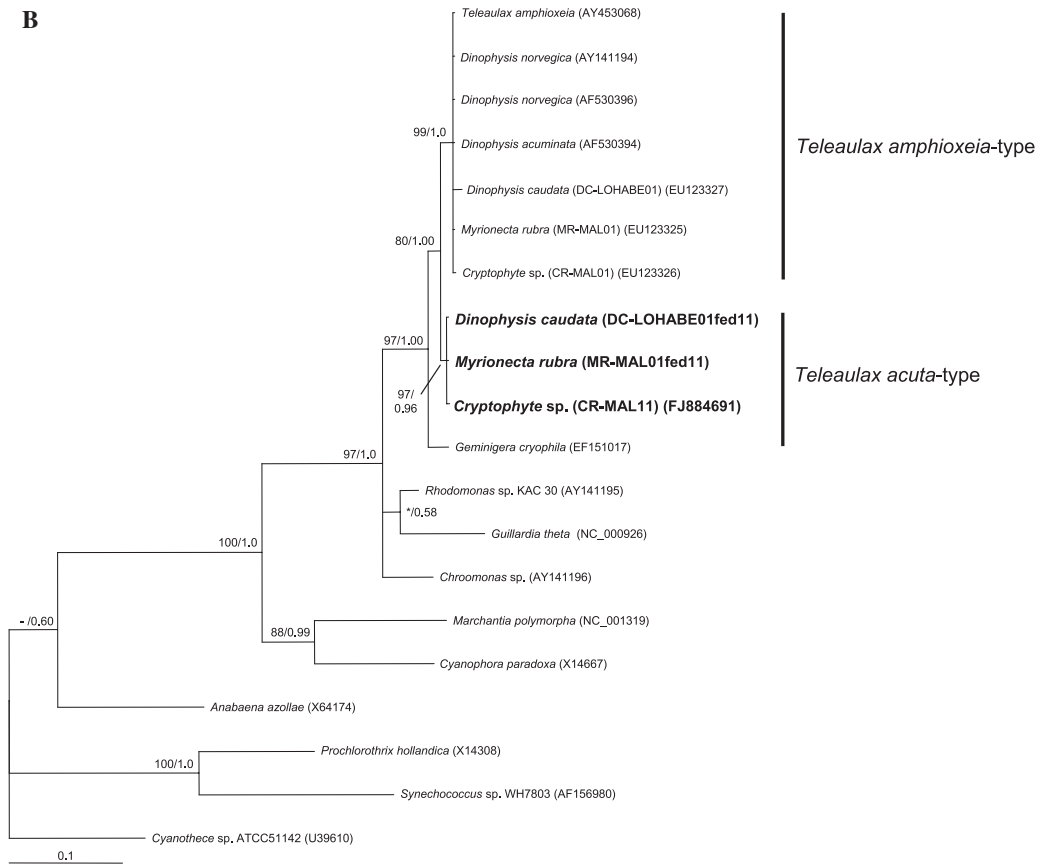
The *psbA* gene sequences from *D. caudata* (DC-LOHABE01fed11), *M. rubra* (MR-MAL01fed11), and the cryptophyte strain CR-MAL11 were almost identical to one another, showing 98.11%–99.65% similarity in sequences. The *psbA* sequences from *D. caudata* (DC-LOHABE01fed11), *M. rubra* (MR-MAL01fed11), and 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. amphioxieia* (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), *Myrionecta 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%.

**A**



**B**





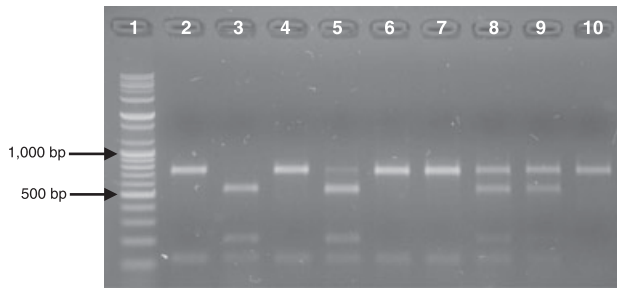


FIG. 3. RFLP patterns produced from SfaNI digestion of the *psbA* gene amplified from cryptophytes, *Myrionecta rubra*, and *Dinophysis caudata*. Lane 1: size marker; lane 2: cryptophyte strain CR-MAL01; lane 3: cryptophyte strain CR-MAL11; lane 4: *M. rubra* strain MR-MAL01; lane 5: *M. rubra* (MR-MAL01fed11) 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 lanes 9 and 10: 2-weeks- and 1-month-starved *D. caudata* (DC-LOHABE01fed11) after feeding on *M. rubra* (MR-MAL01fed11).

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.

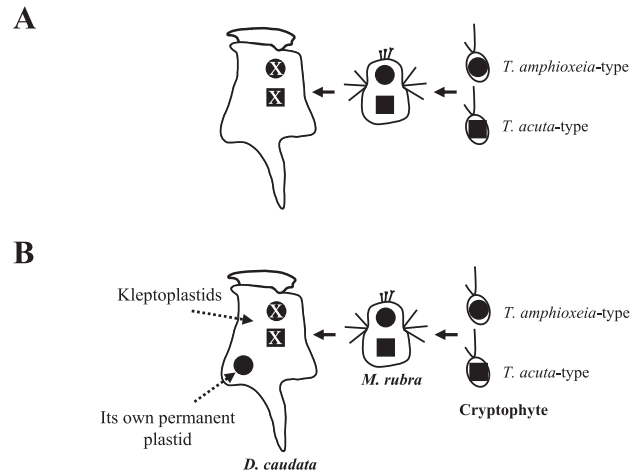


FIG. 4. Possible scenarios related to the fate of plastids taken up by *Dinophysis caudata* from different cryptophytes via the ciliate prey *Myrionecta rubra*. (A) *D. caudata* without its own permanent plastid. (B) *D. caudata* with its own permanent plastid. Filled circles and squares represent the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

#### 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, Minnhagen and Janson (2006) also reported the occurrence of polymorphic plastids in *Dinophysis* spp. from the Greenland Sea, where *Dinophysis* cells contained either the common *Teauleaux amphioxeia*-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 *psbA* gene from our study revealed that *D. caudata* can also have polymorphic plastids, as shown in previous studies. Interestingly, while *D. caudata* lost one (*Teauleaux acuta*-type plastid; CR-MAL11) of the two types of plastids with increasing starvation time, it continued to keep one plastid type (*Teauleaux amphioxeia*-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 *Teleaulax amphioxieia*-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 prey 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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