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Successful cultivation of the toxic dinoflagellate *Dinophysis caudata* (Dinophyceae)

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Abstract: Recently, Park et al. (2006) succeeded in cultivating the toxic dinoflagellate *Dinophysis acuminata* and maintaining them by feeding the ciliate *Myrionecta rubra* grown with a cryptophyte *Teleaulax* sp. After this report, the present study is the second report of propagation of a *Dinophysis* species (*Dinophysis caudata*) under laboratory conditions and describes the maintenance of several clonal strains kept at high abundance (>5,000 cells mL^{-1}) for a relatively long period (>4 months) when fed on *M. rubra* with the addition of *Teleaulax amphioxeia*. We confirmed that *D. caudata* swam actively around its ciliate prey and inserted its peduncle (feeding tube) into the ciliate. Thereafter, the prey became immobile and rounded. *Dinophysis caudata* actively ingested the cytoplasm of the prey through the peduncle. *Dinophysis caudata* grew at a growth rate of 1.03 divisions day^{-1} when supplied with *M. rubra* as prey, reaching a maximum concentration of ca. 5,000 cell $well^{-1}$ (810 μL) during a 9 day growth experiment. In contrast, a culture of *D. caudata* was not able to be established in the absence of the ciliate or when provided with *T. amphioxeia* only, suggesting that *D. caudata* can not directly utilize *T. amphioxeia* as prey.

Key words: culture, diarrhetic shellfish poisoning (DSP), *Dinophysis caudata*, *Myrionecta rubra*, *Teleaulax amphioxeia*

Introduction

In the dinoflagellate genus *Dinophysis*, some species are known to cause diarrhetic shellfish poisoning (DSP). The physiological and ecological characteristics of this genus are not yet fully understood due to difficulties in culturing the organisms.

Dinophysis caudata Saville-Kent is one of the toxic species that causes DSP. Okadaic acid (OA) and dinophysistoxin-1 (DTX1) were detected from *D. caudata* cells (<76.3 pg $cell^{-1}$ of OA and DTX1 in total) in Sapian Bay, the Philippines (Marasigan et al. 2001). This species is widely distributed in tropical and temperate waters and can appear abundantly in coastal waters, with a red tide of this species associated with mass mortalities of fish being reported in the Seto Inland Sea, Japan (Okaichi 1967). Holmes et al. (1999) reported that *D. caudata* was the main species causing DSP in green mussels in Singapore. Thus, *D. caudata* might be one of the main causative species of DSP in the future, especially in tropical regions. The establishment of cultures is crucial to study the physiology and toxicology of this species. Recently, Park et al. (2006) succeeded in cultivating *Dinophysis acuminata* Claparède et Lachmann at high cell densities (>11,000 cell mL^{-1}) and maintained them for a long period (>6 months) by feeding the ciliate *Myrionecta rubra* (Lohmann 1908) grown with a cryptophyte *Teleaulax* sp. In this report, we followed their experimental design, and succeeded in cultivating *D. caudata*. We report here the conditions necessary for cultivation of *D. caudata* and describe their feeding strategy.

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Materials and Methods

The marine ciliate *Myrionecta rubra* and the cryptophyte *Teleaulax amphioxeia* (Conrad) Hill were isolated from Inokushi Bay (131°53′E, 34°47′N) at the end of February 2007 in Oita Prefecture, Japan. *Myrionecta rubra* and *T. amphioxeia* were identified by their morphology and sequence data from the nuclear small subunit rDNA. The sequences are deposited in GenBank under accession num-

bers AB364286 for *M. rubra* and AB364287 for *T. amphioxeia*. The culture of *M. rubra* was maintained by re-inoculating once a week 50 mL of the culture (7,000–9,000 cells mL^{-1}) into 100 mL of a modified f/2 medium (Guillard 1975, Nagai et al. 2004) in 250 mL capacity polycarbonate Erlenmeyer flasks (Corning, NY, USA). The culture medium was made up with 1/3 nitrate, phosphate and metals and 1/10 vitamins, based on enrichment of natural seawater collected from the same location of Inokushi Bay (salinity adjusted to 30 psu) with the addition of 100 μL of *T. amphioxeia* culture (containing 3,000 cells) as prey for *M. rubra*. They were maintained at a temperature of 18°C under an irradiance of 100–150 μmol photon m^{-2} s^{-1} provided by cool-white fluorescent lamps with a 12 : 12 h L : D cycle. Several *Dinophysis caudata* were isolated from Inokushi Bay in June 2007 and established as a clonal culture with feeding on *M. rubra*. The culture of *D. caudata* was maintained by re-inoculating 150 μL of the culture (2,500–3,000 cells mL^{-1}) into 750 μL of *M. rubra* culture (containing 3,000–3,500 cells) at 25°C under the same light conditions as above. All cultures were non-axenic but they were all clonal. Observations of feeding behaviour and binary fission of *D. caudata* were carried using the maintenance culture with an inverted microscope (Nikon TE-300).

A strain (DC0706YTS01) of *D. caudata* was used for the following experiments. The growth experiment on *D. caudata* was conducted under two different initial concentrations of *M. rubra*. In short, a culture of *M. rubra* grown until the late logarithmic growth phase (ca. 8,500 cells mL^{-1}, *T. amphioxeia* was not included) was diluted with fresh culture medium to give initial concentrations of 1,500 and 5,000 cells $well^{-1}$ and 750 μL of the diluted cultures were inoculated into all wells of a 48 well microplate (Iwaki, Chiba, Japan). Thereafter, 60 μL of a *D. caudata* culture was added into the *M. rubra* culture to give an initial concentration of 50 cells per well. Cells of *D. caudata*, which were cultivated without the prey ciliate for two days in a maintenance culture, were used in this experiment. The growth experiment was conducted for 11 days under the same conditions as for the maintenance culture of *D. caudata* (=25°C). As controls, only *M. rubra* (1,500 or 5,000 cells $well^{-1}$), and *D. caudata* (50 cells $well^{-1}$) with *T. amphioxeia* (1,500 cells $well^{-1}$) were incubated under the same conditions as above (=25°C).

To examine the growth potential of *D. caudata* without the presence of the prey ciliate, after feeding heavily on *M. rubra*, 48 cells of *D. caudata* (the same clonal strain as used in the above experiments) that appeared fully expanded by the active ingestion of prey, were picked up from the maintenance culture by micropipetting each separately into each well of a 48 well microplate, containing 800 μL culture medium. These cells of *D. caudata* were maintained under the same conditions as above. In all growth experiments, three wells of the cultures (500 μL) that were randomly selected (i.e. in triplicate) were sampled after gentle pipetting for agitation, and fixed with glutaraldehyde (final conc. 1%). The cell densities of *D. caudata*, *M. rubra* and *T. amphioxeia* were counted using an inverted microscope. The growth rates (divisions day^{-1}) of *D. caudata*, *M. rubra* and *T. amphioxeia*, determined to be in the exponential growth phase, were calculated using the method of Guillard (1973).

Results and Discussion

Observations of the feeding process

Similar to the report for *Dinophysis acuminata* by Park et al. (2006), *Dinophysis caudata* was able to feed on the ciliate *Myrionecta rubra* (Figs. 1, 2). *Dinophysis caudata* used a peduncle, which extends from around the flagellar pore, to extract the cell contents of *M. rubra* (Fig. 1A–D), as has been previously reported for *Dinophysis rotundata* Claparède et Lachmann (Hansen 1991) and *D. acuminata* (Park et al. 2006). Judging from the photographs shown in Fig. 1A–D, the peduncle of *D. caudata* was much narrower than that reported in *D. acuminata* (Park et al. 2006) and the length and width of the peduncle of *D. caudata* was about 20 μm and 2 μm, respectively. No instances of the peduncle being extended outside of the cell in *D. caudata* were observed, indicating that *D. caudata* keeps the peduncle inside of the cell and it only appears outside of the cell immediately before capturing prey. A prong-like structure was found at the edge of the narrow peduncle (Fig. 1A). Soon after *D. caudata* inserted the peduncle into the cell of *M. rubra* (Fig. 1C, D), the ciliate became immobile and their cilia were shed from the cell within one minute (Fig. 2A), suggesting that *D. caudata* injects some kind of toxin into the cell of the ciliate using its peduncle. *Dinophysis caudata* kept the prey captured around the flagellar pore (Fig. 2A) and actively ingested the cytoplasm of its prey through the peduncle. The transfer of small portions of cytoplasm into the cell of *D. caudata* was observed through the transparent peduncle. *Dinophysis caudata* fed heavily on *M. rubra* i.e. even when the cell was fully expanded by the active ingestion of prey, active feeding behavior still continued. Propagation of *D. caudata* was observed by frequent binary fission (Fig. 2B), and sequential binary fission was often observed before cell separation from the previous cell division had occurred (Fig. 2C). These cells were connected at cingular lists and were still able to swim actively. A large number of cells were harvested by sieving *D. caudata* cultures with nylon mesh (10 μm, in diameter), providing successful cultivation of *D. caudata* (Fig. 2D).

With an increase in the cell density of *D. caudata* (>100 cells $well^{-1}$), *M. rubra* cells tended to form many clumps, intertwine with each other by their cilia, swim helicoidally or rotate in the same position on the bottom of the microplate (Fig. 2E), suggesting that some kind of allelopathic chemical was released from *D. caudata* cells. *Dinophysis caudata* aggregated around these abnormally acting *M. rubra* and actively fed on them (Fig. 2F). Various aspects of the feeding behaviour of *D. caudata*, i.e. the ability

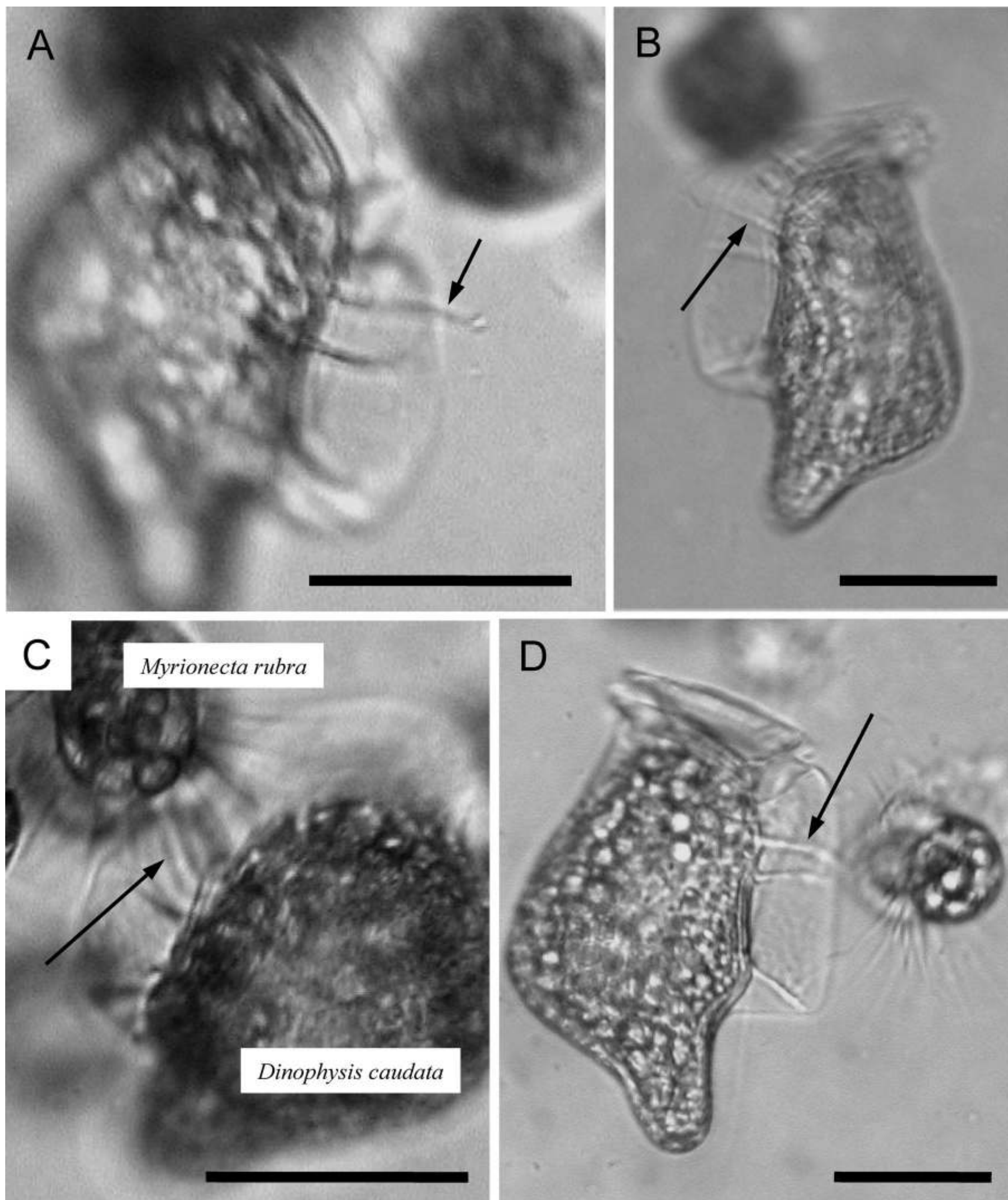

Fig. 1. Observations of the peduncle in *Dinophysis caudata*. A, A peduncle of *D. caudata* having a prong-like structure at the edge (arrow); B, An actively swimming cell trying to capture ciliate prey by its peduncle (arrow); C, A *D. caudata* cell that has just inserted its peduncle into a *Myrionecta rubra* cell. An arrow indicates the peduncle; D, A *D. caudata* cell trying to stick its peduncle into a *M. rubra* cell. Arrows indicate the peduncle. All scale bars$=30\ \mu$m.

to capture *M. rubra*, remain to be clarified as although *M. rubra* is an organism that can move rapidly, *D. caudata* was still able to capture it without any apparent difficulty. We sometimes observed that *M. rubra* cilia were intertwined with the *Dinophysis* cell surface due to mucilaginous secretions released on the cell surface of *D. caudata*, suggesting that *D. caudata* has various feeding strategies to increase the chances for capturing ciliate prey.

During maintenance or growth experiments on *D. caudata*, the formation of small cells was sometimes observed (Fig. 3A, B). These dwarfish cells tended to be produced as a result of depauperating division, especially when entering their stationary phase (old culture) and the shape was similar to that of *Dinophysis diegensis* Kofoid and was clearly

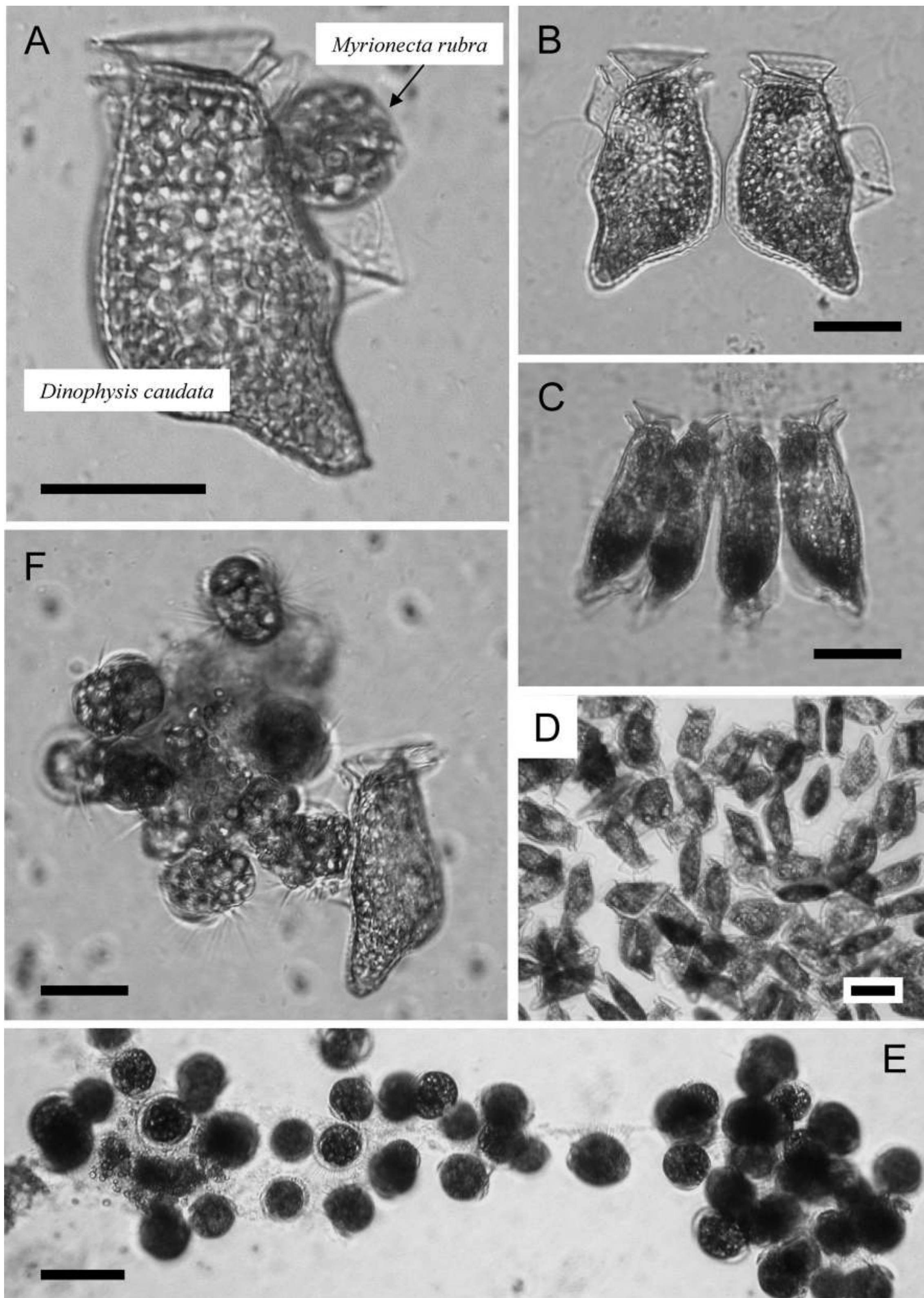

Fig. 2. Observations of feeding and propagation in *Dinophysis caudata* seen during the growth experiment. A, A *D. caudata* cell actively ingesting prey, showing the round shape and loss of cilia in the prey; B, Vegetative cell division of *D. caudata* by binary fission; C, A sequential binary fission of *D. caudata* seen without cell separation from the previous cell division; D, Harvested cells of *D. caudata* after growth experiments, showing successful cultivation; E, Clumped *Myrionecta rubra* cells, which may be caused by the release of some kind of allelopathic chemical from *D. caudata* cells; F, A *D. caudata* cell feeding on clumped *M. rubra* cells. All scale bars$=30\,\mu$m.

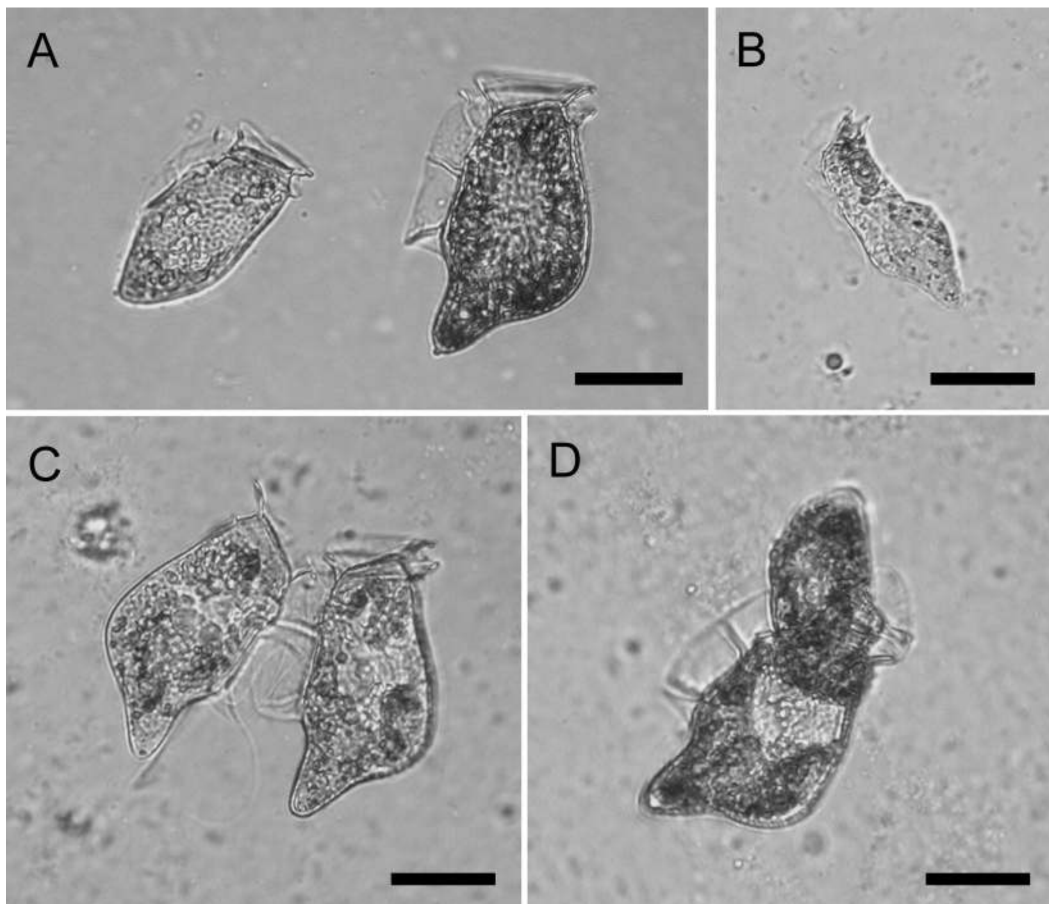

Fig. 3. Various stages in *Dinophysis caudata* observed in the maintenance cultures. A, A dwarfish cell produced as the result of depauperating division (cell shape was similar to that of *Dinophysis diegensis*, left) and a normal size cell (right); B, A small cell having a different shape from the cell shown in Fig. 3A (left); C, A couplet of *D. caudata* joined at the ventral side; D, Fusion in *D. caudata*. All scale bars=30 μm.

different from the normal vegetative cell (Fig. 3A), this being a potential cause of species misidentification in natural samples. The appearance of small cells in trials of laboratory culture have also been reported in *Dinophysis acuta* Ehrenberg (Reguera et al. 2004), *D. caudata* (Nishitani et al. 2003, Reguera et al. 2004), *Dinophysis fortii* Pavillard (Uchida et al. 1999), *Dinophysis pavillardi* Schröder (Giacobbe & Gangemi 1997) and *Dinophysis sacculus* Stein (Delgado et al. 1996). Small cells have been shown to be able to grow again to a large size in *D. acuminata* (Reguera & González-Gil 2001), although we have never observed the phenomenon in cultures of *D. caudata*. Dwarfish cells formed couplets with normal vegetative cells and cell fusion, associating with sexual conjugation, were observed 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 and fusion of *D. caudata* were also observed during the maintenance (Fig. 3C, D), suggesting sexual conjugation within a clonal strain (homothallism).

Growth experiment

The number of cells of *D. caudata* increased exponentially until Day 10 with a growth rate of 1.03 (divisions day^{-1}) during Days 2–5 (Fig. 4A). Initial abundance of *M. rubra* was ca. 1,500 cells well^{-1} and grew until reaching a peak of ca. 8,900 cells well^{-1} on Day 4 (0.74 divisions day^{-1}). After the peak, the number of cells of *M. rubra* declined rapidly and disappeared by Day 8 due to active feeding by *D. caudata* and natural death. Even after the disappearance of *M. rubra*, *D. caudata* continued to increase in number until Day 10 and reached a maximum cell density of ca. 5,200±550 cells well^{-1} (mean±SD).

The number of cells of *D. caudata* increased until Day 9 with a growth rate of 0.93 (divisions day^{-1}) during Days 1–4 (Fig. 4B) and the initial abundance of *M. rubra* was ca. 5,000 cells well^{-1}. *Myrionecta rubra* grew until reaching a peak of ca. 8,260 cells well^{-1} on Day 4 (0.28 divisions day^{-1}). After the peak, cell numbers of *M. rubra* declined rapidly and it disappeared by Day 8. Even after the disappearance of *M. rubra*, the number of *D. caudata* continued

to increase until Day 9 and reached a maximum cell density of ca. 2,500±320 cells $well^{-1}$ (mean±SD). The maximum yields of *D. caudata* at *M. rubra* densities of 1,500 cells $well^{-1}$ were significantly higher than that at *M. rubra* densities of 5,000 cells $well^{-1}$ ($p<0.01$, *t*-test). Perhaps, the lower yield of *D. caudata* was caused by nutrient competition or allelopathy from *M. rubra*.

The growth rates of *D. caudata* obtained in this study were very high in comparison with previous reports concerning *D. caudata*, being estimated at 0.28 divisions day^{-1} in field observations (Reguera et al. 1996) and 0.22 divisions day^{-1} in a cultivation trial under laboratory conditions

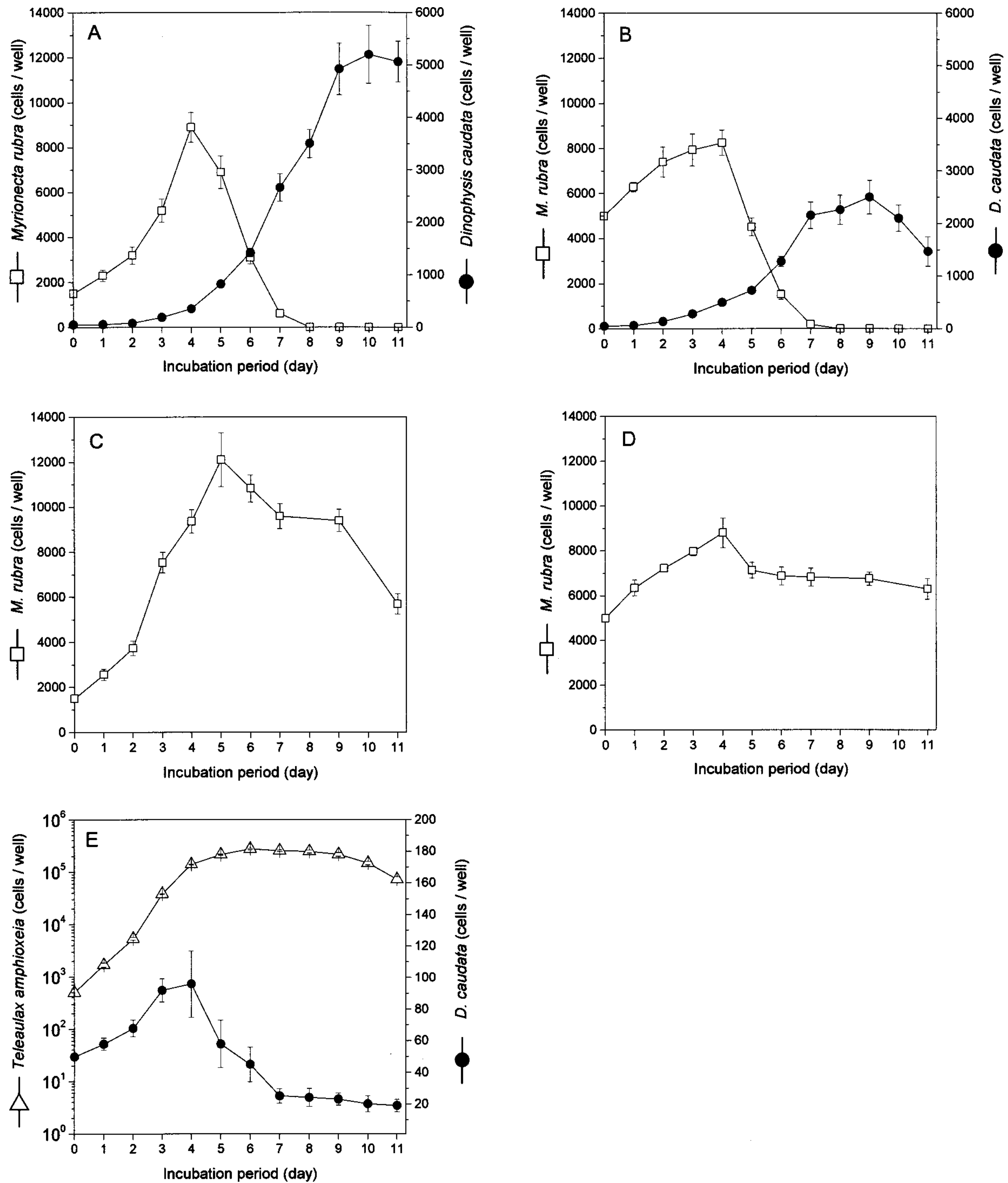

Fig. 4. Growth experiments on *Dinophysis caudata*. A, B, Changes in the number of cells per well of *D. caudata* and *Myrionecta rubra* (A, 1,500 cells $well^{-1}$ as the initial concentration of *M. rubra*; B, 5,000 cells $well^{-1}$ as the initial concentration of *M. rubra*). *Teleaulax amphioxeia* was not included; C, D, Growth of *M. rubra* in cultures without *D. caudata* (C, 1,500 cells $well^{-1}$ as the initial concentration; D, 5,000 cells $well^{-1}$ as the initial concentration). E: Growth of *D. caudata* cultured with *T. amphioxeia* as the only prey. Averages of counts of different triplicate wells and the standard deviation are plotted.

(Nishitani et al. 2003). Park et al. (2006) also reported a high growth rate of 1.37 divisions day^{-1} in *D. acuminata* grown with *M. rubra* as prey under laboratory conditions. It is assumed that these *Dinophysis* species are able to grow as rapidly as other red-tide forming species.

In the control plate without *D. caudata*, *M. rubra* grew for the first 4–5 days, and over the first 3 days at a growth rate of 0.78 and 0.27 divisions day^{-1} at the initial concentrations of 1,500 and 5,000 cells well^{-1}, respectively (Fig. 4C, D). The number of cells of *M. rubra* declined after Days 4–5 due to natural death but many cells (>5,500 cells well^{-1}) survived until the end of the experiments (Day 11).

An increase in the number of cells of *D. caudata* without the presence of ciliate prey but in the presence of the cryptophyte *Teleaulax amphioxeia* was observed for the first four days, reaching a maximum of twice the initial concentration (Fig. 4E). However, the cell numbers of *D. caudata* declined slightly thereafter, and decreased until reaching only 20 cells well^{-1} by the end of the incubation, suggesting that *D. caudata* can not directly utilize *T. amphioxeia* as prey. Cultures of *D. acuminata* and *D. norvegica* were also not able to be established when *Teleaulax* was provided as the only prey (Park et al. 2006, Carvalho et al. 2008). Exponential growth of *T. amphioxeia* was observed until Day 4 at a growth rate of 1.57 divisions day^{-1} and continued to grow until Day 6, reaching a maximum yield of 2.8×10^5 cells well^{-1}. The number of cells remained constant thereafter.

In the control plate without prey, the number of cells of *D. caudata* increased until Day 4 and the average number of cells was 6.5 ± 1.4 cells well^{-1} (mean$\pm$SD), with a growth rate of 0.68 (divisions day^{-1}) during Days 0–4 (Fig. 5). Therefore, cells of *D. caudata*, after feeding heavily on *M. rubra*, could divide at least 3 times without further feeding on the prey. It is assumed that they were able to grow for the first few days utilizing the accumulated surplus of nutrients gained by ingestion of ciliate prey during the previous incubation (Fig. 4E).

Recent molecular analyses of several *Dinophysis* species; *D. acuminata*, *D. acuta*, *D. fortii*, *Dinophysis norvegica* Claparède et Lachmann and *Dinophysis tripos* Gourret, using plastid sequences such as *psbA*, 16S-rDNA and *rbcL* genes, have shown that the plastid specifically originates from the cryptophytes *T. amphioxeia* or *Geminigera cryophila* Hill (Takishita et al. 2002, Hackett et al. 2003, Janson & Granéli 2003, Janson 2004, Takahashi et al. 2005, Minnhagen & Janson 2006). Park et al. (2006) and our present data clearly show that *D. acuminata* and *D. caudata* require *M. rubra* grown with *Teleaulax* as prey for their propagation. Although further examples are clearly required, this evidence strongly suggests that the growth of the photosynthetic *Dinophysis* species are based on the prey-predator interactions occurring among *Dinophysis*, *M. rubra* and *Teleaulax*, in short, culture strains of *Dinophysis* species could potentially be established and maintained by feeding the ciliate prey grown with *Teleaulax*.

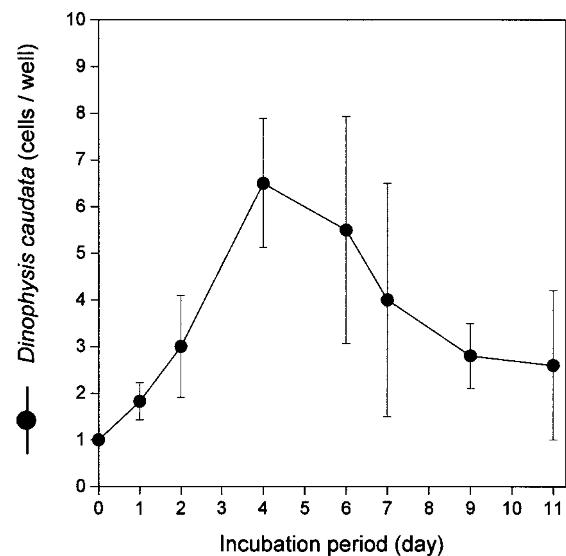

Fig. 5. A growth experiment on *Dinophysis caudata* without the ciliate prey after feeding heavily on *Myrionecta rubra*. Forty-eight cells of *D. caudata* were picked up by micropipetting each separately into each well of a 48 well microplate (1 cell/well). These cells of *D. caudata* were cultivated under the same conditions as in the maintenance culture.

Little is known about the ecophysiology, toxicology and blooming mechanism of *Dinophysis* species, as studies have been hampered by the inability to culture them (Sampayo 1993, Jacobson & Andersen 1994, Maestrini 1998, Nishitani et al. 2003). However, from the clarification of the food web between *Dinophysis*, *M. rubra* and *T. amphioxeia*, significant progress in research on DSP caused by toxic *Dinophysis* species can be expected in the near future.

Acknowledgements

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