Multiple Plastids Collected by the Dinoflagellate *Dinophysis mitra* through Kleptoplastidy

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Kleptoplastidy is the retention of plastids obtained from ingested algal prey, which may remain temporarily functional and be used for photosynthesis by the predator. We showed that the marine dinoflagellate *Dinophysis mitra* has great kleptoplastid diversity. We obtained 308 plastid *rbcL* sequences by gene cloning from 14 *D. mitra* cells and 102 operational taxonomic units (OTUs). Most sequences were new in the genetic database and positioned within Haptophyceae (227 sequences [73.7%], 80 OTUs [78.4%]), particularly within the genus *Chrysochromulina*. Others were closely related to Prasinophyceae (16 sequences [5.2%], 5 OTUs [4.9%]), Dictyochophyceae (14 sequences [4.5%], 5 OTUs [4.9%]), Pelagophyceae (14 sequences [4.5%], 1 OTU [1.0%]), Bolidophyceae (3 sequences [1.0%], 1 OTU [1.0%]), and Bacillariophyceae (1 sequence [0.3%], 1 OTU [1.0%]); however, 33 sequences (10.8%) as 9 OTUs (8.8%) were not closely clustered with any particular group. Only six sequences were identical to those of *Chrysochromulina simplex*, *Chrysochromulina hirta*, *Chrysochromulina* sp. TKB8936, *Micromonas pusilla* NEPCC29, *Micromonas pusilla* CCMP491, and an unidentified diatom. Thus, we detected >100 different plastid sequences from 14 *D. mitra* cells, strongly suggesting kleptoplastidy and the need for mixotrophic prey such as *Laboea*, *Tontonia*, and *Strombidium*-like ciliates, which retain numerous symbiotic plastids from different origins, for propagation and plastid sequestration.

Dinoflagellates are a diverse group of unicellular flagellate eukaryotic algae which widely inhabit fresh and marine waters of the world and serve as significant primary producers. Many dinoflagellate species are photosynthetic. The diversity of plastids (chloroplasts) in dinoflagellates results from the acquisition of different types of photosynthetic endosymbionts (3, 10, 23, 49, 51, 54), heterotrophic nutrition (4, 36, 46), or both, i.e., mixotrophy (50, 60). The types of plastids found in dinoflagellates to date are classified into five categories: (i) a type containing chlorophyll *a* plus *c* (*a/c*) and fucoxanthin derived from a diatom, (ii) a chlorophyll *a/b* type derived from a chlorophyte, (iii) a chlorophyll *a/c* and fucoxanthin derivative type derived from a haptophyte, (iv) a chlorophyll *a/c* and phycobilin type derived from a cryptophyte (51), and (v) kleptoplastids (12, 49, 51). In addition to dinoflagellates with permanent plastids (permanent endosymbioses), there are several taxonomic groups of dinoflagellates having kleptoplastids (stolen plastids) (49), which are temporary but functional plastids captured from prey (6, 13, 20, 22, 29, 37, 40, 41, 44, 52, 57). *Amphidinium poecilochroum* (22), *Amphidinium latum* (13), *Amylax buxus* and *Amylax triacantha* (21), *Gymnodinium aeruginosum* (52), *Gymnodinium acidotum* (6, 62), *Gymnodinium gracilentum* (53), *Cryptoperidiniopsis* sp. (5), *Pfiesteria piscicida* (29), and mixotrophic *Dinophysis* species (35, 37, 40, 41, 44, 57) rob plastids from cryptophyte prey directly or indirectly.

Diarrhetic shellfish poisoning (DSP) is a severe gastrointestinal disease caused by the consumption of shellfish that are contaminated with DSP toxins (64). DSP toxins are derived from dinoflagellates belonging to the genera *Dinophysis* and *Prorocentrum* (28, 63). At present, 11 species of *Dinophysis* are known to be toxic (28, 30). Despite extensive studies in the last 2 decades, little is known about the ecophysiology, toxicology, and bloom mechanisms of *Dinophysis* species, primarily because of the inability to culture

them (15, 30, 38, 48). Recently, feeding by myzocytosis was revealed in *Dinophysis acuminata*, *Dinophysis caudata*, *Dinophysis fortii*, and *Dinophysis infundibulus*, with successful culturing of the species at high cell densities (0.8×10^3 to 11.0×10^3 cells ml⁻¹) (37, 40, 41, 44). The four *Dinophysis* species feed on the marine ciliate *Myrionecta rubra* (*Mesodinium rubrum*) (Mesodiniidae: Litostomatea) grown with the cryptophyte *Teleaulax*. The experimental data clearly showed that these species could not grow by ingestion of only the *Teleaulax* sp. Instead, the *Dinophysis* species required *M. rubra* as prey for enabling their vegetative growth and sequestered the ciliate plastids in order to utilize them as kleptoplastids (37).

Dinophysis mitra is known to be one of the toxic *Dinophysis* species (28) and a kleptoplastidic species, and on the basis of transmission electron microscopy (TEM) and molecular analysis, it is speculated that the kleptoplastids have originated from haptophytes (20). Usually, *D. mitra* retains a number of plastids in a cell (Fig. 1); these plastids are robbed from other microalgae, and they retain their photosynthetic activity in *D. mitra* cells (20). However, in this regard, only one study has been conducted thus far; therefore, plastid diversity in *D. mitra* and precise origins from prey organisms remain unknown. Naturally, no *D. mitra* culture has been established to date because of lack of prey information. In the present study, we analyzed the plastid *rbcL* (encoding the large

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FIG 1 Micrographs of a vegetative cell of *Dinophysis mitra* obtained from a natural sample in bright field (left) and with fluorescence under blue light excitation (right). Plastids in the *D. mitra* cell are emitting red autofluorescence. Scale bar, 30 μ m.

subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase [RuBisCO]) sequences in natural *D. mitra* cells isolated from Japanese coastal waters to reveal the origins of kleptoplastids in *D. mitra* and reported a previously unknown type of kleptoplastidy as the cause of the diversity of acquired plastids.

MATERIALS AND METHODS

Isolation of *D. mitra* and DNA extraction. A total of 14 natural *D. mitra* cells were isolated by micropipetting from seawater samples collected in the Mutsu Bay, (40°56'N, 140°51'E) in August 2009, Seto Inland Sea (33°53'N, 132°10'E) in July 2009, and Yatsushiro Sea (32°27'N, 130°29'E) in July 2009, in Japan (Fig. 2). Species identification was based on the morphological characteristics observed by light microscopy (1) and molecular analysis. Each cell of *D. mitra* was washed by several transfers in filtered (pore size, 0.1 μ m) seawater, separately inoculated into 0.2-ml tubes containing 15 μ l of 10% Chelex suspension (Bio-Rad Laboratories

Inc., Richmond, CA), and frozen at -30°C until use. DNA was extracted by heating at 95°C for 20 min, according to Richlen and Barber (47).

PCR and cloning of nuclear 18S rRNA and plastid *rbcl* genes in *D. mitra* cells. For amplifying the nuclear 18S rRNA gene of *D. mitra*, three primer pairs (18S-1F1/18S-1R632, 18S-2F576/18S-2R1209, and 18S-3F1129/18S-R1772) were used (Table 1), and the gene sequences were determined according to the method of Nishitani et al. (39, 41). In order to check for any contamination by symbiotic microorganisms within the *D. mitra* cells or any ingested prey species in food vacuoles, partial nuclear 18S rRNA gene sequences were also amplified from the DNA extracts of the *D. mitra* cells by using a universal primer pair (18S-F1289/18S-R1772) (Table 1), and 5 to 11 sequences (6.9 ± 1.8 , average $\pm$ standard deviation [SD]) were determined from each *D. mitra* cell (total number of sequences, 96).

For amplifying the plastid *rbcl* (encoding the RuBisCO large subunit) gene of *D. mitra*, two primer pairs (*rbcl*-F118/*rbcl*-R876 and *rbcl*-F184/*rbcl*-R704) were designed as universal primers to cover chromophyte microalgae (Bolidophyceae, Chrysophyceae, Cryptophyceae, Bacillariophyceae, Dictyochophyceae, Eustigmatophyceae, Haptophyceae, Pelagophyceae, Pinguiphyceae, and Raphidophyceae) and chlorophyte microalgae (Euglenophyceae, Chlorarachniophyceae, Prasinophyceae, Pedinophyceae, Chlorophyceae, and Trebouxiophyceae), respectively (Table 1). PCR was performed on a thermal cycler in a reaction mixture (50.0 μ l) containing 2.0 μ l of template DNA, 0.2 mM each deoxynucleoside triphosphate (dNTP), $1\times$ PCR buffer, 1.5 mM Mg^{2+} , 1.0 U of KOD-Plus, version 2 (Toyobo), that has intensive 3' $\rightarrow$ 5' exonuclease activity, and 1.0 μ M each primer. The PCR cycling conditions were as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles at 94°C for 15 s, at 50°C for 30 s, and at 68°C for 1 min. Results of PCR amplification were checked on 1.5% agarose gels, with ethidium staining.

PCR products were purified and concentrated using Suprec-PCR (Takara Bio, Shiga, Japan). Following an additional A-overhang elongation step, the purified PCR products were ligated to a pGEM-T Easy Vector (Promega, Madison, WI) and then transformed into ECOS competent

FIG 2 Sampling locations of natural *D. mitra* cells. The numbers in parentheses refer to the numbers of isolated cells.

TABLE 1 Details of the primers used in this study^a

Target region	Target organism	Primer name	Sequence (5'–3')	Annealing site (nt) ^b
Nuclear 18S rRNA	Marine dinoflagellate	18S-1F1	AACCTGGTTGATYCTGCCAG	1–20 ^c
		18S-1R632	ACTACGAGCTTTTAAACYGCARC	610–632 ^c
		18S-2F576	GGTAATTCAGCTCYAATRG	576–595 ^c
		18S-2R1209	AAGTTTCCCGTGTGARTC	1190–1209 ^c
		18S-3F1129	GCTGAAACTTAAAGRAATTGACGGA	1129–1153 ^c
		18S-F1289	TGGAGTGATTGTCTGGTTRATTCCG	1289–1314 ^d
Plastid <i>rbcL</i>	Marine eukaryotic microalga	18S-R1772	TCACCTACGAAACCTGTGTACG	1749–1772 ^d
	Marine eukaryotic microalga	<i>rbcL</i> -F118	ACWTGGACWGTWGTWGTGGAC	118–137 ^e
		<i>rbcL</i> -R876	CATCCAYTTACARATWACACGG	855–876 ^e
	Marine chlorophyte microalga	<i>rbcL</i> -F184	TCWACWGGWACWTGGACWAC	184–203 ^f
		<i>rbcL</i> -R704	AYTTCRCWGTTCWGHHTG	685–704 ^f

^a All primers were designed in the course of the present study.^b nt, nucleotides.^c *Dinophysis acuta* (AJ506973).^d *Chlamydomonas reinhardtii* (M32703).^e *Imantonia rotunda* (AB043696).^f *Ostreococcus tauri* (CR954199).

Escherichia coli DH5 α (Nippon Gene, Tokyo, Japan). Twenty-four white colonies were randomly chosen from each library and picked for sequencing. To check whether 24 colonies from a single *D. mitra* cell were enough to investigate the plastid diversity within the *D. mitra* cells, more than 100 white colonies were picked from a *D. mitra* cell (0908MUT01) and sequenced. The presence of the desired gene insert in the plasmid was verified by colony PCR with a U19 forward primer (5'-GGTTTCCCAGTCACGACG-3') and pUC/M13 reverse primer (5'-TCACACAGGAAACA GCTATGAC-3'). DNA sequences were determined using a DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) and analyzed on a DNA sequencer (ABI 3100; Applied Biosystems). The forward and reverse sequences were aligned using GENETYX software (Genetyx Corporation), and the aligned sequences were checked against the GenBank database by using the nucleotide Basic Local Alignment Search Tool (BLASTN).

Phylogenetic analysis. Partial sequences of the nuclear 18S rRNA and plastid *rbcL* genes were aligned with the sequences of other related species obtained from GenBank using the Clustal W algorithm (59) by careful checking manually, and neighbor-joining (NJ) trees were generated using the Molecular Evolutionary Genetics Analysis (MEGA) software, version 4.0 (58), with the default setting. Bootstrapping with 1,000 replications was used to estimate the reliability of the phylogenetic trees. Sequences

were regarded as the same operational taxonomic units (OTUs) according to McDonald et al. (31) if they had more than 99% similarity.

Nucleotide sequence accession numbers. Sequences obtained in this study for the nuclear 18S rRNA genes and plastid *rbcL* genes have been deposited in the DDBJ under the accession numbers shown in Fig. 3 to 5 for the respective genes.

RESULTS AND DISCUSSION

Analysis of the nuclear 18S rRNA gene in *D. mitra*. We determined the nuclear 18S rRNA gene sequences (1,740 bp) of the 14 *D. mitra* isolates to identify the exact species although there is no available data for this species in the GenBank at present. All the sequences matched completely and were closest to the sequence of *Phalacroma rapa* (Fig. 3), which is the most morphologically similar species to *D. mitra*, and 29 bp (including insertions/deletions of 4 bp) were different from those in the sequence of *Phalacroma rapa* EU780655. Phylogenetic analysis and observations of cell morphology indicated that the 14 isolated cells were certainly those of *D. mitra*. Using the universal primers for eukaryotic phytoplanktons, we also determined the partial nuclear 18S rRNA

FIG 3 A neighbor-joining tree inferred from the analysis of nuclear 18S rRNA genes. There were 1,667 informative sites in the final data set. A sequence of *D. mitra* obtained in this study is highlighted in gray. Bootstrap values of >50% are given as percentage of 1,000 bootstrap replicates at the respective nodes. *Prorocentrum micans* was used as the outgroup. The scale bar represents the number of substitutions per site.

Plastid *rbcL* gene

- Mutsu Bay
- ▲ Seto Inland Sea
- Yatsushiro Sea

Haptophyceae

Dictyochophyceae

Unknown (clade 1)

Diatoms

Bolidophyceae

Unknown (clade 2)

Chrysophyceae

Unknown (clade 3)

Pelagophyceae

TABLE 2 Number of plastid *rbcL* clones sequenced in each cell

Isolation area	Cell no.	No. of clones assigned to: ^a									Total no. of clones sequenced	No. of OTUs
		Bolido	Bacillario	Dictyocho	Hapto	Pelago	Prasino	Unknown, clade 1	Unknown, clade 2	Unknown, clade 3		
Mutsu Bay	0908MUT01	3	1	4	62	9			4	17	100	23
	0908MUT02				22		16	1		1	40	26
	0908MUT03				5	3		1		1	10	6
	0908MUT04				13	1					14	13
	0908MUT05				14			2			16	13
	0908MUT06			4	9						13	10
	0908MUT07			1	20			2		1	24	23
	0908MUT08				13						13	11
	0908MUT09			1	10	1					12	12
	0908MUT10			1	7			1	1	1	11	10
Seto Inland Sea	0907SET01				13						13	7
	0907SET02			2	12						14	11
Yatsushiro Sea	0907YAT01				15						15	12
	0907YAT02			1	12						13	11
Total		3	1	14	227	14	16	7	5	21	308	102

^a Bolido, Bolidophyceae; Bacillario, Bacillariophyceae; Dictyocho, Dictyochophyceae; Hapto, Haptophyceae; Pelago, Pelagophyceae; Prasino, Prasinophyceae; Unknown, unknown algae.

gene sequences in the DNA extracts of the *D. mitra* cells to check for any contamination by other microorganisms. No other species except *D. mitra* were detected in the 96 sequences detected from the 14 *D. mitra* cells, indicating no contamination in the DNA extracts of the *D. mitra* cells and no symbiotic microorganisms within the cells. In addition, these data also clearly showed that any nuclear DNAs, originated from microorganisms ingested in the food vacuoles, were not detected in the *D. mitra* cells.

Kleptoplastid diversity in *D. mitra* cells. When we used the primer pair *rbcL*-F118/*rbcL*-R876 targeting chromophyte microalgae, a total of 292 plastid *rbcL* sequences (717 bp) were determined by gene cloning from the 14 cells of *D. mitra*, and 97 operational taxonomic units were obtained (Fig. 4 and Table 2). Namely, 76, 17, and 21 OTUs were detected from the clone libraries in Mutsu Bay (10 cells of *D. mitra*), Seto Inland Sea (2 cells), and Yatsushiro Sea (2 cells), respectively. Most of the plastid *rbcL* sequences detected from the 14 *D. mitra* cells were novel ones in the GenBank database and positioned within the lineage of Haptophyceae (227 sequences, 80 OTUs), particularly within the genus *Chrysochromulina*. Other sequences were closely related to Dictyochophyceae (14 sequences, 5 OTUs), Pelagophyceae (14 sequences, 1 OTU), Bolidophyceae (3 sequences, 1 OTU), and Bacillariophyceae (1 sequence, 1 OTU); however, 33 sequences as 9 OTUs were not closely clustered with any particular group, and therefore these OTUs were provisionally named as unknown (clade 1, clade 2, and clade 3) (Fig. 4).

About 500 species are now recognized in Haptophyceae; however, the total number of species has been estimated at ca. 2,000, which is four times those currently known (2). This implies that

haptophyte diversity remains to be fully described. Recent genetic studies have suggested the existence of hidden diversity in Haptophyceae (7, 31), detecting a number of novel sequences from environmental clone libraries. Therefore, it is always possible that environmental samples contain previously undescribed species. However, we obtained four plastid *rbcL* sequences identical to those of *Chrysochromulina simplex* (Haptophyceae: Prymnesiales), *Chrysochromulina hirta* (Haptophyceae: Prymnesiales), *Chrysochromulina* sp. TKB8936 (Haptophyceae: Prymnesiales), and an unidentified diatom (Bacillariophyceae). The unidentified diatom is reported to be an endosymbiont in the marine dinoflagellate *Peridinium quinquecorne* (14). Nuclear 18S rRNA gene analysis indicated that the diatom species was closely related to *Chaetoceros* sp. (14), and plastid *rbcL* gene analysis showed a close phylogenetic affiliation with *Chaetoceros socialis* (Fig. 4).

When the primer pair *rbcL*-F184/*rbcL*-R704 was used for chlorophyte microalgae, a very thin PCR band was obtained only for the cell of *D. mitra* 0908MUT02. Five different OTUs from 16 plastid *rbcL* sequences (481 bp) were obtained (Fig. 5 and Table 2). All the OTUs were positioned within the lineage of Prasinophyceae; four of the five OTUs were positioned in the *Micromonas pusilla* clade, and the remaining one OTU was positioned in the *Pyramimonas* clade. Chlorophyte plastids appear to be markedly less abundant than chromophyte plastids in the natural *D. mitra* cells.

In the plastid *rbcL* gene analysis, 10 to 40 sequences (16.0 ± 4.9 , average $\pm$ standard deviation [SD]) as 6 to 26 OTUs (13.4 ± 6.1 , average $\pm$ SD) were successfully determined in 13 *D. mitra* cells when 24 or 48 (in the case of *D. mitra* 0908MUT02) white colonies

FIG 4 Plastid diversity in chromophyte microalgae detected from natural *D. mitra* cells, determined using the primer pair *rbcL*-F118/*rbcL*-R876. The tree topology is based on the neighbor-joining method and was constructed with MEGA, version 4.0. There were 627 informative sites in the final data set. The plastid *rbcL* sequences detected from the *D. mitra* cells are indicated in colored letters. The numbers in parentheses refer to the total number of clones in each OTU. Bootstrap values of $>50\%$ are given as a percentage of 1,000 bootstrap replicates. *Palmaria palmata* was used as the outgroup. The scale bar represents the number of substitutions per site. *, sequence of a diatom endosymbiont in the marine dinoflagellate *Peridinium quinquecorne* (14).

FIG 5 Plastid diversity in chlorophyte microalgae detected from a *D. mitra* cell (0908MUT02), determined using the primer pair *rbcL*-F184/*rbcL*-R704. The tree topology is based on the neighbor-joining method and was constructed with MEGA, version 4.0. There were 447 informative sites in the final data set. The plastid *rbcL* sequences detected from the *D. mitra* cell are highlighted in gray (the Mutsu Bay sample). The numbers in parentheses refer to the total clone numbers in each OTU. Bootstrap values of >50% are given as a percentage of 1,000 bootstrap replicates. *Chlorarachnion reptans* was used as the outgroup. The scale bar represents the number of substitutions per site.

were picked after cloning; these sequences were clustered into four algal classes and three unknown groups (Table 2). On the other hand, 100 sequences as 23 OTUs were detected from a single cell of *D. mitra* (0908MUT01) and positioned closely to six algal classes with two unknown algal groups. Comparison of these data indicates that selection of 24 white colonies was probably not good enough to cover the whole plastid diversity in a single *D. mitra* cell. Plastid *rbcL* sequences obtained from the *D. mitra* cells in the Mutsu Bay were widely distributed in several taxonomic groups, whereas those obtained in the Seto Inland Sea and Yatsushiro Sea were distributed only in Haptophyceae and Dictyochophyceae; this was mainly caused by differences in the numbers of *D. mitra* cells and sequences examined. Naturally, the diversity and composition of plastids in a *D. mitra* cell may vary considerably depending on individual cells and environmental conditions for the growth of *D. mitra* itself or the prey species.

Koike et al. (20) demonstrated that six sequences of the plastid 16S rRNA gene detected from approximately 30 cells of *D. mitra* were all positioned within the lineage of Haptophyceae. Moreover, we obtained in total 102 OTUs assigned to several taxonomic groups from 14 natural cells of *D. mitra*. Thus, we provided novel information on such a previously unknown type of kleptoplastidy in *D. mitra*, showing a remarkable difference from *D. acuminata*/*D. caudata*/*D. fortii*/*D. infundibulus*, whose plastids originated from only a few particular cryptophytes.

Kleptoplastidy in *Dinophysis*. Nonphotosynthetic species of *Dinophysis* feed by myzocytosis, a process in which the peduncle (or feeding tube) sucks the cytoplasm from the prey, leaving behind the plasmalemma (11). Photosynthetic species share this structure; food vacuoles are often found in their cytoplasm, clearly

indicating mixotrophy (15, 19). Recent molecular analyses of several *Dinophysis* species (except for *D. mitra*) by using plastid sequences such as *psbA*, 16S rRNA, and *rbcL* gene sequences have also showed high-level congruence of the plastid sequences among *Dinophysis* species and the cryptophytes *Teleaulax*, *Geminigera*, and *Plagioselmis* (16, 17, 35, 39, 56, 57). This has led to the suggestion that the plastid of *Dinophysis* might be a kleptoplastid, a temporary but functional plastid captured from a prey (16, 33, 35). In a recent study, mixotrophy in *D. acuminata*/*D. caudata*/*D. fortii*/*D. infundibulus* was clearly showed by demonstrating that *Myrionecta rubra* grown on *Teleaulax* is required as prey for propagation (8, 18, 37, 40, 41, 43, 44). Although further examples are clearly required, this evidence strongly suggests that propagation of *Dinophysis* species, which are regarded as mixotrophic species, depends on the predator-prey interactions occurring with *M. rubra* and *Teleaulax*/*Plagioselmis*/*Geminigera*. Observation of the sequestration process of the chloroplasts ingested from *M. rubra* by *D. acuminata* (S. Nagai, unpublished data), *D. fortii* (37), and *D. infundibulus* (41) has indicated that the chloroplasts of *M. rubra* are ingested and dispersed in the *Dinophysis* cells before the ingestion of other cell contents to prevent them from being digested in the food vacuoles, clearly showing that the ingested chloroplasts can function as kleptoplastids. Plastid DNA content of *D. norvegica* cells in different stages of cell division was analyzed to directly test the possibility of plastid replication in this species in order to obtain a direct evidence of whether the ingested plastids are functional as permanent plastids or are simply retained as kleptoplastids; no replication signal of plastids was detected, which strongly suggests kleptoplastidic behavior (34). In addition, monitoring of photosynthetic activities by ^{13}C method in *D. fortii*

TABLE 3 List of possible kleptoplastidic dinoflagellates and plastid origins

Dinoflagellate species	Habitat	Species of plastid origin	Source of plastid ^a	Description of retained plastids detected by:		Reference or source
				TEM	PCR	
<i>Gymnodinium acidotum</i>	Freshwater	<i>Chroomonas</i> spp. (Cryptophyceae)	Direct ingestion	Plastids with cytoplasm		6, 61
<i>Amphidinium latum</i>	Marine	Cryptophyceae	NA	Plastids with cytoplasm		13
<i>Amphidinium poecilochroum</i>	Marine	<i>Chroomonas</i> (Cryptophyceae)	Direct ingestion	Plastids with cytoplasm		22
<i>Amylax buxus</i>	Marine	<i>Teleaulax amphioxeia</i> (Cryptophyceae)	NA	Plastids with cytoplasm		21
<i>Amylax triacantha</i>	Marine	<i>Teleaulax amphioxeia</i> (Cryptophyceae)	NA	Plastids with cytoplasm		21
<i>Cryptoperidiniopsis</i> sp.	Marine (estuary)	<i>Storeatula major</i> (Cryptophyceae)	Direct ingestion	Plastids		5
<i>Dinophysis acuminata</i>	Marine	<i>Geminigera criophila</i> (Cryptophyceae)	NA		Plastids	57
<i>Dinophysis acuminata</i>	Marine	<i>Teleaulax amphioxeia</i> (Cryptophyceae)	<i>M. rubra</i>		Plastids	16
<i>Dinophysis acuminata</i>	Marine	<i>Teleaulax amphioxeia</i> (Cryptophyceae)	<i>M. rubra</i>	Plastids		S. Nagai, unpublished data
<i>Dinophysis acuta</i>	Marine	<i>Geminigera criophila</i> (Cryptophyceae)	NA		Plastids	57
<i>Dinophysis caudata</i>	Marine	<i>Teleaulax amphioxeia</i> (Cryptophyceae)	<i>M. rubra</i>		Plastids	45
<i>Dinophysis fortii</i>	Marine	<i>Geminigera criophila</i> (Cryptophyceae)	NA		Plastids	57
<i>Dinophysis fortii</i>	Marine	<i>Teleaulax amphioxeia</i> (Cryptophyceae)	<i>M. rubra</i>		Plastids	35
<i>Dinophysis fortii</i>	Marine	<i>Teleaulax amphioxeia</i> (Cryptophyceae)	<i>M. rubra</i>	Plastids		37
<i>Dinophysis infundibulus</i>	Marine	<i>Teleaulax amphioxeia</i> (Cryptophyceae)	<i>M. rubra</i>		Plastids	40
<i>Dinophysis norvegica</i>	Marine	<i>Geminigera criophila</i> (Cryptophyceae)	NA		Plastids	57
<i>Dinophysis norvegica</i>	Marine	<i>Teleaulax amphioxeia</i> (Cryptophyceae)	<i>M. rubra</i>		Plastids	16, 34
<i>Dinophysis triplos</i>	Marine	<i>Teleaulax amphioxeia</i> (Cryptophyceae)	<i>M. rubra</i>		Plastids	T. Kamiyama, unpublished data
<i>Dinophysis mitra</i>	Marine	Haptophyceae	NA	Plastids		20
<i>Dinophysis mitra</i>	Marine	Bacillariophyceae	Unknown ciliates		Plastids	This study
<i>Dinophysis mitra</i>	Marine	Bolidophyceae	Unknown ciliates		Plastids	This study
<i>Dinophysis mitra</i>	Marine	Dictyochophyceae	Unknown ciliates		Plastids	This study
<i>Dinophysis mitra</i>	Marine	Haptophyceae	Unknown ciliates		Plastids	This study
<i>Dinophysis mitra</i>	Marine	Pelagophyceae	Unknown ciliates		Plastids	This study
<i>Dinophysis mitra</i>	Marine	Prasinophyceae	Unknown ciliates		Plastids	This study
<i>Gymnodinium aeruginosum</i>	Marine	Cryptophyceae	Direct ingestion	Plastids with cytoplasm		51, 52
<i>Gymnodinium gracilentum</i>	Marine	<i>Rhodomonas salina</i> (Cryptophyceae)	Direct ingestion	Plastids		53
<i>Pfiesteria piscicida</i>	Marine (estuary)	<i>Rhodomonas</i> sp. (Cryptophyceae)	Direct ingestion	Plastids		29
Unidentified dinoflagellate (W5-1)	Marine	<i>Phaeocystis antarctica</i> (Haptophyceae)	Direct ingestion	Plastids with nucleus		9
Unidentified dinoflagellate (RS24)	Marine	<i>Phaeocystis antarctica</i> (Haptophyceae)	Direct ingestion	Plastids with nucleus		9

^a Unknown ciliates were detected from another specimen of *Dinophysis mitra*, indicating the mixotrophy of *D. mitra* and sequestration of plastids through ingestions of ciliate prey. NA, not available.

without the prey ciliate after feeding heavily on *M. rubra* clearly indicated that the activities decreased rapidly within 1 week (S. Nagai, unpublished data). However, on the basis of TEM and laboratory culture experiments, Garcia-Cuetos et al. (8) found that several plastid features separated *D. acuminata* from both the cryptophyte *Teleaulax amphioxeia* and the ciliate *M. rubra*. Their interpretation of the data was that *D. acuminata* harbors permanent plastids of cryptophyte origin, not kleptoplastids. Therefore, it remains debatable whether the plastids of the dinoflagellate *Dinophysis* are temporarily sequestered (kleptoplastids) or permanently established (8, 43, 45). In this study, however, we detected more than 100 different plastid sequences from 14 *D. mitra* cells, which strongly suggested kleptoplastidic behavior. This would also be supported by the fact that natural *D. mitra* cells without plastids have occasionally been observed (20).

There has been accumulated information on kleptoplastidic dinoflagellate species and kleptoplastid diversities (Table 3). Most of the kleptoplastidic dinoflagellates seem to prefer to retain kleptoplastids derived from cryptophytes, especially *Teleaulax amphioxeia*, as observed in several mixotrophic *Dinophysis* species and *Amylax* species, with a few exceptions such as an unidentified Antarctic dinoflagellate that retains plastids from *Phaeocystis antarctica* (Haptophyta) (9) and *D. mitra* that retains many plastids of different origins (Fig. 4 and Table 3). Interestingly, in several dinoflagellate species such as *Amylax* species and *Gymnodinium acidotum*, cryptophycean symbionts (*Teleaulax amphioxeia* and *Chroomonas* sp.) with a nearly full complement of organelles are retained inside the cells (21, 61). However, in species such as *Dinophysis* species and *Gymnodinium gracilentum*, only plastids are retained inside the cells, and other organelles are digested soon

after they are ingested, possibly showing the evolutionary process of trial and selection to utilize kleptoplastids more functionally. Members of the genus *Dinophysis* are perhaps in the earliest stages of plastid acquisition (10). What “benefit” do some dinoflagellate species derive from kleptoplastids? The presence of starch indicates that photosynthate is produced (29). It has been assumed that the starch produced by cryptophyte kleptoplastids is an available carbon source for the host (23, 50), implying that photosynthesis is a critical mechanism for supplementing an energy source to survive starvation periods when food is scarce. In any case, we revealed that *D. mitra* has the novel type of kleptoplastidy.

Origin of plastids in *D. mitra*. Photosynthetic *Dinophysis* species generally contain plastids of cryptophyte origin; however, *D. mitra* cells show red fluorescent, non-phycoerythrin-containing plastids, indicating that the plastids are not of cryptophyte origin (Fig. 1). The molecular phylogenetic position of *D. mitra*, inferred from the plastid sequences, indicates that the *D. mitra* plastid is originated mainly of haptophytes. This is also supported by the morphological features of the plastids as observed using TEM; however, no other organelles such as nuclei and Golgi bodies were observed (20). Measurements of photosynthetic activity by ^{14}C incorporation method clearly showed the activity in the retained plastids, and analysis of the plastid 16S rRNA gene revealed heterogeneity in the sequences of six clones obtained from different *D. mitra* cells, which strongly suggests that the kleptoplastidy originated from several haptophyte species (20).

The presence of food vacuoles has been confirmed in natural *D. mitra* cells (20, 42), indicating predation of some unknown prey organisms by myzocytosis. In this study, we obtained more than 100 different plastid sequences from 14 cells of *D. mitra*; these sequences were assigned to several microalgal classes such as Bolidophyceae, Bacillariophyceae, Dictyochophyceae, Haptophyceae, Pelagophyceae, and Prasinophyceae. This suggests that *D. mitra* is a unique dinoflagellate, which can easily acquire functional plastids from many and different microalgal origins by feeding on several ciliate prey species, in comparison with other *Dinophysis* species possessing kleptoplastids, originating from only one or two particular cryptophytes obtained by ingestion of a ciliate prey, *M. rubra*.

It is well known that the ciliates *Laboea*, *Strombidium*, and *Tontonia* retain hundreds of plastids (symbiotic plastids) (24, 25, 26, 27, 55) from different origins, i.e., Chrysophyceae, Bacillariophyceae, Dinophyceae, Euglenophyceae, Haptophyceae, and Ulvophyceae, as inferred on the basis of cytological characteristics observed by TEM (25, 27, 55) and PCR (32). Plastids are always situated at the periphery of ciliate cells and not surrounded by the dense endoplasmic reticulum (i.e., present outside the digestive system) to be retained and utilized as functional plastids (27). Our data strongly suggest mixotrophy in *D. mitra*, which requires prey such as *Laboea*, *Tontonia*, and *Strombidium*-like ciliates for its propagation and carries out the sequestration of robbed plastids, in a manner similar to that of other *Dinophysis* species (37, 41). Establishing laboratory cultures would also further understanding of the ecophysiology and toxicology of toxic dinoflagellate species.

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