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High-Level Congruence of *Myrionecta rubra* Prey and *Dinophysis* Species Plastid Identities as Revealed by Genetic Analyses of Isolates from Japanese Coastal Waters[▽]

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We analyzed cryptophyte nucleomorph 18S rRNA gene sequences retained in natural *Myrionecta rubra* cells and plastid 16S rRNA gene and *psbA* sequences retained in natural cells of several *Dinophysis* species collected from Japanese coastal waters. A total of 715 nucleomorph sequences obtained from 134 *M. rubra* cells and 564 plastid 16S rRNA gene and 355 *psbA* sequences from 71 *Dinophysis* cells were determined. Almost all sequences in *M. rubra* and *Dinophysis* spp. were identical to those of *Teleaulax amphioxeia*, suggesting that *M. rubra* in Japanese coastal waters preferentially ingest *T. amphioxeia*. The remaining sequences were closely related to those of *Geminigera cryophila* and *Teleaulax acuta*. Interestingly, 37 plastid 16S rRNA gene sequences, which were different from *T. amphioxeia* and amplified from *Dinophysis acuminata* and *Dinophysis norvegica* cells, were identical to the sequence of a *D. acuminata* cell found in the Greenland Sea, suggesting that a widely distributed and unknown cryptophyte species is also preyed upon by *M. rubra* and subsequently sequestered by *Dinophysis*. To confirm the reliability of molecular identification of the cryptophyte *Teleaulax* species detected from *M. rubra* and *Dinophysis* cells, the nucleomorph and plastid genes of *Teleaulax* species isolated from seawaters were also analyzed. Of 19 isolates, 16 and 3 clonal strains were identified as *T. amphioxeia* and *T. acuta*, respectively, and no sequence variation was confirmed within species. *T. amphioxeia* is probably the primary source of prey for *M. rubra* in Japanese coastal waters. An unknown cryptophyte may serve as an additional source, depending on localities and seasons.

The marine dinoflagellate genus *Dinophysis* comprises photosynthetic and nonphotosynthetic members and is globally distributed in coastal and oceanic waters (12, 29, 30). Several members of the genus *Dinophysis* produce potent polyether toxins that can accumulate in filter-feeding bivalves, leading to a syndrome known as diarrhetic shellfish poisoning (DSP) in humans who consume tainted shellfish. These toxic algal species are important not only for their potential impact on public health but also from an ecological point of view because of their dual role as primary and secondary producers in complex microbial food webs. Despite extensive studies over the last 2 decades, little is known about the ecophysiology, toxicology, and bloom mechanisms of DSP-causing species of *Dinophysis*, primarily due to an inability to culture them. Since the first successful cultivation of *Dinophysis acuminata* by Park et al. (42), the understanding of *Dinophysis* biology and ecology has progressed considerably. Three other species (*Dinophysis caudata*, *Dinophysis fortii*, and *Dinophysis infundibulus*) are now available in culture, and it has become clear that these different *Dinophysis* species require the presence of both cryptophytes and the marine ciliate *Myrionecta rubra* to grow and proliferate (36, 37, 38). When presented with the marine ciliate *M. rubra* as prey, the four *Dinophysis* species mentioned above exhibited phagotrophic behaviors, sequestering the cytoplasm and plastids from within the ciliate through a feeding tube called a peduncle. *M. rubra* individuals prey upon cryptophytes, harboring their plastids, nuclei, and nucleomorphs; they also utilize the cryptophyte plastids for their own photosynthesis (21, 22, 23, 54). Hence, plastids from the cryptophyte are first transferred to the ciliate *M. rubra* and then transferred again to the dinoflagellate *Dinophysis* while their photosynthetic machinery remains functional as kleptoplastids (34). However, whether the plastids of *Dinophysis* are temporarily sequestered (kleptoplastid) or permanently established is still controversial (9, 34, 35, 41, 43, 50), as is the case of plastids in *M. rubra* (13, 23). Minnhagen et al. (34) reported that there was no significant difference in plastid DNA content between the G_1 and G_2 phases in natural *Dinophysis norvegica* cells, suggesting that this is consistent with kleptoplastidy. On the other hand, Garcia-Cuetos et al. (9) found that several plastid features separated *D. acuminata* from both the cryptophyte *Teleaulax amphioxeia* and the ciliate *M. rubra* based on observations by

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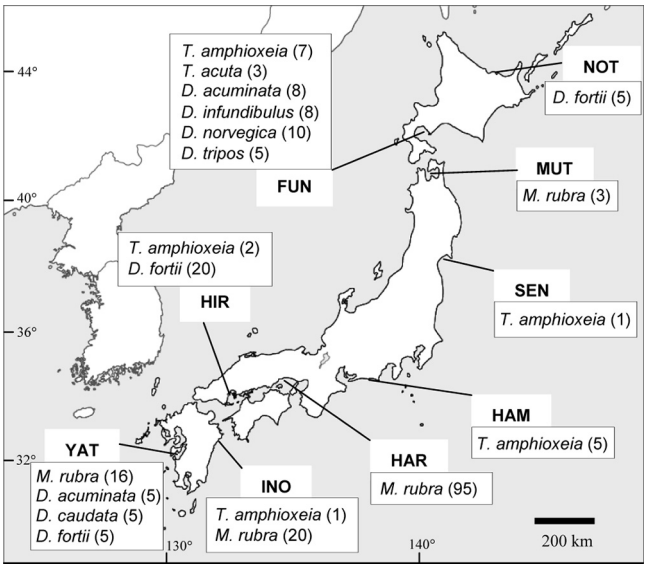

FIG. 1. Sampling locations of *M. rubra*, *Dinophysis* spp., and *Teleaulax* spp. Numbers in parentheses refer to the number of natural *M. rubra* or *Dinophysis* cells isolated and the number of *Teleaulax* strains in culture (background map courtesy of the Itsuki Corporation; reproduced with permission).

transmission electron microscopy (TEM) in laboratory culture experiments. Their interpretation of the data was that *D. acuminata* harbors permanent plastids of cryptophyte origin, not kleptoplastids.

In recent years, it has become clear that plastid sequences of the 16S rRNA gene and *psbA* (encoding the photosystem II [PSII] reaction center protein D1) in natural *Dinophysis* cells (except *Dinophysis mitra*) (27) originate from the cryptophyte genus complex *Teleaulax*/*Geminigera*/*Plagioselmis* (19, 35, 49, 50). Minnhagen and Janson (35) have also reported an exception to this finding in that natural *D. acuminata* cells isolated from the Greenland Sea contained a plastid 16S rRNA gene sequence that was more closely related to *Geminigera cryophila*. These characteristics are probably caused by the cryptophyte prey preference of *M. rubra* in natural environments. Under laboratory conditions, *M. rubra* can be cultivated by providing the cryptophyte *T. amphioxeia* (36, 37, 38), *Teleaulax acuta* (22), or *G. cryophila* (23), but its prey preference in natural environments has never been investigated.

Therefore, in this study, we investigated the prey organisms of *M. rubra* and the plastid identities of several *Dinophysis* species in Japanese coastal waters to reveal their ecological relationships in natural environments. The prey organisms of *M. rubra* were determined by sequencing the cryptophyte nucleomorph 18S rRNA gene in natural *M. rubra* cells; this genetic region has high substitution rates and facilitates species identification (16). The origins of plastids in *Dinophysis* spp. were revealed by sequencing the plastid 16S rRNA gene and *psbA* in natural *Dinophysis* cells. We also isolated several *Teleaulax*-like cryptophytes from Japanese coastal waters and sequenced their nuclear 18S rRNA genes, nucleomorph 18S rRNA genes, plastid 16S rRNA genes, and *psbA* in order to compare these genes with those in naturally occurring *M. rubra* and *Dinophysis* cells.

MATERIALS AND METHODS

Cryptophyte isolation, cultivation, and identification. Morphologically *Teleaulax*-like cryptophytes, which have a tail-like protrusion, were isolated from Japanese coastal waters (Fig. 1 and Table 1) and cultured according to the method of Nishitani et al. (38). Nuclear 18S rRNA gene sequences of each cryptophyte were determined for species identification, and the nucleomorph 18S rRNA gene, plastid *psbA*, and plastid 16S rRNA gene sequences were also determined to compare with those of *M. rubra* and *Dinophysis* spp. All sequences of cryptophytes were determined using DNA extracted from cultured cells.

Sample collection of natural *M. rubra* and *Dinophysis* cells. Sampling locations for *M. rubra* and *Dinophysis* spp. used in this study are shown in Fig. 1 and Table 1. *M. rubra* and *Dinophysis* cells were not collected all together at the same sites except for the Yatsushiro Sea because of the different peaks of blooms between the two species and the limited bloom seasons in Japan. In addition, living *Myrionecta* cells were very fragile when subjected to oscillation during transportation from the sampling sites to our laboratory. Seawater samples were collected at the surface layer. Single living cells were isolated, washed in several steps in drops of sterile seawater, and transferred into PCR tubes containing 10 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) by micropipetting under an

TABLE 1. The sampling sites and analyzed organisms

Sampling site	Abbreviation	Position	Sample date	Organism
Notoro saline lake	NOT	44.061°, 144.148°	7 July 2007	*D. fortii*
Funka Bay	FUN	42.553°, 140.680°	27 September 2008	*T. amphioxeia*
Funka Bay	FUN	42.553°, 140.680°	27 September 2008	*T. acuta*
Funka Bay	FUN	42.553°, 140.680°	8 June 2008	*D. acuminata*
Funka Bay	FUN	42.553°, 140.680°	8 June 2008	*D. infundibulus*
Funka Bay	FUN	42.553°, 140.680°	8 June 2008	*D. norvegica*
Funka Bay	FUN	42.553°, 140.680°	20 August 2007	*D. tripos*
Mutsu Bay	MUT	40.945°, 140.863°	19 August 2008	*M. rubra*
Sendai Bay	SEN	38.372°, 141.086°	10 June 2006	*T. amphioxeia*
Hamana saline lake	HAM	34.721°, 137.563°	15 April 2007	*T. amphioxeia*
Harima-nada	HAR	34.760°, 134.591°	1 November 2007	*M. rubra*
Hiroshima Bay	HIR	34.275°, 132.267°	7 February 2009	*T. amphioxeia*
Hiroshima Bay	HIR	34.275°, 132.267°	8 June 2007	*D. fortii*
Inokushi Bay	INO	32.803°, 131.892°	25 February 2007	*T. amphioxeia*
Inokushi Bay	INO	32.803°, 131.892°	28 January 2008	*M. rubra*
Yatsushiro Sea	YAT	32.171°, 130.317°	19 October 2007	*M. rubra*
Yatsushiro Sea	YAT	32.171°, 130.317°	12 July 2007	*D. acuminata*
Yatsushiro Sea	YAT	32.171°, 130.317°	12 July 2007	*D. caudata*
Yatsushiro Sea	YAT	32.171°, 130.317°	12 July 2007	*D. fortii*

TABLE 2. Primers used in this study

Primer	Sequence (5′–3′)	Annealing site bases[f]	Reference or source
S1F	AACCTGGTTGATCCTGCCAG	1–20[a]	38
S1R	CTACGAGCTTTTTAACTGCAACAA	571–594[b]	38
S2F	CTGAGAAACGGCTACCACATC	359–380[b]	38
S2R	TGGTAAGTTTTCCCGTGTTGAGTC	1146–1169[b]	38
S3F	AGCTTGCGGCTTAATTTGACTC	1129–1150[b]	38
S3R	CTACGGAAACCTTGTTACGAC	1707–1727[b]	38
Nmorph-F	CATAGGAAGGATTGACAGAT TGAG	1231–1254[c]	This study
Nmorph-R	AAGGCATTCCTCGTTCAAGATG	1578–1599[c]	This study
pSSU-F	TCGCGTCTGATTAGCTAGTTG	100–120[e]	This study
pSSU-R	ATGCACCACCTGTGTTCAC	882–900[e]	This study
psbAFdino	AGCACTGACAACCGTTTATAC	1–21[d]	11
psbAR2	TCATGCATWACTTCCATACCT	912–928[d]	11

[a] *G. theta* sequence X57162 (6).
[b] *T. amphioxeia* sequence AJ007287 (31).
[c] *T. amphioxeia* sequence AJ421146 (16).
[d] *D. norvegica* sequence AF530396 (11).
[e] *D. norvegica* sequence AF530388 (11).
[f] Annealing sites indicate primer positions from the 5′ termini of the sequences.

inverted microscope. Species identifications were based on morphological characteristics observed by light microscopy.

Primer design. For the amplification of nucleomorph 18S rRNA and plastid 16S rRNA genes, specific primers were designed to amplify the target regions in almost all cryptophytes with sequences registered in GenBank (Table 2). For the amplification of nuclear the 18S rRNA gene and plastid *psbA*, the primers were used as reported by Nishitani et al. (38) and Hackett et al. (11), respectively (Table 2).

DNA extraction, PCR amplification, cloning, and sequencing. PCR tubes, each containing 10 μl of TE buffer and single cells of *Myrionecta* or *Dinophysis*, were boiled at 99°C for 10 min to extract the DNA. In the extraction of cryptophyte DNA, 100 to 1,000 cells/strain collected from the culture were boiled as above. Boiled samples (1.0 μl) were used as templates to amplify the target regions. All PCRs were performed on a PCR thermal cycler (PC-816; Astec, Fukuoka, Japan) in a reaction mixture (20 μl) containing 1.0 μl of template DNA, a 0.2 mM concentration of each deoxynucleoside triphosphate (dNTP), 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl [pH 8.3]; Applied Biosystems, Foster City, CA), 2.5 mM Mg^{2+}, 0.25 U of Ampli*Taq* Gold (Applied Biosystems), and a 0.2 μM concentration of each primer. For amplifying the nuclear 18S rRNA gene in cryptophytes, three primer pairs (S1F/S1R, S2F/S2R, and S3F/S3R) (Table 2) were used, and the PCR cycling conditions were as follows: 5 min at 94°C, followed by 38 cycles of 30 s each at 94°C, 30 s at 56°C, 1 min at 72°C, with a final elongation for 5 min at 72°C. For amplification of the cryptophyte nucleomorph 18S rRNA gene, the Nmorph-F and Nmorph-R primer pair (Table 2) was used, and the PCR cycling conditions were the same as those mentioned earlier for the nuclear 18S rRNA gene. For amplifications of the plastid 16S rRNA gene, a specifically designed primer pair (pSSU-F and pSSU-R) (Table 2) was used, and the PCR cycling conditions were the same as those of the nuclear 18S rRNA gene, as mentioned above. For amplification of the plastid *psbA*, the primer pair psbAFdino and psbAR2 (11) was used, and the PCR cycling conditions were the same as those used for the nuclear 18S rRNA gene, except that the annealing temperature was 50°C. Results of the PCRs were checked on ethidium-stained 1.5% agarose gels, and PCR products were then transformed into ECOS Competent *Escherichia coli* DH5α cells (Nippon Gene, Tokyo, Japan) after ligation into the pGEM T-Easy Vector (Promega, Madison, WI). DNA sequences were determined using a Dynamic ET Terminator Cycle Sequencing Kit (GE Healthcare, Little Chalfont, United Kingdom) with a U19 forward primer (5′-GGTTTTCCCAGTCACGACG-3′) or pUC/M13 reverse primer (5′-TCACACAGGAAACAGCTATGAC-3′) and analyzed on a DNA sequencer (ABI3100; Applied Biosystems). The forward and reverse sequences were aligned in GENETYX software (Software Development, Tokyo, Japan), and the sequences were checked against GenBank using BLASTN (1).

Checking the effectiveness of primers and PCR bias. To confirm the effectiveness of all the primers (Table 2), we performed PCRs using the following eight extracted DNAs: *Chroomonas mesostigmatica* (CCMP269), *G. cryophila* (CCMP2564), *Guillardia theta* (CCMP327), *Hanusia phi* (CCMP325), *Hemiselmis rufescens* (CCMP644), *Proteomonas sulcata* (CCMP1175), *Rhodomonas salina* (CCMP1170), and *T. amphioxeia* (Japanese strain). Successful amplification was confirmed with the eight cryptophyte templates.

To check any bias during PCR and subcloning steps, we conducted a nucleomorph PCR and subcloning experiment using combined DNAs extracted from two cryptophyte species of *G. cryophila* and *T. amphioxeia* as a template. The ratio of DNA concentrations was 1:1 in the first test and 1:9 in the second. Sequences were determined under the same conditions as mentioned earlier, and the sequence ratios between the species were roughly reproduced in a total of 35 nucleomorph sequences.

Phylogenetic analysis. The partial sequences of the nuclear 18S rRNA gene, nucleomorph 18S rRNA gene, plastid 16S rRNA gene, and plastid *psbA* were aligned using the Clustal W algorithm (52) in MEGA software, version 4.0 (51). Maximum-likelihood analyses were performed with PhyML (10) using an input tree generated by BIONJ with a general time-reversal model (44) that incorporated invariable sites and a discrete gamma distribution (eight categories) (GTR+I+Γ). PhyML bootstrap trees (1,000 replicates) were constructed using the same parameters as the individual maximum-likelihood trees. Posterior probabilities of Bayesian trees were also estimated using MrBayes, version 3.1.1 (17, 46), under the GTR+I+Γ model. One cold and three heated Markov chain Monte Carlo (MCMC) chains were run for 500,000 generations to sample log likelihoods and trees at 100-generation intervals (5,000 trees were saved during MCMC with a burn-in of 1,250 trees).

RESULTS AND DISCUSSION

Genetic analyses in established clonal cultures of cryptophytes. In total, we established 19 clonal strains of *Teleaulax*-like species from Japanese coastal waters (Fig. 1) and identified 16 strains as *T. amphioxeia* and three as *T. acuta*, respectively. These two species were identified based on morphological characteristics (14, 15) and on their nuclear and plastid sequences. The partial nuclear 18S rRNA gene sequences determined in this study were 1,705 bp in *T. amphioxeia* and 1,706 bp in *T. acuta*. Sequences of *T. amphioxeia* in the 16 Japanese strains were identical to each other and showed 0.2% divergence from the sequence of *T. amphioxeia* (SCCAP K0434) found in Denmark, as reported by Marin et al. (31). These two sequences shared high sequence similarity with those of the cryptophyte species (CR-MAL01, CR-MAL02, and CR-MAL05) reported by Park et al. (40) and formed a clade with five operational taxonomic units (OTUs) (Fig. 2). All nuclear 18S rRNA gene sequences in the three strains of *T. acuta* were identical to the sequence of the *T. acuta* MUCC088 strain reported by Deane et al. (5) and formed a sister clade with *T. amphioxeia* and *Plagioselmis prolonga*. In the partial nucleomorph 18S rRNA gene (369 bp) sequences of 16 strains of *T. amphioxeia* and 3 strains of *T. acuta* isolated from Japanese coastal waters, no intraspecific sequence variations were detected in the two species (Fig. 3). Although we detected several sequence variations (4 to 10 bp) of the nuclear 18S rRNA gene among the Japanese *T. amphioxeia* strain, the Danish *T. amphioxeia* strain (K0434), and the Korean cryptophyte species (CR-MAL01), the strains had identical sequences in the plastid 16S rRNA gene (801 bp) and plastid *psbA* (890 bp) (Fig. 2, 4, and 5), thereby suggesting that these three isolates were the same species and that the sequence variations in the nuclear 18S rRNA genes were likely to be intraspecific variations.

Genetic analyses in natural *M. rubra* cells. In consideration of the possibility that different cryptophyte species coexist in a single *M. rubra* cell, we checked for a bias in PCR amplification efficiency by using a mixture of nucleomorph primers for DNAs from two species, *G. cryophila* and *T. amphioxeia*, at different ratios. When the ratio for *G. cryophila* and *T. am-*

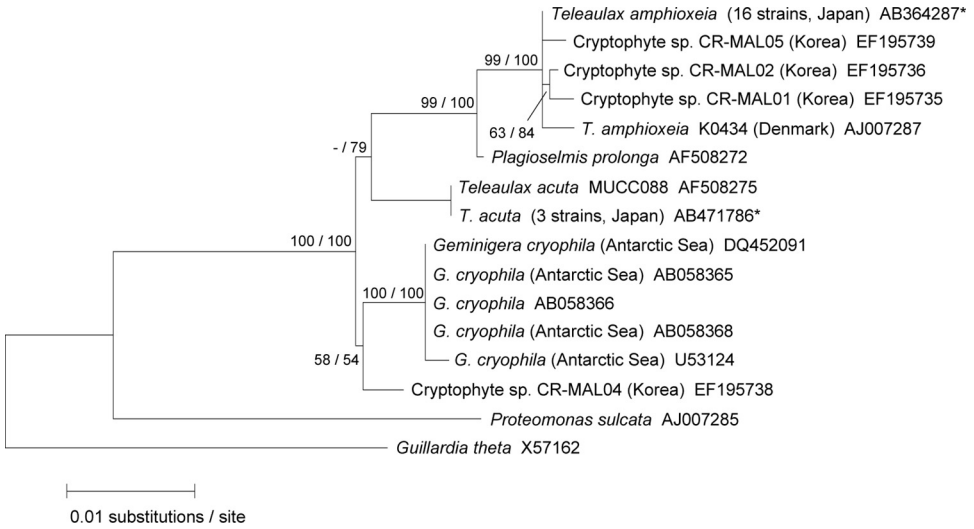

FIG. 2. A maximum-likelihood tree inferred from the cryptophyte nuclear 18S rRNA gene. Sequences obtained in this study are indicated by asterisks. The values at left are in the form bootstrap support values/posterior probabilities. −, a value less than 50.

phioxeia DNAs was 1:1, 18 and 17 sequences out of a total of 35 were detected, respectively; i.e., the sequence ratio was 1.0:1.1. Similarly, when the ratio of DNAs was 1:9, 4 and 31 sequences out of 35 were obtained, respectively; i.e., the sequence ratio was 1.0:7.8, which clearly showed that our PCR and cloning procedures provided highly reliable information on the detection of cryptophyte species.

The partial nucleomorph 18S rRNA gene sequences determined in this study comprised 369 bp and showed 8-bp substitutions between *T. amphioxeia* and *G. cryophila*. The percentages of cells of those isolated from natural *M. rubra* that were successfully PCR amplified varied and depended mostly on the sampling locations, ranging from 33% in Mutsu Bay to 100% in Harima-Nada (Table 3). A possible explanation for this might be that there were some artificial errors in the DNA extraction step and/or the PCR amplification. We determined a total of 715 nucleomorph sequences from 114 PCR products of 134 *M. rubra* cells. Of these, 713 sequences were identical to the sequence of *T. amphioxeia* (99.7%), while the other 2 sequences (AB471788 and AB471789, representing 0.3%), which were detected from the Harima-Nada station *M. rubra* cells (cell identifier [ID] HAR12 and HAR76), were closely related to those of *G. cryophila* and *T. acuta* (Table 3 and Fig. 3). These two *M. rubra* cells contained two different cryptophyte sequences in each cell; in each of these cells the sequences detected occurred in the proportion of seven sequences of *T. amphioxeia* to one other sequence. Knowledge of the availability of suitable prey for *M. rubra* is very limited, but up to now, *M. rubra* has been successfully cultured using *T. amphioxeia* (36, 37, 38), *T. acuta* (22), and *G. cryophila* (23) as prey. In addition, Park et al. (40) have isolated several *T. amphioxeia*-like cryptophyte species inferred from the nuclear 18S rRNA gene phylogeny and have cultured *M. rubra* by addition of those cryptophytes as prey. Considering these facts, the genera *Teleaulax* and *Geminigera* seem to be preferred prey for *M. rubra.* In this study, however, the nucleomorph sequences of *T. acuta* and *G. cryophila* were never detected from natural cells of *M. rubra*, suggesting that the two species are unlikely to become the prey of *M. rubra*, at least in Japanese coastal waters. Accordingly, this study found that *T. amphioxeia* is

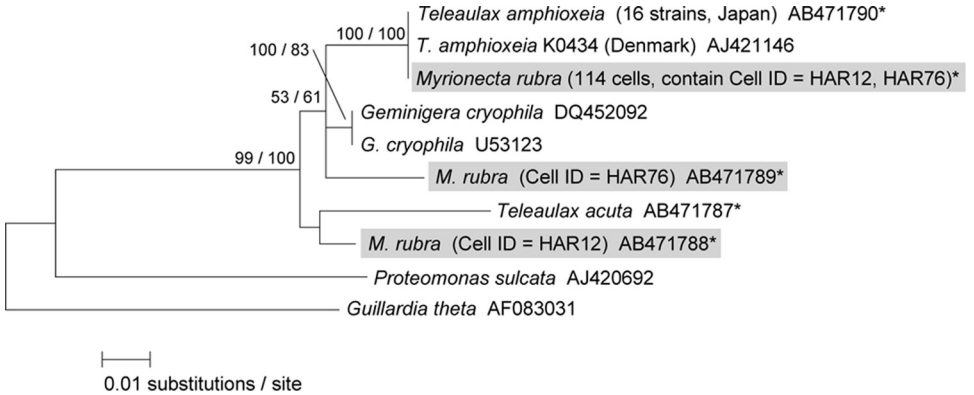

FIG. 3. A maximum-likelihood tree inferred from the cryptophyte nucleomorph 18S rRNA gene. Sequences obtained in this study are indicated by asterisks, and sequences detected from natural *M. rubra* cells are further indicated by gray boxes. The values at left are in the form bootstrap support values/posterior probabilities.

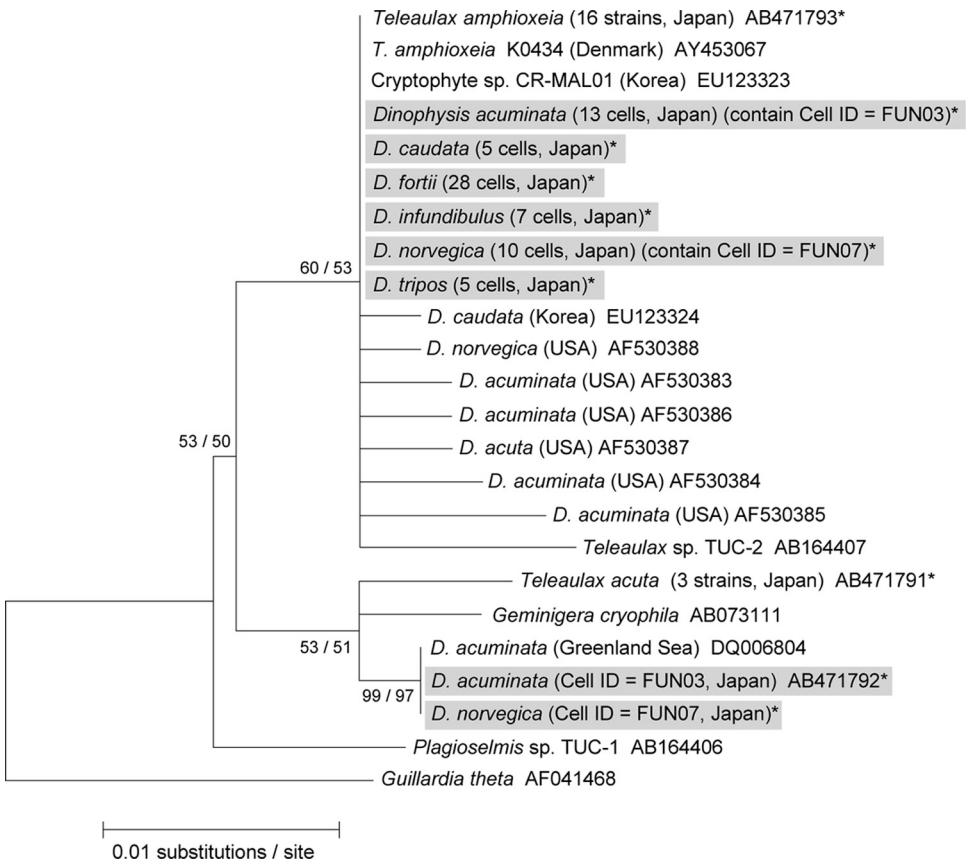

FIG. 4. A maximum-likelihood tree inferred from the plastid 16S rRNA gene. Sequences obtained in this study are indicated by asterisks, and sequences detected from natural *Dinophysis* cells are further indicated by gray boxes. The values at left are in the form bootstrap support values/posterior probabilities.

probably the most important species for *M. rubra* as a prey source. On the other hand, the nucleomorph analysis of *M. rubra* in this study also detected two sequences other than *T. amphioxeia* that were closely related to the sequences of *G. cryophila* and *T. acuta* (Fig. 3). The fact suggests that unknown cryptophytes exist and that they serve as an additional prey source for *M. rubra*. In fact, the possibility exists that unknown cryptophytes serve as a primary prey source for *M. rubra*, depending on localities and seasons in which *T. amphioxeia* is less abundant. Thus, it is important to isolate the unknown cryptophytes and conduct further culture experiments with *M. rubra*. In a recent examination of *M. rubra* based on ultrastructure by TEM and on the dynamics of photosynthesis, ingestion, and growth rates under laboratory conditions, Hansen and Fenchel (13) presented evidence showing that *M. rubra* harbors a permanent cryptophyte endosymbiont that has different and larger chloroplasts than the *Teleaulax* added as prey. Hence, it is possible that the different sequence may have originated from a permanent cryptophyte endosymbiont.

Currently, 17 genera of cryptophytes are recognized (4). The group contains about 100 freshwater species and a similar number of marine representatives. However, the total number of species has been estimated at about 1,200, which is six times more than are currently known (2), implying that cryptophyte diversity is far from being fully described. Therefore, it is always possible that environmental samples will contain previously undescribed species. Recently, several surveys of environmental sequence diversity using universal primers uncovered the hidden diversity of cryptophytes (8, 32, 33, 45, 48). McDonald et al. (33) detected several novel 16S plastid rRNA gene sequences from environmental clone libraries from the Gulf of Naples that were closely related to *G. cryophila* (AB073111) or *Plagioselmis* sp. (AB164406) and represent unknown species. Thus, diverse cryptophyte species have been reported in natural environments. However, in our study we found that only a few cryptophyte species (i.e., some members of the genera *Teleaulax* and *Geminigera*) were ingested by *M. rubra*, suggesting their clear prey selectivity. Studies of natural distribution and abundances of cryptophytes have been undertaken throughout the year using fluorescence in situ hybridization (FISH) probes that specifically bind to *Dinophysis* plastids (26). Their results have shown that abundances of *Teleaulax* and *Geminigera* were much lower than the total abundance of cryptophytes in Iwate Prefecture in northern Japan. In view of the low abundance of *Teleaulax and Geminigera* in natural environments, why does *M. rubra* prefer to ingest these cryptophyte species? How does *M. rubra* they distinguish *Teleaulax*/*Geminigera* from other cryptophytes? Food selectivity based on prey size, surface properties, and chemosensory factors has been reported for some herbivorous ciliates (3, 7, 24, 25, 53). Any of these factors may greatly influence the prey selectivity of *M. rubra*. There may also be some other unknown factors

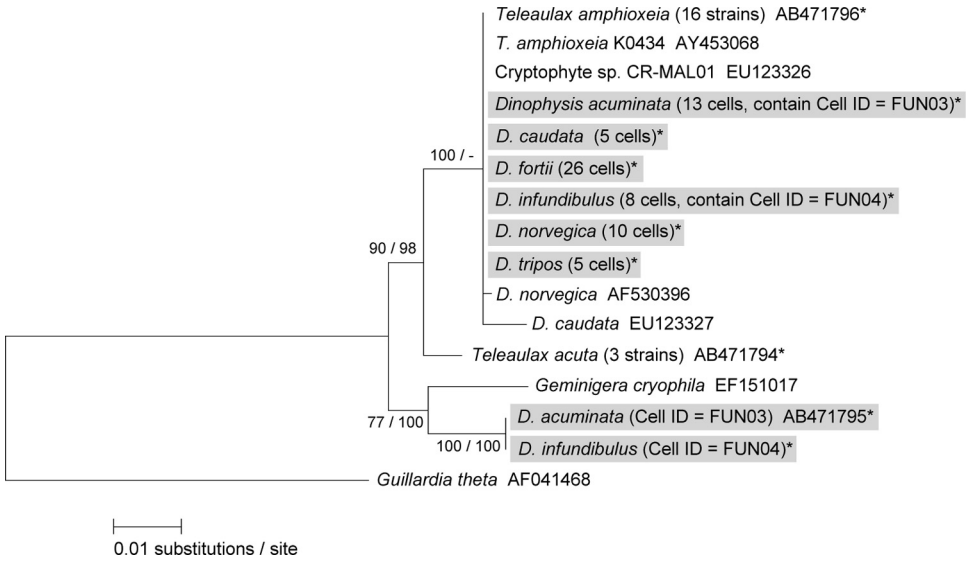

FIG. 5. A maximum-likelihood tree inferred from the plastid *psbA*. Sequences obtained in this study are indicated by asterisks, and sequences detected from natural *Dinophysis* cells are further indicated by gray boxes. The values at left are in the form bootstrap support values/posterior probabilities.

associated with the easier acquirement of *Teleaulax*/*Geminigera* plastids as kleptoplastids by *M. rubra*. Kleptoplastidy of *T. amphioxeia* by other dinoflagellates, such as *Amylax buxus* and *Amylax triacantha*, has also been reported although the vestigial plastid, nucleomorph, and mitochondria were still kept (28). These findings suggest that the process of plastid sequestration by kleptoplastidy is still insufficiently understood.

Plastid genes in natural *Dinophysis* cells. PCR efficiencies of plastid 16S rRNA genes ranged from 80% to 100% (mean, 96%), depending on the sampling location (Table 3). The partial plastid 16S rRNA gene sequences of *Dinophysis* spp. determined in this study were 801 bp, and we determined 564 sequences from the successfully amplified PCR products (n = 68). Of these, 527 sequences were identical to the sequence of *T. amphioxeia* (93.4%). No variation among the sequences was confirmed although Hackett et al. (11) previously detected several variations (less than 16 bp out of ca. 1,200 bp) in plastid 16S rRNA gene sequences in *Dinophysis* spp. isolated from the East Coast of North America (Fig. 4). The remaining 37 sequences obtained in this study (5.6%), which were all identical and were detected only from the Funka Bay sample (*D. acuminata*, cell ID FUN03; *D. norvegica*, cell ID FUN07), showed a close phylogenetic affiliation and a divergence of 0.7% and 0.9% compared with sequences of *G. cryophila* and *T. acuta*, respectively (Table 3 and Fig. 4). These *Dinophysis* cells contained two different cryptophyte sequences in a single cell.

TABLE 3. Summary of the sequence analyses of the cryptophyte nucleomorph gene in natural *M. rubra* cells and of plastid genes in natural *Dinophysis* cells detected by single-cell PCR

Species	Site[c]	No. of cells isolated[b]	Sequence analysis (no. of cells [no. of sequences analyzed])[a]								
			Nucleomorph			Plastid 16S rRNA			*psbA*		
			PCR	*T. amphioxeia*	Other	PCR	*T. amphioxeia*	Other	PCR	*T. amphioxeia*	Other
M. rubra	MUT	3	1	1 (45)	0						
M. rubra	HAR	95	95	95 (604)	2 (2)						
M. rubra	INO	20	11	11 (37)	0						
M. rubra	YAT	16	7	7 (27)	0						
D. fortii	NOT	5				5	5 (75)	0	5	5 (88)	0
D. acuminata	FUN	8				8	8 (64)	1 (31)	8	8 (38)	1 (1)
D. infundibulus	FUN	8				7	7 (43)	0	8	8 (45)	1 (2)
D. norvegica	FUN	10				10	10 (75)	1 (6)	10	10 (36)	0
D. tripos	FUN	5				5	5 (26)	0	5	5 (18)	0
D. fortii	HIR	20				19	19 (135)	0	17	17 (53)	0
D. acuminata	YAT	5				5	5 (39)	0	5	5 (25)	0
D. caudata	YAT	5				5	5 (38)	0	5	5 (26)	0
D. fortii	YAT	5				4	4 (32)	0	4	4 (23)	0
Total			114	114 (713)	2 (2)	68	68 (527)	2 (37)	67	67 (352)	2 (3)

[a] Values are from successful PCR amplifications. Sequences not identified as belonging to *T. amphioxeia* are grouped as other.
[b] A total of 134 and 71 cells were isolated from *M. rubra* and *Dinophysis* spp., respectively.
[c] See Table 1 for identification of the sampling sites.

Four sequences of *T. amphioxeia* and 31 other sequences were detected from the *D. acuminata* cell (cell ID FUN03), and two sequences of *T. amphioxeia* and six other sequences were detected from the *D. norvegica* cell (cell ID FUN07). Interestingly, the other sequence (AB471792) was identical to that of a *D. acuminata* cell isolated from the Greenland Sea (35) although the cryptophyte species was not identified. The plastid 16S rRNA gene sequences of *T. acuta* and *G. cryophila* were never detected in natural cells of *Dinophysis* spp. throughout this study.

The result of the plastid *psbA* analysis was similar to that of the plastid 16S rRNA gene (Table 3). Depending on the sampling locations, PCR efficiencies ranged from 80% to 100% (mean, 94%). The partial plastid *psbA* sequences determined in this study were 890 bp, and 355 *psbA* sequences were determined from the successfully amplified PCR products. Of these, 352 sequences were identical to the sequence of *T. amphioxeia* (99.4%), and no variation was observed. As in the analysis of the plastid 16S rRNA gene, another three sequences (AB471795), which were all identical and detected only from the Funka Bay sample (*D. acuminata*, cell ID FUN03; *D. infundibulus*, cell ID FUN04) (0.6%), showed a close phylogenetic affiliation and a 2.6% divergence from that of *G. cryophila* (Table 3 and Fig. 5). Six sequences of *T. amphioxeia* and one other sequence were detected from the *D. acuminata* cell (cell ID FUN03), and five sequences of *T. amphioxeia* and two other sequences were detected from the *D. infundibulus* cell (cell ID FUN04). This unknown cryptophyte may occur in relatively cold waters at high latitudes since this unique sequence was detected from Greenland (35), and the water temperature in Funka Bay when we isolated the *Dinophysis* cells was ca. 7°C. Because there is an inflow of cold water from around the Sea of Okhotsk into Funka Bay every spring (18, 39, 47), the above unknown cryptophyte may have been transferred from the northern regions into the bay through the coastal Oyashio Current. Thus, it will be necessary to isolate and establish cultures in order to investigate further the role of the unknown cryptophyte as an additional prey species for *M. rubra*.

The high congruence of *M. rubra* prey (cryptophyte *T. amphioxeia*) and *Dinophysis* plastid identities obtained in this study probably support the kleptoplastidy concept in *Dinophysis* species. This is primarily because plastid sequences identical with *T. amphioxeia* sequence were obtained from all *Dinophysis* cells (*D. acuminata*, *D. caudata*, *D. fortii*, *D. infundibulus*, *D. norvegica*, and *Dinophysis tripos*) collected from Japanese coastal waters, and they are all identical with those collected from the Baltic Sea (20, 35), the North Sea (20, 35), the North Atlantic Ocean (20), a Norwegian fjord (35), and the North American East Coast (11). Second, the other sequences that differ from *T. amphioxeia* were detected from natural *Dinophysis* cells in this study (Fig. 4 and 5). If these sequences originated from permanently established plastids in *Dinophysis*, the sequences should be detected from not only the Funka Bay sample but also the other sampling areas. Although the high congruence can also be interpreted as the result of recent acquisition of plastids (41), this idea is invalidated by the fact that, based on TEM observations, each plastid is lined by only two complete membranes, with remains of a third membrane in *D. acuminata* (9). Third, we observed the disappearance of relatively large chloroplasts (>5 μm in length) from *D. fortii* after >4 weeks of incubation without the ciliate prey; only a few small chloroplasts (0.5 to 2 μm in length) remained in the marginal region of the cells, especially in small cells (36). Fourth, Minnhagen et al. (34) reported that there was no significant difference in plastid DNA content between the G_1 and G_2 phases in natural *D. norvegica* cells, showing no duplication of plastid genomes coupled with the cell cycle, as expected with kleptoplastidy. In addition, data obtained from monitoring photosynthetic activities by ^{13}C labeling in *D. fortii* without the ciliate prey clearly indicated that the activities decreased rapidly within 1 week after heavy feeding on *M. rubra* (S. Nagai et al., unpublished data).

One hypothesis is that *D. fortii* might ingest *M. rubra* as merely a heterotrophic nutrient source and utilize the stolen chloroplasts for an ancillary function because growth of *D. fortii* was observed only during the first 7 days after the removal of prey (36). In observations of *Dinophysis* cells that were incubated without the ciliate prey for ca. 2 months, cells still retained a few small chloroplasts, implying the possibility of permanent plastids (36, 43). Are they retained kleptoplastids or permanent plastids, or both? To definitively answer these contradictory questions, further study is needed by several approaches such as tracing the cryptophyte plastids in cells of *Dinophysis* via *M. rubra* and/or analyzing plastid genomes using more variable regions.

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