Vol. 52: 209–221, 2008
doi: 10.3354/ame01233

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Printed September 2008
Published online August 21, 2008

Growth characteristics and phylogenetic analysis of the marine dinoflagellate *Dinophysis infundibulus* (Dinophyceae)

Goh Nishitani[1], Satoshi Nagai[1,*], Yoshihito Takano[2], Sanae Sakiyama[1], Katsuhisa Baba[3], Takashi Kamiyama[4]

[1]Harmful Algal Bloom Division, National Research Institute of Fisheries and Environment of Inland Sea, 2-17-5 Maruishi, Hatsukaichi, Hiroshima 739-0452, Japan

[2]Faculty of Fisheries, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

[3]Hokkaido Hakodate Fisheries Experimental Station, 1-2-66 Yunokawa, Hakodate, Hokkaido 042-0932, Japan

[4]Tohoku National Fisheries Research Institute, Fisheries Research Agency, 3-27-5 Shinhama, Shiogama, Miyagi 985-0001, Japan

ABSTRACT: We report the successful growth of the toxic dinoflagellate *Dinophysis infundibulus* under laboratory conditions, when fed the marine ciliate *Myrionecta rubra* grown with the cryptophyte *Teleaulax amphioxeia*; this study follows previous studies on *D. acuminata*, *D. caudata*, and *D. fortii*, showing the mixotrophy. We succeeded in maintaining several clonal strains of *D. infundibulus* for a relatively long period of time (>8 mo). Growth rates in *D. infundibulus* ranged from 0.40 to 0.94 divisions d^{-1}, reaching a maximum concentration of 1.2 to 2.3×10^3 cells ml^{-1}. The rate was 0.36 divisions d^{-1} when apparently fully expanded cells, resulting from the active ingestion of *Myrionecta rubra*, were cultivated without the ciliate prey. In contrast, the culture of *D. infundibulus* was not established in the absence of the ciliate or when provided only with the cryptophyte *T. amphioxeia*, suggesting that *D. infundibulus* cannot directly use *T. amphioxeia* as prey. We also determined the sequences of nuclear small subunit (SSU) rRNA, 5.8S rRNA with internal transcribed spacers (ITS1 and ITS2), and large subunit (LSU) rRNA regions in *D. infundibulus*. Phylogenetic relationships revealed that they are non-identical with regard to any of the DNA sequence data of *Dinophysis* deposited in GenBank, and are distinguishable from other *Dinophysis* species for species identification.

KEY WORDS: Culture · Dinoflagellate · *Dinophysis infundibulus* · *Myrionecta rubra* · *Teleaulax amphioxeia*

Resale or republication not permitted without written consent of the publisher

INTRODUCTION

Some species of the genus *Dinophysis* cause diarrhetic shellfish poisoning (DSP). To resolve the ecophysiology, toxicology, and blooming mechanisms of this genus, many scientists have attempted to culture the toxic species of *Dinophysis* by using various culture media, but have thus far had little success (Ishimaru et al. 1988, Sampayo 1993, Maestrini et al. 1995, Delgado et al. 1996, Nishitani et al. 2003). Recently, Park et al. (2006) revealed the feeding mechanism of myzocytosis in *D. acuminata* and completely succeeded in culturing it at a high cell density (>1.1×10^4 cells ml^{-1}). Nishitani et al. (2008) and Nagai et al. (2008) followed their work and succeeded in establishing cultures of *D. caudata* and *D. fortii*, respectively. These 3 *Dinophysis* species were fed on the marine ciliate *Myrionecta rubra* (=*Mesodinium rubrum*) (Mesodiniidae: Litostomatea) grown with the cryptophyte *Teleaulax*. Their experimental data clearly showed that these species could

*Corresponding author. Email: snagai@affrc.go.jp

© Inter-Research 2008 · www.int-res.com

not grow by ingestion of only the *Teleaulax* sp. Instead, *Dinophysis* require *M. rubra* as prey to enable their vegetative growth, and they sequester the ciliate plastids in order to use them as kleptoplastids (Nagai et al. 2008). Recent molecular studies suggest that the plastid DNA sequences of several photosynthetic species of *Dinophysis* are identical and closely related to those of *T. amphioxeia*, *Plagioselmis* sp., or *Geminigera cryophila* (Takishita et al. 2002, Janson 2004, Koike et al. 2005, Takahashi et al. 2005, Minnhagen & Janson 2006). Therefore, we speculated that many photosynthetic *Dinophysis* species had a similar nutrient requirement as *D. acuminata*/*caudata*/*fortii*. In this study, we first confirmed whether the sequence of plastid DNA (*psbA* gene) in naturally occurring *D. infundibulus* cells is identical to that of *T. amphioxeia*. The analyzed data clearly showed that the sequence matched that of *T. amphioxeia*. Therefore, we attempted to establish cultures of *D. infundibulus*, with addition of the ciliate prey *M. rubra* grown with *T. amphioxeia*.

Schiller (1933) distinguished *Dinophysis infundibulus* from *D. parva* by the presence of more distinctly formed cingular lists in the former. However, the 2 species bear a very close resemblance to each other not only with regard to size but also with regard to the shape of the body. Therefore, they are thought to be synonymous (Abe 1967). Occurrences of *D. infundibulus* have been reported in Japan (Abe 1967), British Columbia, Canada (Taylor & Haigh 1996), the Adriatic Sea (Sidari et al. 1998), the Sea of Okhotsk (Orlova et al. 2004), and Korea (Shin et al. 2004), suggesting its wide distribution throughout the temperate regions in both the Atlantic and Pacific Oceans. Thus far, *D. infundibulus* as a research object has not received much interest because this species is considered to be nontoxic. As a result, little information is available regarding its ecophysiology and molecular biology. Recently, however, pectenotoxin-2 (PTX2) was detected in naturally occurring cells of *D. infundibulus* sampled from Hokkaido, Japan, using liquid chromatography-tandem mass spectrometry (LC-MS/MS; Suzuki et al. 2006, Miyazono et al. 2008). Therefore, this species should be added as a target species in DSP monitoring programs, and more information should be accumulated on its ecophysiology and toxicology. In the present study, we show that *D. infundibulus*, similar to *D. acuminata*/*caudata*/*fortii*, requires *Myrionecta rubra* that was maintained with the addition of *Teleaulax amphioxeia* for its propagation. We report the growth characteristics of *D. infundibulus* in the laboratory and the phylogenetic relationships within the genus *Dinophysis*, as inferred on the basis of nuclear ribosomal RNA genes.

MATERIALS AND METHODS

Isolation of clonal strains. *Myrionecta rubra* and *Teleaulax amphioxeia* were isolated from Inokushi Bay (131° 53′ E, 34° 47′ N) at the end of February 2007 in Oita Prefecture, Japan (Nishitani et al. 2008). The *M. rubra* culture was maintained by mixing 50 ml of the culture (7.0 to 9.0×10^3 cells ml^{-1}) with 100 ml of a modified f/2 medium (Guillard 1975, Nagai et al. 2004). The culture medium was prepared with 1/3 nitrate, phosphate, and metals and 1/10 vitamins based on the enrichment of natural seawater collected from the same location of Hiroshima Bay (salinity adjusted to 30 psu). Transfers were made once a week, with the addition of 100 µl of *T. amphioxeia* culture (containing 1.0 to 2.0×10^4 cells), and they were maintained at a temperature of 16°C under a photon irradiance of 100 µmol m^{-2} s^{-1}, provided by cool-white fluorescent lamps, with a 12:12 h light:dark cycle. The *T. amphioxeia* culture was also maintained by reinoculating 0.3 ml of the culture (7.0 to 8.0×10^4 cells ml^{-1}) into 150 ml of the modified f/2 medium, under the same conditions as those for *M. rubra*.

Five single cells of *Dinophysis infundibulus* were isolated by micropipetting from a seawater sample collected from Hiroshima Bay, Japan (132° 15′ E, 34° 16′ N), in May 2007, and incubated in individual wells of a 48-well microplate (Iwaki). Each single cell was grown in 0.5 ml of the culture medium, containing ca. 500 and 20 cells of the marine ciliate *Myrionecta rubra* and the cryptophyte *Teleaulax amphioxeia*, respectively, as prey species. *D. infundibulus* cells were incubated under the abovementioned conditions. After 1 mo of incubation, 3 clonal strains were established and maintained by reinoculating 50 µl of the culture (containing 50 to 100 cells) into 950 µl of the *M. rubra* culture (containing 3.0 to 3.5×10^3 cells) in a 48-well microplate (Iwaki) at 16°C, under the abovementioned light conditions. The feeding behavior and binary fission of *D. infundibulus* were observed in the maintenance culture, using an inverted microscope (Nikon TE-300).

To verify whether *Dinophysis infundibulus* sequesters the chloroplasts ingested from *Myrionecta rubra* without digesting it in the food vacuoles, we conducted a feeding experiment. In order to monitor the transfer of orange autofluorescence derived from the chloroplasts of *M. rubra*, *D. infundibulus* cells containing only a few chloroplasts were required. Therefore, a clonal strain of *D. infundibulus* was incubated for 51 d under starved conditions (without the addition of the ciliate prey). The *D. infundibulus* culture (200 µl) containing ca. 2.0×10^2 cells was mixed with 1 ml of the *M. rubra* culture (containing ca. 4.0×10^3 cells), and incubated in a well of a 12-well microplate (Iwaki) on the

inverted microscope at room temperature. A small proportion of the *D. infundibulus* cells started capturing *M. rubra* within 7 h of adding the prey. *D. infundibulus* cells that captured the first ciliate prey were then micropipetted into individual wells of a 48-well microplate filled with 500 µl of fresh culture medium. At 0, 2, 15, 30 min, the *D. infundibulus* cells were inoculated into the fresh medium, and 3 cells (3 wells) were fixed with glutaraldehyde (at a final concentration of 0.1%) for each experimental time. In addition, the *D. infundibulus* cells, which were incubated with *M. rubra* for 2 d and fed heavily on the ciliate prey, were fixed and observed. The process by which *D. infundibulus* ingests chloroplasts from *M. rubra* was observed using epifluorescence microscopy with blue light excitation. Therefore, this is not a series of sequential observations, and the cells shown in Fig. 2A,C,E,G,I are not the same cell.

Growth experiments. The *Myrionecta rubra* and *Teleaulax amphioxeia* cultures grown until the late logarithmic growth phase (ca. 8.5×10^3 and 2.4×10^5 cells ml^{-1}, respectively) were diluted with fresh culture media to give initial concentrations of ca. 1.5×10^3 and 1.3×10^3 cells ml^{-1}, respectively. Three *Dinophysis infundibulus* clonal strains (10 µl) were added to the *M. rubra* and *T. amphioxeia* cultures to give an initial concentration of 8 cells ml^{-1}, and 1.0 ml aliquots of the mixed culture were inoculated into 48-well microplates (Iwaki). The growth experiment was conducted for 25 d under the same light and temperature conditions used for maintaining the culture of *D. infundibulus*. As controls, only *M. rubra* and *D. infundibulus* with *T. amphioxeia* were incubated. Briefly, 200 µl of *M. rubra* (1.5×10^3 cells) were inoculated into 800 µl of the fresh culture medium, and 10 µl of the *T. amphioxeia* (1.3×10^3 cells) and *D. infundibulus* (8 cells) cultures were inoculated into 1.0 ml of the culture medium. The control cultures were incubated for 25 d under the same conditions used for the maintenance cultures. In the above experiments, we used *D. infundibulus* cells that were cultivated for an additional 2 wk after they had consumed all prey ciliates in the maintenance culture.

To examine the growth potential of *Dinophysis infundibulus* in the absence of ciliate prey, after feeding heavily on *Myrionecta rubra*, 48 cells of *D. infundibulus* (strain 1) that appeared fully expanded by active ingestion of the prey were micropipetted into individual wells of 48-well microplates (Iwaki) containing 1.0 ml aliquots of the culture medium (1 cell ml^{-1}). These cells of *D. infundibulus* were incubated for 100 d in the absence of *M. rubra* and *Teleaulax amphioxeia* under the abovementioned conditions.

In all growth experiments, 3 wells of cultures (500 µl), randomly selected (triplicate), were sampled after gentle pipetting for agitation, and fixed with glutaraldehyde (final concentration, 1%). The cell densities of *Dinophysis infundibulus*, *Myrionecta rubra*, and *Teleaulax amphioxeia* were counted using an inverted microscope. The growth rates (divisions d^{-1}) of *D. infundibulus*, *M. rubra*, and *T. amphioxeia*, determined to be in the exponential growth phase, were calculated using the method of Guillard (1973).

DNA extraction, PCR amplification, sequencing, and phylogenetic analysis. To compare the plastid *psbA* gene sequence of *Teleaulax amphioxeia* to that of *Dinophysis infundibulus*, we determined the sequence of naturally occurring cells of *D. infundibulus* collected from Hiroshima Bay, Hiroshima, Japan (132° 15′ E, 34° 16′ N), in May 2007, and from Funka Bay, Hokkaido, Japan (140° 20′ E, 42° 16′ N), in June 2007 (Table 1). A single cell of *D. infundibulus* in each area was micropipetted by mouth and inoculated into 10 µl of Tris-EDTA (TE) buffer. The samples were then boiled at 99°C for 10 min in order to extract the DNA. For the PCR primers and conditions for amplifying the *psbA* gene, we followed the method of Hackett et al. (2003).

Table 1. *Dinophysis infundibulus.* Isolation details for cells used in this study. SSU: small subunit; LSU: large subunit; ITS: internal transcribed spacer

Strain	Location	Sampling date (2007)	Region analyzed	Accession no.
Natural cell	Hiroshima Bay, Japan (132° 15′ E, 34° 16′ N)	May	*psbA*	AB376283
0705HIR01	Hiroshima Bay, Japan	May	SSU	AB366002
			LSU	AB366003
			ITS	AB366004
0705HIR02	Hiroshima Bay, Japan	May	LSU	AB374253
			ITS	AB374255
0705HIR03	Hiroshima Bay, Japan	May	LSU	AB374986
			ITS	AB374987
Natural cell	Funka Bay, Japan (140° 20′ E, 42° 16′ N)	Jun	*psbA*	AB376284
			LSU	AB374254
			ITS	AB374256

To determine the rRNA gene, single *Dinophysis infundibulus* cells from the cultures (Hiroshima Bay: strains 1, 2, and 3) and a single natural cell from Funka Bay obtained in June 2007 (Table 1) were picked up under an inverted microscope and inoculated into 10 µl of TE buffer. The samples were then boiled at 99°C for 10 min to extract the DNA. Boiled samples (1 µl) were used as templates to amplify the small subunit (SSU) rRNA gene (only 1 sample, i.e. strain 1 from Hiroshima Bay), the internal transcribed spacer regions (ITS1-5.8S rRNA gene-ITS2), and the large subunit (LSU; D1-D2 region) rRNA gene. All PCR reactions were performed on a PCR thermal cycler (PC-808; ASTEC) in a reaction mixture (10 µl) containing 1 µl of template DNA, 0.2 mM of each dNTP, 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl (pH 8.3); Applied Biosystems), 2.5 mM of Mg^{2+}, 0.25 U of Ampli*Taq* Gold (Applied Biosystems), and 0.5 µM of primers. For amplification of the SSU rDNA region, the following primers were used: S1F (5′-AACCTGGTTGATYCTGCCAG-3′) + S1R (5′-CTACGAGCTTTTTAACCGCAACAA-3′), S2F (5′-CTGAGAAACGGCTACCACATC-3′) + S2R (5′-TGGTAAGTTTTCCCGTGTTGAGTC-3′), and S3F (5′-AGCTTGCGGCTTAATTTGACTC-3′) + S3R (5′-CTACGGAAACCTTGTTACGAC-3′). The ITS regions and LSU rDNA (D1-D2 region) were amplified according to Adachi et al. (1994) and Scholin et al. (1994), respectively. The PCR cycling conditions were as follows: 5 min at 94°C, 40 cycles of 30 s each at 94°C, 30 s at 56°C, 1 min at 72°C, and a final elongation for 5 min at 72°C. All amplified fragments were cloned into the pGEM-T Easy Vector Systems (Promega) and subsequently transformed into *Escherichia coli*, following the standard protocol. Sequences were determined using the DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare) and an ABI PRISM 3100 genetic analyzer (Applied Biosystems). All new sequences have been submitted to the DDBJ/EMBL/GenBank databank.

For *Dinophysis infundibulus*, the SSU, the ITS regions, and the LSU D1-D2 regions, excluding the primer regions, were aligned with the sequences of other *Dinophysis* species and *Karenia mikimotoi* (outgroup), which were obtained from GenBank by using the ClustalX package (Thompson et al. 1997), and edited manually. Neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) methods were implemented by PAUP version 4.0b10 (Swofford 2002), for the phylogenetic analyses. The distance matrix was calculated using Kimura 2-parameter distances (Kimura 1980), and the distance tree was constructed using the NJ method (Saitou & Nei 1987). The MP analysis was performed using the heuristic search option with random addition of sequences (1000 replicates) and a branch-swapping algorithm (tree bisection-reconnection; TBR). All characters were weighted equally, and gaps were treated as missing. The program Modeltest version 3.04 (Posada & Crandall 1998) was used to explore the model of sequence evolution that best fits the dataset, based on the Akaike Information Criterion (AIC). In the SSU sequences, a likelihood score (–lnL = 3026.46573) was obtained under the TrN+I model with the following parameters: assumed nucleotide frequencies of A = 0.2689, C = 0.1963, G = 0.2568, and T = 0.2780; substitution rate matrix with AC = 1, AG = 2.8761, AT = 1, CG = 1, CT = 7.1414, and GT = 1; proportion of sites assumed to be invariable = 0.8160; rates for variable sites assumed to follow a gamma distribution with shape parameter = equal, as estimated by Modeltest 3.04. Parsimony analysis of the SSU alignment set resulted in 1 most parsimonious tree of 109 steps (consistency index [CI] = 0.963; retention index [RI] = 0.909). In the ITS with 5.8S rDNA sequences, a likelihood score (–lnL = 2398.0178) was obtained under the GTR+G model with the following parameters: assumed nucleotide frequencies of A = 0.2137, C = 0.2167, G = 0.2562, and T = 0.3134; substitution rate matrix with AC = 1.9440, AG = 3.1297, AT = 1.6803, CG = 0.3555, CT = 4.9885, and GT = 1; proportion of sites assumed to be invariable = 0.7255; rates for variable sites assumed to follow a gamma distribution with shape parameter = equal, as estimated by Modeltest 3.04. Parsimony analysis of the ITS alignment set resulted in 1 most parsimonious tree of 383 steps (CI = 0.945; RI = 0.967). In the LSU sequences, a likelihood score (–lnL = 1999.7334) was obtained under the TrN+G model with the following parameters: assumed nucleotide frequencies of A = 0.2515, C = 0.1723, G = 0.2979, and T = 0.2783; substitution rate matrix with AC = 1, AG = 2.1236, AT = 1, CG = 1, CT = 4.1765, and GT = 1; proportion of sites assumed to be invariable = 0; rates for variable sites assumed to follow a gamma distribution with shape parameter = 1.3074, as estimated by Modeltest 3.04. Parsimony analysis of the LSU alignment set resulted in 6 most parsimonious trees, which were equally parsimonious, of 259 steps (CI = 0.977; RI = 0.989). The proportion of invariable sites, the gamma distribution shape parameter, base frequencies, and substitution parameters were estimated from the dataset by Modeltest. ML was performed using the heuristic search option with a branch-swapping algorithm (TBR). Starting trees were obtained by stepwise random addition of sequences (10 replicates). Bootstrap analyses with 1000 replicates for NJ, MP, and ML analyses were applied to examine the robustness and statistical reliability of the topologies (Felsenstein 1985). For MP bootstrap analyses, the heuristic search option with random addition of sequences (10 replicates) and a branch-swapping algorithm (TBR) were applied. For ML bootstrap analyses, we used the

heuristic search option with a branch-swapping algorithm (TBR) and starting trees obtained by NJ.

RESULTS AND DISCUSSION

Cell observations

The *Dinophysis infundibulus* cells isolated from Hiroshima Bay were 40 to 50 µm long, and the lateral outline of the body was broadly ovate and more circular than that of *D. acuminata* (Fig. 1A). Naturally occurring *D. infundibulus* cells showed a yellow-orange autofluorescence under blue light excitation, suggesting the presence of phycobilin (Lessard & Swift 1986, Hallegraeff & Lucas 1988, Schnepf & Elbrächter 1988, Geider & Gunter 1989, Imai & Nishitani 2000) and phycoerythrin (Geider & Gunter 1989, Vesk et al. 1996, Hewes et al. 1998). We succeeded in establishing 3 clonal cultures of 5 single isolates with the addition of the marine ciliate *Myrionecta rubra* and the cryptophyte *Teleaulax amphioxeia* as the prey species. Similar to the reports for *D. acuminata/caudata/fortii* by Park et al. (2006), Nishitani et al. (2008), and Nagai et al. (2008), *D. infundibulus* was able to feed on *M. rubra*. *D. infundibulus* uses its peduncle (Fig. 1B), which extends from around the flagellar pore, to capture *M. rubra* cells and ingest the cell contents, as has been previously reported for *D. rotundata* (Hansen 1991), *D. acuminata* (Park et al. 2006), *D. caudata*

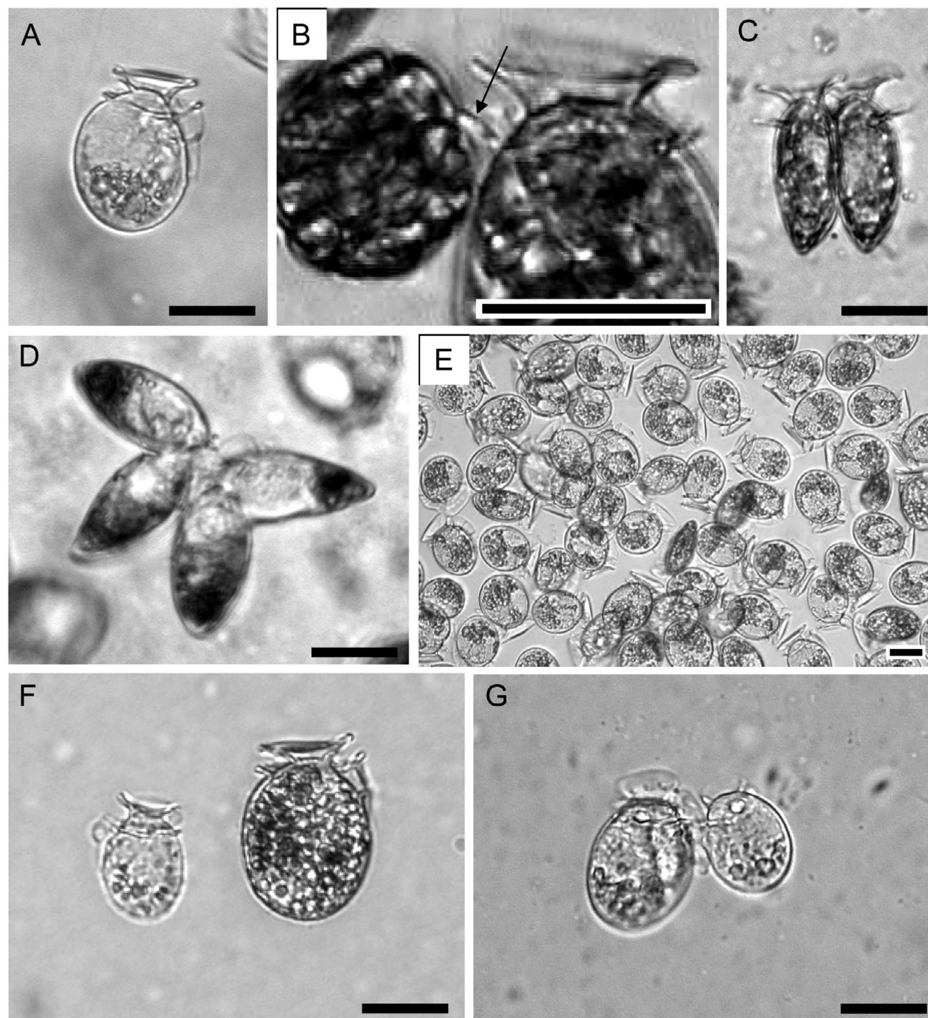

Fig. 1. *Dinophysis infundibulus*. Observations of feeding and propagation during maintenance culture or growth experiment. (A) A natural cell collected from Hiroshima Bay, Japan. (B) A cell actively ingesting prey, showing the round shape and loss of cilia in the prey. The arrow indicates the peduncle. (C) Vegetative cell division by binary fission. (D) Sequential binary fission observed without the separation of cells from the previous cell division. (E) Harvested cells after growth experiments, showing their successful cultivation. (F) A small cell (left) and a cell after feeding on *Myrionecta rubra* (right). (G) A couplet joined at the ventral side. All scale bars = 20 µm

(Nishitani et al. 2008), and *D. fortii* (Nagai et al. 2008), indicating myzocytosis as the feeding mechanism. Soon after *D. infundibulus* cells captured the ciliates, the ciliates became immobile and their cilia were shed within 1 to 5 min. During feeding, the prey was closely tethered around the flagellar pore region of *D. infundibulus*, and the cytoplasm of the prey was actively ingested through the peduncle (Fig. 1B).

Propagation of *Dinophysis infundibulus* was observed by frequent vegetative cell divisions (Fig. 1C), and a sequential division was sometimes observed without separation of the cells from the previous cell division (Fig. 1D). These 4 daughter cells were united by their cingular lists and were still able to swim actively. A large amount of cells was harvested by sieving *D. infundibulus* cultures through a nylon mesh (pore diameter of 10 µm), demonstrating the successful cultivation of *D. infundibulus* (Fig. 1E).

The formation of small cells was sometimes observed in the maintenance cultures of *Dinophysis infundibulus* (Fig. 1F). These small cells tended to be produced particularly when entering the stationary phase of growth, and were clearly different from normal vegetative cells. The appearances of small cells in trials in laboratory cultures have also been reported in *D. acuta* (Reguera et al. 2004), *D. caudata* (Nishitani et al. 2003, Reguera et al. 2004), *D. fortii* (Uchida et al. 1999, Nagai et al. 2008), *D. pavillardi* (= *D. sacculus*, see Zingone et al. 1998, Delgado et al. 1996, Giacobbe & Gangemi 1997). Small cells forming couplets with normal vegetative cells by associating via sexual conjugation or cannibalism have been reported in *D. fortii* (Uchida et al. 1999, Koike et al. 2006), *D. pavillardi* (Giacobbe & Gangemi 1997), *D. caudata* and *D. rotundata* (Reguera & González-Gil 2001). In our cultures, couplets of *D. infundibulus* were also observed during maintenance, particularly in the old culture (Fig. 1G).

To demonstrate the sequestration process by which chloroplasts from *Myrionecta rubra* were ingested by *Dinophysis infundibulus* cells, a time-series observation of intracellular autofluorescence in *D. infundibulus* is shown in Fig. 2; however, these observations are

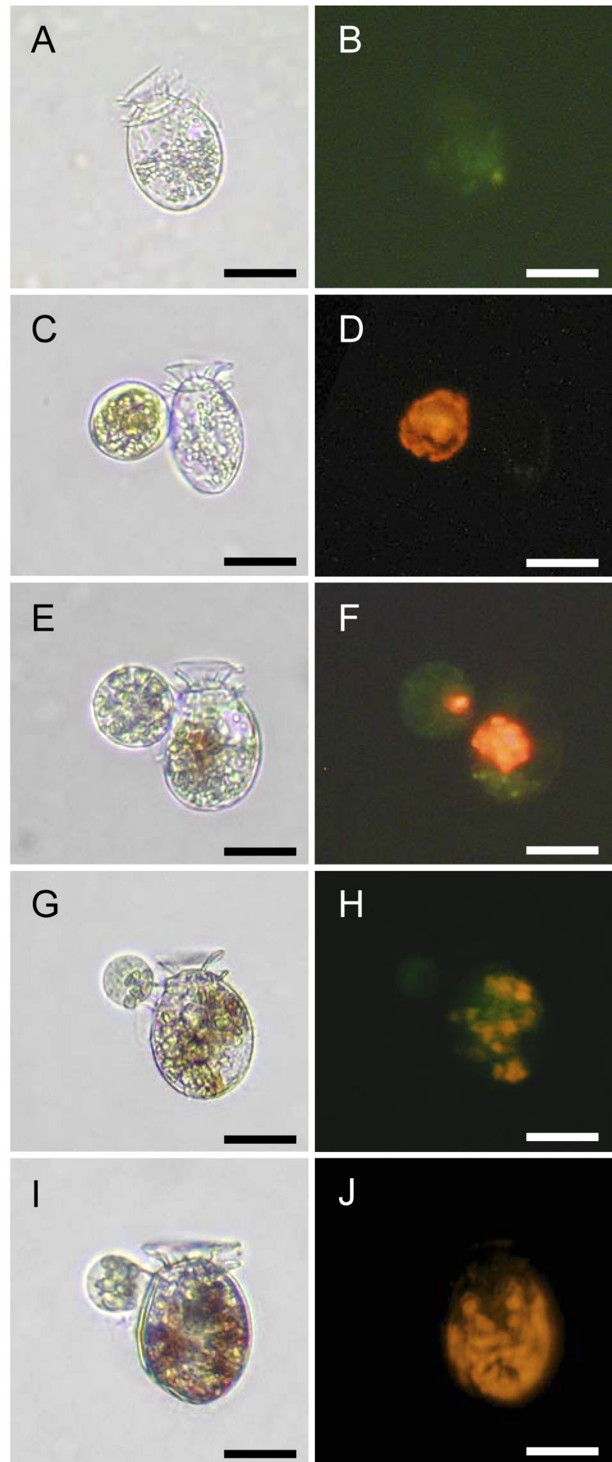

Fig. 2. *Dinophysis infundibulus.* Observations of the sequestration process of the chloroplasts ingested from *Myrionecta rubra*. The left- and right-hand columns show micrographs by an inverted normal light microscope and an inverted epifluorescence microscope, respectively. This is not a series of sequential observations, and the *D. infundibulus* cells shown in the left-hand micrographs are not the same cell. (A,B) A cell cultivated for 51 d without the addition of *M. rubra* and *Teleaulax amphioxeia*. (C,D) 2 min after the cell captured *M. rubra*. Ingestion of the chloroplasts through the peduncle had not started. (E,F) 15 min after the cell captured *M. rubra*. Most of the chloroplasts were ingested. Accumulation and concentration of the ingested chloroplasts were observed in the center of the cell. (G,H) 30 min after the cell captured *M. rubra*. Apparently, all the chloroplasts were ingested, and the beginning of the dispersion of the chloroplasts was observed. (I,J) The *D. infundibulus* cell 2 d after *M. rubra* was provided; the cell has certainly ingested some ciliates and shows accumulation of the ingested chloroplasts, possibly functioning as kleptoplastids. All scale bars = 20 µm (all at the same magnification)

not in sequence. After 51 d of starvation, chloroplasts in most *D. infundibulus* cells were reduced in number and size, and the cells became colorless, suggesting disappearance of the chloroplasts during the long starvation period (Fig. 2A,B). A small proportion of the *D. infundibulus* cells started capturing *M. rubra* within 7 h of adding the prey. In the observation of intracellular fluorescence in *D. infundibulus,* 2 min after *D. infundibulus* captured *M. rubra*, ingestion of the chloroplasts via the peduncle had not started, and most cell content containing the chloroplasts still remained in the *M. rubra* cell (Fig. 2C,D). After 15 min, most of the chloroplasts were ingested, while most other cell contents still remained in the *M. rubra* cell (Fig. 2E,F). After 30 min, 80 to 90 % of the cell contents of *M. rubra* were ingested, and dispersion of the chloroplasts toward the marginal region of the *D. infundibulus* cell was observed (Fig. 2G,H). After 2 d, accumulation of the chloroplasts sequestered from *M. rubra* was confirmed in *D. infundibulus* cells (Fig. 2I,J).

Observations of *Dinophysis fortii* cells by transmission electron microscopy (TEM) revealed that no chloroplast-like particles were contained in the food vacuoles of *D. fortii* cells that had fully fed on the prey, despite membrane-like structures and/or mitochondria-like particles that were confirmed in the well-developed food vacuoles (Nagai et al. 2008). This suggests that chloroplasts of *M. rubra* are ingested and dispersed in *D. fortii* cells in advance of the ingestion of the other cell contents in order to spare the chloroplasts from digestion so that they may function as kleptoplastids. In our study, the sequestration process of chloroplasts by *D. infundibulus* was very similar to that by *D. fortii*.

When exposed to high *Dinophysis fortii* cell densities (>500 cells ml^{-1}), *Myrionecta rubra* cells tended to form clumps, become entangled with each other via their cilia, swim helicoidally, or rotate in the same position at the bottom of the microplate, suggesting the release of an allelopathic chemical from *D. fortii* cells (Nagai et al. 2008). The apparently allelopathic interactions have also been observed with *D. acuminata* (S. Nagai et al. unpubl. data) and *D. caudata* (Nishitani et al. 2008); however, in our observations, the clumping of *M. rubra* was never observed at high *D. infundibulus* cell densities (>2000 cells ml^{-1}).

Growth experiments

Three strains of *Dinophysis infundibulus* grew actively when *Myrionecta rubra* cells were added as prey (Fig. 3A–C). The number of *D. infundibulus* (strains 1, 2, and 3) cells increased exponentially with a growth rate of 0.94 (Days 1 to 5), 0.87 (Days 1 to 4), and 0.71 (Days 0 to 3) divisions d^{-1}, respectively (Fig. 3A–C). *M. rubra* cells were initially abundant (ca. 1.5×10^3 cells ml^{-1}) and increased until they reached peaks of 4.5×10^3 cells ml^{-1} (mean) on Day 7, 3.5×10^3 cells ml^{-1} on Day 4, and 3.4×10^3 cells ml^{-1} on Day 6. The growth rates were calculated as 0.44 (Days 2 to 4), 0.59 (Days 0 to 2) and 0.27 (Days 1 to 4) divisions d^{-1}, respectively. After reaching the peaks, the number of cells of *M. rubra* declined rapidly and disappeared by Day 16 due to natural death and active feeding by *D. infundibulus*; additional deaths may have been caused by a decline in water quality. Even after the disappearance of *M. rubra*, *D. infundibulus* (strains 1, 2, and 3) continued to increase in number until Days 19 to 22, and the cell densities reached their maximum yields, i.e. 1.4×10^3, 2.3×10^3, and 1.2×10^3 cells ml^{-1} (mean), respectively. The number of *Teleaulax amphioxeia* cells continued to remain steady or slightly declined with the increase in the number of *M. rubra* cells and increased rapidly with the decrease in the number of *M. rubra* cells; *T. amphioxeia* finally reached concentrations of 1.2×10^5, 1.3×10^5 and 5.1×10^5 cells ml^{-1} (mean), respectively, at the end of the experiment (Fig. 3A–C).

In this study, the maximum yields of *Dinophysis infundibulus* were variable among the 3 strains. *D. fortii* (Nagai et al. 2008) showed a similar variation in the maximum yields (4.17×10^2 to 2.55×10^3 cells ml^{-1}, n = 48) after 1 mo of incubation of a single cell. In addition to the variability of feeding activity among strains and individuals in *D. infundibulus*, there is a possibility of growth competition by the release of allelopathic chemicals, nutrient uptake, and changes in pH, leading to the different growth rates and maximum yields. The growth suppression of *D. infundibulus*, shown in Fig. 3C, may be due to an increase in pH by active growth of *Teleaulax amphioxeia*, which approaches the pH limits for the growth of *D. infundibulus* suggested by Hansen (2002), Pedersen & Hansen (2003), Hansen & Fenchel (2006), and Park et al. (2006).

The growth rates of *Dinophysis infundibulus* obtained in this study (0.40 to 0.94 divisions d^{-1}) were similar to those obtained in the other *Dinophysis* cultures to which *Myrionecta rubra* was added as prey, i.e. 0.41 to 0.85 divisions d^{-1} in *D. fortii* (Nagai et al. 2008), 0.95 to 1.03 divisions d^{-1} in *D. caudata* (Nishitani et al. 2008), and a slightly higher growth rate of 1.37 divisions d^{-1} in *D. acuminata* (Park et al. 2006). These data suggest that these *Dinophysis* species have the potential to grow as fast as other red tide-forming species, such as *Chattonella antiqua*, *C. marina* (Yamaguchi et al. 1991), and *Karenia mikimotoi* (=*Gymnodinium mikimotoi*; Yamaguchi & Honjo 1989), if sufficient quantities of the ciliate prey are provided to these *Dinophysis* cultures.

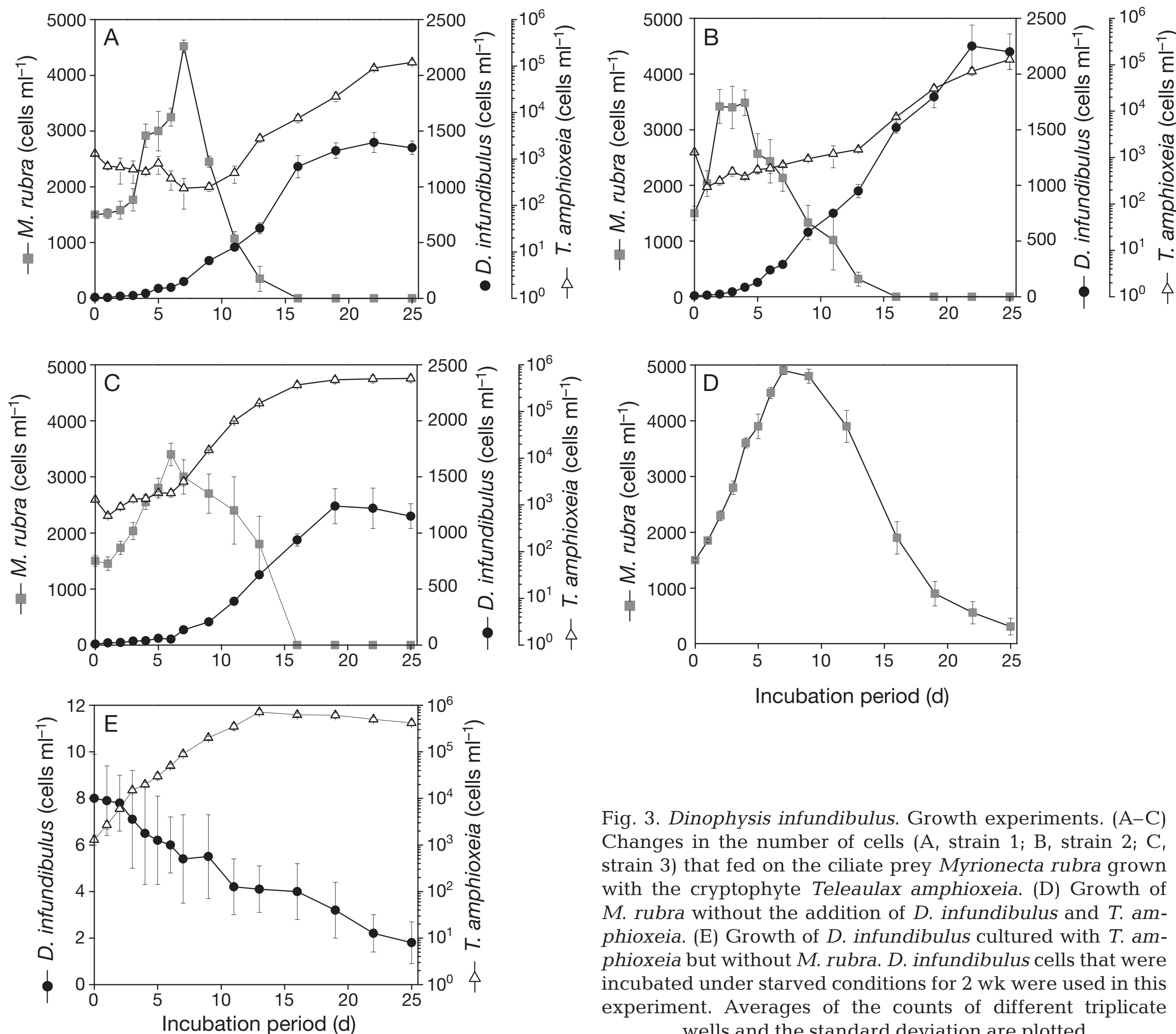

Fig. 3. *Dinophysis infundibulus*. Growth experiments. (A–C) Changes in the number of cells (A, strain 1; B, strain 2; C, strain 3) that fed on the ciliate prey *Myrionecta rubra* grown with the cryptophyte *Teleaulax amphioxeia*. (D) Growth of *M. rubra* without the addition of *D. infundibulus* and *T. amphioxeia*. (E) Growth of *D. infundibulus* cultured with *T. amphioxeia* but without *M. rubra*. *D. infundibulus* cells that were incubated under starved conditions for 2 wk were used in this experiment. Averages of the counts of different triplicate wells and the standard deviation are plotted

Due to the absence of the predator in the control plate of *Myrionecta rubra*, *M. rubra* grew exponentially at a growth rate of 0.32 divisions d^{-1} from Day 1 to Day 4 (Fig. 3D), until it reached a peak of $4.9 \times 10^3 \pm 2.0 \times 10^2$ cells ml^{-1} (mean ± SD) on Day 7. The number of *M. rubra* cells declined after Day 7 due to death caused by unknown reasons, but many cells survived (310 cells ml^{-1}) until the end of the experiments (Day 25).

The control plates of *Dinophysis infundibulus* contained *Teleaulax amphioxeia* but not the ciliate prey (Fig. 3E); in this control plate, the cell numbers of *D. infundibulus* declined slightly until they reached ca. one-fourth their original numbers during the 25 d incubation, showing that *D. infundibulus* cannot directly use *T. amphioxeia* as prey. In contrast, *T. amphioxeia* exhibited an exponential growth until Day 13, with a growth rate of 1.18 divisions d^{-1} from Day 0 to Day 4, and the number of cells was saturated thereafter. Similarly, *D. acuminata*, *D. fortii*, and *D. norvegica* cultures did not grow when only *Teleaulax* was provided as prey (Park et al. 2006, Carvalho et al. 2008, Nagai et al. 2008).

To examine the growth potential of *Dinophysis infundibulus*, the cells that appeared fully expanded by active ingestion of *Myrionecta rubra* were cultivated without the ciliate prey. The numbers of *Dinophysis infundibulus* cells increased with a growth rate of 0.36 divisions d^{-1} from Day 2 to Day 6, until they reached a peak of 9.0 ± 3.7 cells ml^{-1} (mean ± SD) on Day 13 (Fig. 4); thereafter, they declined gradually. Surprisingly, a cell of *D. infundibulus* survived without prey until Day 100 after the start of incubation; however, the cell did not grow when the ciliate prey was added. Therefore, after feeding heavily on *Myrionecta rubra*, cells of *D. infundibulus* could divide at least

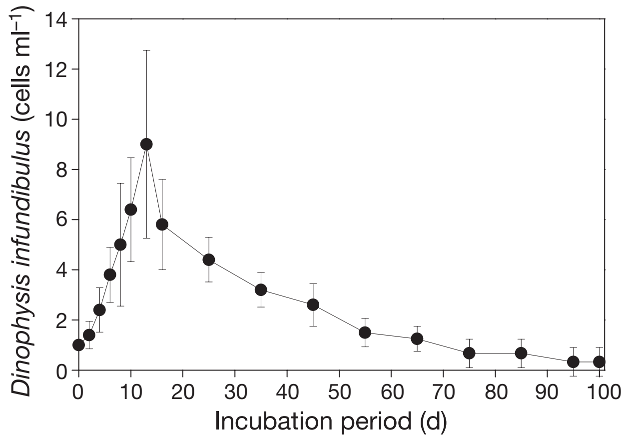

Fig. 4. *Dinophysis infundibulus.* Growth experiment without the prey ciliate, after heavy feeding on *Myrionecta rubra*. Cells (n = 48) of *D. infundibulus* were individually picked up by micropipetting and inoculated into each well of a 48-well microplate. These cells were cultivated under the same conditions used for the maintenance culture

4 times without further feeding, and survive for more than 100 d. It is assumed that *D. infundibulus* cells were able to grow for the first 2 wk by using accumulated surplus nutrients and chloroplasts sequestered by ingestion of the ciliate prey during the previous incubation.

Origin of the plastids in *Dinophysis infundibulus*

Both the plastid *psbA* sequences of *Dinophysis infundibulus* cells collected from Hiroshima Bay and Funka Bay (accession numbers AB376283 and AB376284, respectively; Table 1) were identical to that of the cryptophyte *Teleaulax amphioxeia*. This result strongly suggests that the plastid of *D. infundibulus* originates from *T. amphioxeia* and that the food web of *D. infundibulus* depends on the predator–prey interactions occurring among *Dinophysis*, *Myrionecta rubra*, and *T. amphioxeia*, as reported for *D. acuminata*/*caudata*/*fortii* (Park et al. 2006, Nagai et al. 2008, Nishitani et al. 2008).

Phylogenetic analysis

From a culture strain of *Dinophysis infundibulus*, 1801 nucleotides of the almost complete SSU rRNA gene, 642 nucleotides of the ITS regions, and 735 nucleotides of the partial LSU rRNA gene (D1-D2) were successfully amplified and determined. The sequence information is provided in Table 1. In the SSU alignment set, 8 operational taxonomic units (OTUs) of the *Dinophysis* species and 1 OTU of *Karenia mikimotoi* were used. All 3 analytical methods (NJ, MP, and ML) yielded the same topology. In the ITS regions alignment set, 22 OTUs of the *Dinophysis* species and 2 OTU of *K. mikimotoi* were used. All 3 analytical methods yielded the same topology. In the LSU alignment set, 21 OTUs of the *Dinophysis* species and 3 OTUs of *K. mikimotoi* were used. All 3 analytical methods yielded almost the same topology. Only the ML tree is presented, and bootstrap values from NJ, MP, and ML analyses are indicated on these trees (Fig. 5, SSU; Fig. 6, ITS; Fig. 7, LSU).

The SSU rDNA sequence of *Dinophysis infundibulus* was very similar to that of *D. fortii* and *D. acuta*; however, 1 and 2 base pair substitutions were detected. In the SSU phylogenetic trees (Fig. 5), *D. infundibulus* formed a clade with *D. fortii* AB073118; this was supported by low bootstrap values (71/61/64% = NJ/MP/ML). This clade was sister to *D. acuta* AJ506973, supported by high bootstrap values (100/97/98% = NJ/MP/ML). The ITS sequence of *D. infundibulus* has 8 and 21 base pair substitutions compared to those of *D. fortii* and *D. caudata*, respectively. The phylogenetic tree showed that *D. infundibulus* formed a clade with *D. fortii* AB355142 and AB355143, supported by high bootstrap values (95/94/82% = NJ/MP/ML). This clade was sister to *D. caudata* AY040584, supported by high bootstrap values (90/98/92% = NJ/MP/ML). The partial LSU rDNA sequence of *D. infundibulus* has 4 to 11 base pair substitutions compared to that of *D. fortii*, *D. acuta*, *D. tripos*, and *D. odiosa*. In the partial LSU phylogenetic trees, *D. infundibulus* was sister to a clade that includes sequences of *D. fortii* and *D. acuta*, sup-

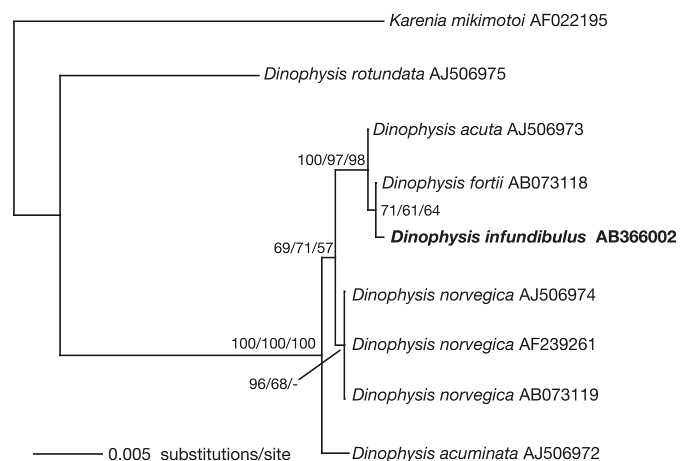

Fig. 5. Maximum likelihood tree using the sequences of nuclear small subunit rRNA regions, showing relationships within the genus *Dinophysis*. *Karenia mikimotoi* was used as an outgroup. Neighbor-joining/maximum parsimony/maximum likelihood (NJ/MP/ML) bootstrap values (>50%) are placed close to each node. *D. infundibulus* sequences from this study are in **bold**

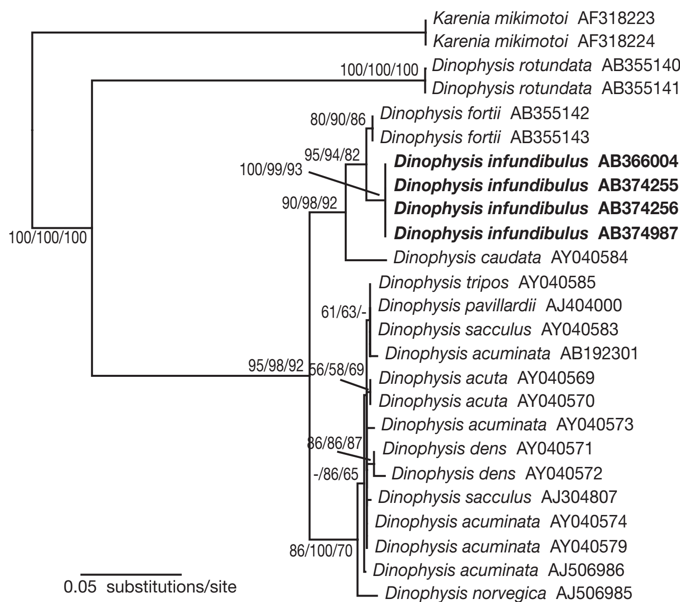

Fig. 6. Maximum likelihood tree using the sequences of nuclear 5.8S rRNA with the ITS regions, showing relationships within the genus *Dinophysis*. *Karenia mikimotoi* was used as an outgroup. NJ/MP/ML bootstrap values (>50%) are placed close to each node. *D. infundibulus* sequences from this study are in **bold**

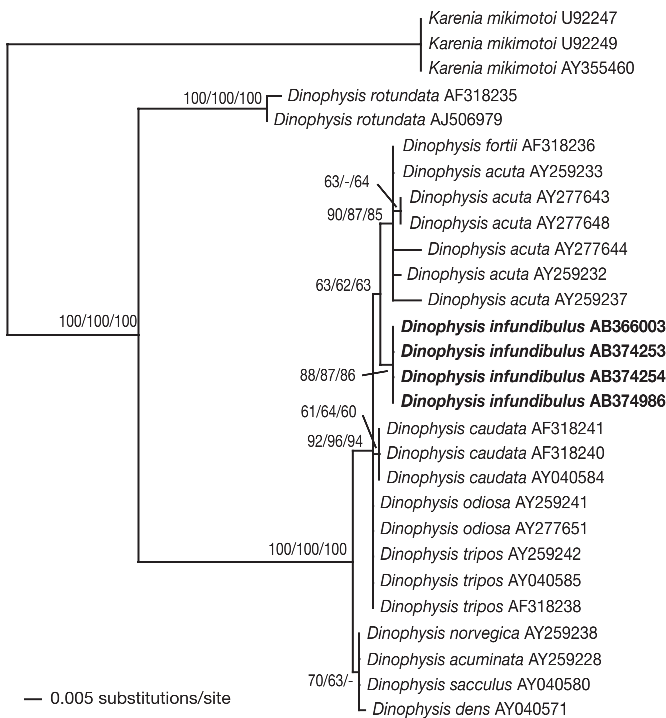

Fig. 7. Maximum likelihood tree using the sequences of nuclear large subunit (D1-D2) rRNA regions, showing relationships within the genus *Dinophysis*. *Karenia mikimotoi* was used as an outgroup. NJ/MP/ML bootstrap values (>50%) are placed close to each node. *D. infundibulus* sequences from this study are in **bold**

ported by moderate bootstrap values (63/62/63% = NJ/MP/ML). Interestingly, these phylogenetic trees suggest that *D. fortii* and *D. acuta* are the closest species to *D. infundibulus*, although their morphology and toxin productivities of DSP are not so similar. The base substitution between the sequences of *D. infundibulus* and the closest species was lowest with regard to SSU (1 base per 1801 nucleotides), intermediate with regard to LSU (4 bases per 735 nucleotides), and highest with regard to the ITS regions (8 bases per 642 nucleotides). However, in *D. infundibulus*, the sequences of both LSU and ITS regions were perfectly matched at the intraspecific level and were distinguishable from those of other species, demonstrating the high utility of both genes for species identification.

Food web among *Dinophysis*, *Myrionecta*, and *Teleaulax*

Non-photosynthetic species of *Dinophysis* feed by myzocytosis, a process whereby the peduncle (or feeding tube) sucks up the cytoplasm from the prey, leaving behind the plasmalemma (Hansen 1991). Photosynthetic species share this structure, and although they have not been observed feeding, food vacuoles are often found in their cytoplasm, clearly indicating mixotrophy (Jacobson & Andersen 1994, Koike et al. 2000). Park et al. (2006), Nishitani et al. (2008), Nagai et al. (2008), and our present data have clearly shown that *D. acuminata*/*caudata*/*fortii*/*infundibulus* use their peduncle to ingest *Myrionecta rubra* grown on *Teleaulax* for their propagation. Interestingly, a contradictory result was obtained in *D. fortii* sampled from the Okhotsk Sea (144° 20′ E, 44° 03′ N): the percentage of isolation success was 26/192 (13.5%; S. Nagai et al. unpubl. data), which was significantly lower than for the Hiroshima Bay population (48/60, 80.0%). Although most of the single cells isolated from the Okhotsk Sea grew once, the maintenance of the culture was unsuccessful due to poor growth after the *Dinophysis* cells were re-inoculated into *M. rubra* culture. In addition, trials for the establishment of clonal cultures in *D. tripos* sampled from the Okhotsk Sea were unsuccessful, although a phylogenetic analysis inferred from a plastid genome supported its chloroplast origin in the cryptophytes *Teleaulax*/*Plagioselmis*/*Geminigera* (Koike et al. 2005, Takahashi et al. 2005). Thus, with regard to *D. fortii* or *D. tripos* in the Okhotsk Sea, the predator and prey cultures may be incompatible or the prey species may be slightly different from those found in the northern part of Japan.

Nagai et al. (2008) observed the disappearance of relatively large chloroplasts (>5 µm in length) from *Dinophysis fortii* after >4 wk 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, particularly in small cells. However, the dinoflagellate survived more than 2 mo of incubation without feeding on prey. Cells of *D. acuminata* (S. Nagai et al. unpubl. data), *D. caudata* (G. Nishitani et al. unpubl. data), and *D. fortii* (Nagai et al. 2008), which were incubated without the prey ciliate for ca. 2 mo, still retained a few small chloroplasts. Whether they are permanent chloroplasts or retained kleptoplastids is debatable. Recently, Hansen & Fenchel (2006) argued that the plastids of *Myrionecta rubra* are not kleptoplastids, but rather permanent endosymbionts, and that feeding on the cryptophyte *Teleaulax amphioxeia* is for acquisition of essential growth factors. Johnson et al. (2007) provided evidence that *M. rubra* sequesters cryptophyte organelles by retainment of transcriptionally active cryptophyte nuclei. Gene sequences of *Dinophysis* and *M. rubra* plastids show striking similarities to those of the cryptophytes *Teleaulax/Plagioselmis/Geminigera* (Takishita et al. 2002, Janson 2004, Koike et al. 2005, Takahashi et al. 2005, Johnson et al. 2006, Minnhagen & Janson 2006), implying that more variable regions in the plastid genome may need to be analyzed to definitively answer this question.

CONCLUSIONS

Recent molecular studies have suggested that the plastid DNA sequences of several photosynthetic species of *Dinophysis* are identical and closely related to those of *Teleaulax amphioxeia*, *Plagioselmis* sp., or *Geminigera cryophila* (Takishita et al. 2002, Janson 2004, Koike et al. 2005, Takahashi et al. 2005, Minnhagen & Janson 2006). Park et al. (2006), Nishitani et al. (2008), Nagai et al. (2008), and our present data clearly showed the mixotrophy of *Dinophysis acuminata/caudata/fortii/infundibulus*, requiring *Myrionecta rubra* grown on *Teleaulax* as prey for their propagation. Although further examples are clearly required, this evidence strongly suggests that *Dinophysis* regarded as photosynthetic species depend on the predator–prey interactions occurring with *M. rubra* and *Teleaulax/Plagioselmis/Geminigera*. Briefly, culture strains of *Dinophysis* species could potentially be established and maintained by feeding the ciliate prey grown on *Teleaulax/Plagioselmis/Geminigera*. In the observation of the sequestration process of the chloroplasts ingested from *M. rubra* by *D. acuminata* (S. Nagai et al. unpubl. data), *D. fortii* (Nagai et al. 2008), and *D. infundibulus* (Fig. 2), chloroplasts of *M. rubra* are ingested and dispersed in these *Dinophysis* cells in advance of the ingestion of the other cell contents to prevent them from being digested in food vacuoles, suggesting that the ingested chloroplasts can function as kleptoplastids. Koike et al. (2005) deduced that *D. mitra* takes up haptophytes myzocytotically and selectively retains the plastid with surrounding plastidal membranes, whereas other haptophyte cell components are degraded. This suggests the possibility of another type of triangular food web among *Dinophysis*, ciliates and haptophytes. Presumably, the food webs revolving around *Dinophysis* are more variable and complicated than we expect at present.

Little is known about the ecophysiology, toxicology, and blooming mechanisms of *Dinophysis* species because studies have been hampered by the inability to culture them (Sampayo 1993, Jacobson & Andersen 1994, Maestrini 1998, Nishitani et al. 2003). However, with the clarification of the food web involving *Dinophysis*, *Myrionecta rubra*, and *Teleaulax amphioxeia*, significant progress in the research on DSP caused by toxic *Dinophysis* species can be expected in the near future. However, as research progresses, it will lead to many new questions.

Acknowledgements. This work was supported by the Global Environment Research Fund (RF-066) by the Ministry of the Environment, Japan.

LITERATURE CITED

Abe TH (1967) The armoured Dinoflagellata: II. Prorocentridae and Dinophysidae (B)—*Dinophysis* and its allied genera. Publ Seto Mar Biol Lab 15:37–78

Adachi M, Sako Y, Ishida Y (1994) Restriction fragment length polymorphism of ribosomal DNA internal transcribed spacer and 5.8S regions in Japanese *Alexandrium* species (Dinophyceae). J Phycol 30:857–863

Carvalho WS, Minnhagen S, Granéli E (2008) *Dinophysis norvegica* (Dinophyceae), more a predator than a producer? Harmful Algae 7:174–183

Delgado M, Garcés E, Camp J (1996) Growth and behaviour of *Dinophysis sacculus* from NW Mediterranean. In: Yasumoto T, Oshima Y, Fukuyo Y (eds) Harmful and toxic algal blooms. IOC of UNESCO, Paris, p 261–264

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Geider RJ, Gunter PA (1989) Evidence for the presence of phycoerythrin in *Dinophysis norvegica*, a pink dinoflagellate. Br Phycol J 24:195–198

Giacobbe MG, Gangemi E (1997) Vegetative and sexual aspects of *Dinophysis pavillardi* (Dinophyceae). J Phycol 33:73–80

Guillard RRL (1973) Division rates. In: Stein JR (ed) Handbook of phycological methods: culture methods and growth measurements. Cambridge University Press, Cambridge, p 289–311

Guillard RRL (1975) Culture of phytoplankton for feeding marine invertebrates. In: Smith WL, Chanley MH (eds) Culture of marine invertebrate animals. Plenum Press, New York, p 26–60

Hackett JD, Maranda L, Yoon HS, Bhattacarya D (2003) Phylogenetic evidence for the cryptophyte origin of the plastid

of *Dinophysis* (Dinophysiales, Dinophyceae). J Phycol 39: 440–448

Hallegraeff GM, Lucas IAN (1988) The marine dinoflagellate genus *Dinophysis* (Dinophyceae): photosynthetic, neritic and non–photosynthetic, oceanic species. Phycologia 27: 25–42

Hansen PJ (1991) *Dinophysis* — a planktonic dinoflagellate genus which can act both as a prey and a predator of a ciliate. Mar Ecol Prog Ser 69:201–204

Hansen PJ (2002) Effect of high pH on the growth and survival of marine phytoplankton: implications for species succession. Aquat Microb Ecol 28:279–288

Hansen PJ, Fenchel T (2006) The bloom-forming ciliate *Mesodinium rubrum* harbours a single permanent endosymbiont. Mar Biol Res 2:169–177

Hewes CD, Mitchell BG, Moisan TA, Vernet M, Reid FMH (1998) The phycobilin signatures of chloroplasts from three dinoflagellate species: a microanalytical study of *Dinophysis caudata, D. fortii*, and *D. acuminata* (Dinophysiales, Dinophyceae). J Phycol 34:945–951

Imai I, Nishitani G (2000) Attachment of picophytoplankton to the cell surface of the toxic dinoflagellates *Dinophysis acuminata* and *D. fortii.* Phycologia 39:456–459

Ishimaru T, Inoue H, Fukuyo Y, Ogata T, Kodama M (1988) Culture of *Dinophysis fortii* and *D. acuminata* with the cryptomonad, *Plagioselmis* sp. In: Aibara K, Kumagai S, Ohtsubo K, Yoshizawa T (eds) Mycotoxins and phycotoxins. Proceedings of the Japanese Association of Mycotoxicology, Tokyo, p 19–20

Jacobson DM, Andersen RA (1994) The discovery of mixotrophy in photosynthetic species of *Dinophysis* (Dinophyceae): light and electron microscopical observations of food vacuoles in *Dinophysis acuminata*, *D. norvegica* and two heterotrophic dinophysoid dinoflagellates. Phycologia 33:97–110

Janson S (2004) Molecular evidence that plastids in the toxin-producing dinoflagellate genus *Dinophysis* originate from the free-living cryptophyte *Teleaulax amphioxeia.* Environ Microbiol 6:1102–1106

Johnson MD, Tengs T, Oldach D, Stoecker DK (2006) Sequestration, performance, and functional control of cryptophyte plastids in the ciliate *Myrionecta rubra* (Ciliophora). J Phycol 42:1235–1246

Johnson MD, Oldach D, Delwiche CF, Stoecker DK (2007) Retention of transcriptionally active cryptophyte nuclei by the ciliate *Myrionecta rubra.* Nature 445:426–428

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Koike K, Koike K, Takagi M, Ogata T, Ishimaru T (2000) Evidence of phagotrophy in *Dinophysis fortii* (Dinophysiales, Dinophyceae), a dinoflagellate that causes diarrhetic shellfish poisoning (DSP). Phycol Res 48:121–124

Koike K, Sekiguchi H, Kobiyama A, Takishita K, Kawachi M, Koike K, Ogata T (2005) A novel type of kleptoplastidy in *Dinophysis* (Dinophyceae): presence of haptophyte-type plastid in *Dinophysis mitra.* Protist 156:225–237

Koike K, Nishiyama A, Saitoh K, Imai K, Koike K, Kobiyama A, Ogata T (2006) Mechanism of gamete fusion in *Dinophysis fortii* (Dinophyceae, Dinophyta): light microscopic and ultrastructural observations. J Phycol 42:1247–1256

Lessard EJ, Swift E (1986) Dinoflagellates from the North Atlantic classified as phototrophic or heterotrophic by epifluorescence microscopy. J Plankton Res 8:1209–1215

Maestrini SY (1998) Bloom dynamics and ecophysiology of *Dinophysis* spp. In: Anderson DM, Cembella AD, Hallegraeff GM (eds) Physiological ecology of harmful algal blooms. NATO ASI Series, Vol G 41, Springer-Verlag, Berlin, p 243–265

Maestrini SY, Berland BR, Grzebyk D, Spanò AM (1995) *Dinophysis* spp. cells concentrated from nature for experimental purposes, using size fractionation and reverse migration. Aquat Microb Ecol 9:177–182

Minnhagen S, Janson S (2006) Genetic analyses of *Dinophysis* spp. support kleptoplastidy. FEMS Microbiol Ecol 57:47–54

Miyazono A, Suzuki T, Baba K, Sugawara R (2008) Occurrence of *Dinophysis* spp., and temporal changes in diarrhetic shellfish poison and lipophilic toxins in *Dinophysis* spp. in Funka Bay, Hokkaido. Bull Plankton Soc Jpn 55: 25–28 (in Japanese with English Abstract)

Nagai S, Matsuyama Y, Oh SJ, Itakura S (2004) Effect of nutrients and temperature on encystment of the toxic dinoflagellate *Alexandrium tamarense* (Dinophyceae) isolated from Hiroshima Bay, Japan. Plankton Biol Ecol 51: 103–109

Nagai S, Nishitani G, Tomaru Y, Sakiyama S, Kamiyama T (2008) Growth characteristics of the toxic dinoflagellate *Dinophysis fortii* fed on a marine ciliate *Myrionecta rubra* and observation of sequestration process of the ingested chloroplasts. J Phycol 44:909–922

Nishitani G, Miyamura K, Imai I (2003) Trying to cultivation of *Dinophysis caudata* (Dinophyceae) and the appearance of small cells. Plankton Biol Ecol 50:31–36

Nishitani G, Nagai S, Sakiyama S, Kamiyama T (2008) Successful cultivation of the toxic dinoflagellate *Dinophysis caudata* (Dinophyceae). Plankton Benthos Res 3:78–85

Orlova TY, Selina MS, Stonik IV (2004) Species structure of plankton microalgae on the coast of the Sea of Okhotsk on Sakhalin Island. Russ J Mar Biol 30:77–86

Park MG, Kim S, Kim HS, Myung G, Kang YG, Yih W (2006) First successful culture of the marine dinoflagellate *Dinophysis acuminata.* Aquat Microb Ecol 45:101–106

Pedersen MF, Hansen PJ (2003) Effect of high pH on a natural marine planktonic community. Mar Ecol Prog Ser 260: 19–31

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 14:817–818

Reguera B, González-Gil S (2001) Small cell and intermediate cell formation in species of *Dinophysis* (Dinophyceae, Dinophysiales). J Phycol 37:318–333

Reguera B, González-Gil S, Delgado M (2004) Formation of *Dinophysis dens* Pavillard and *D. diegensis* Kofoid from laboratory incubations of *Dinophysis acuta* Ehrenberg and *D. caudata* Saville-Kent. In: Steidinger KA, Landsberg JH, Tomas CR, Vargo GA (eds) Harmful algae 2002. IOC of UNESCO, FL, p 440–442

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sampayo MA de M (1993) Trying to cultivate *Dinophysis* spp. In: Smayda TJ, Shimizu Y (eds) Toxic phytoplankton blooms in the sea. Elsevier, Amsterdam, p 807–810

Schiller J (1933) Dinoflagellatae (Peridineae). In: Rabenhorst GL, Kolkwitz R (eds) Kryptogamen-Flora von Deutschland, Österreich und der Schweiz. Akademische Verlagsgesellschaft, Leipzig, p 111–112

Schnepf E, Elbrächter M (1988) Cryptophycean-like double membrane-bound chloroplast in the dinoflagellates, *Dinophysis* Ehrenb.: evolutionary, phylogenetic and toxicological implications. Bot Acta 101:196–203

Scholin CA, Herzog M, Sogin M, Anderson DM (1994) Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). II.

Sequence analysis of a fragment of the LSU rRNA gene. J Phycol 30:999–1011
Shin EY, Park JG, Yeo HG (2004) A taxonomic study of family Dinophysiaceae Stein (Dinophysiales, Dinophyta) in Korean coastal waters. Ocean Polar Res 26:655–668
Sidari L, Nichetto P, Cok S, Sosa S, Tubaro A, Honsell G, Loggia RD (1998) Phytoplankton selection by mussels, and diarrhetic shellfish poisoning. Mar Biol 131:103–111
Suzuki T, Miyazono A, Okumura Y, Kamiyama T (2006) LC-MS/MS analysis of lipophilic toxins in Japanese *Dinophysis* species. PICES 15th Annu Meeting, Book of Abstracts, p 232. Available at: www.pices.int/publications/book_of_abstracts/
Swofford DL (2002) Phylogenetic analysis using parsimony and other methods (Software). Version 4.0 Beta 10, Sinauer Associates, Sunderland, MA
Takahashi Y, Takishita K, Koike K, Maruyama T, Nakayama T, Kobiyama A, Ogata T (2005) Development of molecular probes for *Dinophysis* (Dinophyceae) plastid: a tool to predict blooming and explore plastid origin. Mar Biotechnol 7:95–103
Takishita K, Koike K, Maruyama T, Ogata T (2002) Molecular evidence for plastid robbery (kleptoplastidy) in *Dinophysis*, a dinoflagellate causing diarrhetic shellfish poisoning. Protist 153:293–302
Taylor FJR, Haigh R (1996) Spatial and temporal distributions of microplankton during the summers of 1992-1993 in Barkley Sound, British Columbia, with emphasis on harmful species. Can J Fish Aquat Sci 53:2310–2322
Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882
Uchida T, Matsuyama Y, Kamiyama T (1999) Cell fusion in *Dinophysis fortii* Pavillard. Bull Fish Environ Inland Sea 1: 163–165
Vesk M, Dibbayawan TP, Vesk PA (1996) Immunogold localization of phycoerythrin in chloroplasts of *Dinophysis acuminata* and *D. fortii* (Dinophysiales, Dinophyta). Phycologia 35:234–238
Yamaguchi M, Honjo T (1989) Effects of temperature, salinity and irradiance on the growth of the noxious red tide flagellate *Gymnodinium nagasakiense* (Dinophyceae). Nippon Suisan Gakkaishi 55:2029–2036 (in Japanese with English Abstract)
Yamaguchi M, Imai I, Honjo T (1991) Effects of temperature, salinity and irradiance on the growth of the noxious red tide flagellates *Chattonella antiqua* and *C. marina* (Raphidophyceae). Nippon Suisan Gakkaishi 57:1277–1284 (in Japanese with English Abstract)
Zingone A, Montresor M, Marino D (1998) Morphological variability of the potentially toxic dinoflagellate *Dinophysis sacculus* (Dinophyceae) and its taxonomic relationships with *D. pavillardii* and *D. acuminata.* Eur J Phycol 33:259–273

Editorial responsibility: Patricia Glibert, Cambridge, Maryland, USA

Submitted: February 21, 2008; Accepted: June 10, 2008
Proofs received from author(s): August 18, 2008