クルマエビ中腸腺初代培養に混入した鞭毛菌類の特徴

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Characterization of a Zoosporic Fungus Appearing in Primary Cultures of the Kuruma Prawn Midgut Gland

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Zoosporic fungi which contaminated primary cultures of the Kuruma prawn (*Penaeus japonicus*) midgut gland were consecutively cultured and cloned. An obtained clonal fungus was used as material for characterization. A zoospore of the clonal fungus had two flagella laterally attached to the zoospore body. One flagellum was of the tinsel type. These characters indicated that the clonal fungus should be classified as Oomycetes. The zoospore of the clonal fungus lost the flagella in culture medium and became a spherical vegetative cell having a diameter of about 3 μm. The vegetative cell possessed rhyzoids, gradually increased its diameter to 15 to 20 μm and became a coenocyte. The coenocyte had many mitochondria with tubular cristae, Golgi bodies, two types of granules and well developed rough endoplasmic reticulum in the cytoplasm. Division of the coenocyte into several non-flagellated small cells was confirmed using 16 mm micro-cinematography and electron microscopy. These characters suggest that the clonal fungus belongs to thraustochytrids. Possible routes of the contamination of the primary cultures were discussed.

Key words: midgut gland · *Penaeus japonicus* · primary culture · thraustochytrids · zoosporic fungi.

In the previous study, we initiated several primary cultures of the Kuruma prawn (*Penaeus japonicus*) midgut gland (Machii *et al.* 1988), which produced vigorous proliferation of spherical cells in the first 5 cultures. The cells were certainly neither bacteria nor molds, but their cytological characters were completely different from those of the midgut gland cells of the prawn, as previously described. It was concluded that these cells were contaminants which might be zoosporic fungi, Mastigomycotina (Machii *et al.* 1988).

Further characterization and identification of the fungus would be helpful to prevent fungal contamination in primary cultures of the midgut gland. Thus it would be useful to have information about routes of contamination or methods to prevent their growth when primary cultures are initiated.

This study was conducted to make further identification of the zoosporic fungi which appeared in the primary cultures of the midgut gland of Kuruma prawn.

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Material and Methods

The zoosporic fungi were consecutively cultured and cloned. An obtained clonal fungus was used as material for characterization. Morphology of zoospores and vegetative cells were examined with light and electron microscopy. Cell division of vegetative cells were observed with 16 mm micro-cinematography.

Culture Methods Zoosporic fungi were obtained in 1986 from a primary culture of the midgut gland of Kuruma prawn, *P. japonicus,* details already described elsewhere (Machii *et al.* 1988). The fungi were cultured in medium Pf35 (Machii 1985) at 25°C with T-25 plastic flasks (Nunc 163371), and subcultured 2 to 4 weeks. The pH of the medium was adjusted to 7.5 to 8.0.

Cloning Procedures Preliminary experiments revealed that the contaminant discharged many zoospores within 10 h after displacement from medium Pf35 into balanced salt solution for marine mollusca (MMBSS) (Machii and Wada 1989). Zoospores swimming in the MMBSS could be harvested by gently aspirating the solution, taking care not to disturb the fungi on the bottom of a culture flask. The aspirated zoospores were inoculated in a plastic flask and cultured in Pf35 at 25°C. By the next day, the zoospores had lost their flagella and had become small spherical cells. Single cell suspension of these spherical cells was used for the cloning of the fungi. Cells were diluted to 1 cell/100 μl in Pf35 and inoculated into a 96 well microplate (Corning 25860), each well containing 50 μl of cell suspension. Wells containing a single cell were confirmed with an inverted phase contrast microscope (Olympus IMT-2) and marked. The marked wells were observed daily and wells which had a single colony of proliferating cells were selected from among the previously marked wells. Six days after the inoculation , cells in the selected wells were subcultured to 60 mm glass petri dishes to enlarge the cultures. The procedures were repeated again to obtain fungus clones.

Light Microscopy Zoospores were obtained from a clonal fungus by the method described above. A drop of MMBSS containing zoospores was placed on a glass slide and covered with a cover slip. Swimming zoospores in the preparations were observed with Nomarski differential interference (Olympus Vanox AH-2) and phase contrast microscopes (Olympus Photomax).

Phase contrast micrographs of vegetative cells of the clonal fungus were taken with an inverted phase contrast microscope (Olympus IMT-2).

Electron Microscopy For observation of fine structure of zoospore's flagella, a drop of zoospore suspension obtained from a culture of the clonal fungus was placed on a formvar filmed grid (BIO-RAD 0191) coated with 0.1% poly-L-arginine, and fixed in the vapor of osmic acid (2% OsO_4 in 0.025 M cacodylate buffered sea water, pH 7.5) for 3 min. The medium droplet was carefully drawn off with a filter paper. The grids were rinsed 3 times in distilled water and dried. Preparations were shadowed by gold-palladium and observed by TEM (JEM-100CX).

Vegetative cells of the clonal fungus 2 days after subculture were suspended in the medium by gentle pipetting. The cell suspension was centrifuged (120 × g) for 10 min and the supernatant was discarded. The cells were resuspended in glutaraldehyde (4% glutaraldehyde, 0.28 M sucrose, 0.15 M Na cacodylate, pH 7.5) and fixed for 20 h at 4°C. After fixation, the cells were rinsed twice and centrifuged in rinse buffer (0.025 M cacodylate buffered sea water, pH 7.5), followed by postfixation with

osmic acid (1% OsO_4 in 0.025 M cacodylate buffered sea water, pH 7.5) for 1 h at room temperature. Fixed cells were again rinsed twice and the rinse buffer was removed. A small drop of 1% agarose was added to the tube, the agarose was solidified on a glass slide, and cut into small cubes. These prepared samples were dehydrated in ascending concentrations of ethanol and embedded in Spurr's resin. Ultra thin sections were cut using a diamond knife and a ultramicrotome (Porter-Blum MT-2B). Observation was made with TEM (JEM-1200EX) after double staining with lead citrate and uranyl acetate.

16 mm Micro-cinematography A zoospore suspension obtained from the clonal fungus was inoculated in tissue culture glass flasks (Ikemoto Rika TD-15) containing Pf35 medium. Three hours after the inoculation 16 mm cinematography was begun at room temperature, at 5 min frame intervals using Mini Copy film (Fuji), a phasecontrast microscope (Olympus IMT), a time lapse control unit (Olympus PM-IVM), a motor drive unit (Olympus PM-MDB), an exposure control unit (Olympus PM-CBAD, PM-PBS) and a 16 mm movie camera (BOLEX H16 RX-5, Olympus PM-D 0.4X).

Results

We obtained 12 clones from the above procedure. One of them, Pjmg3-A9C10, was selected as material for further characterization of the fungus. Other clones were successfully stored at $-80°C$ for at least 16 months, adding 10% dimethylsulfoxide to the Pf35 medium as a cryoprotectant (data not shown).

A zoospore of Pjmg3-A9C10 had two oppositely directed flagella that were laterally attached to the zoospore body (Fig. 1A). One of the flagella was of the tinsel type (Sparrow 1973), or a pantoneme flagellum (Perkins 1976), with mastigonemes (Perkins 1976) along both sides of the flagellum (Fig. 1B). The length of the flagella could not be accurately measured but were nearly equal. The zoospore body was ovoid and the length of a longer axis was about 3 μm (Fig. 1). A longitudinal groove on the zoospore body, in which the flagella insert in other zoosporic fungi (Sparrow 1973), could not be observed.

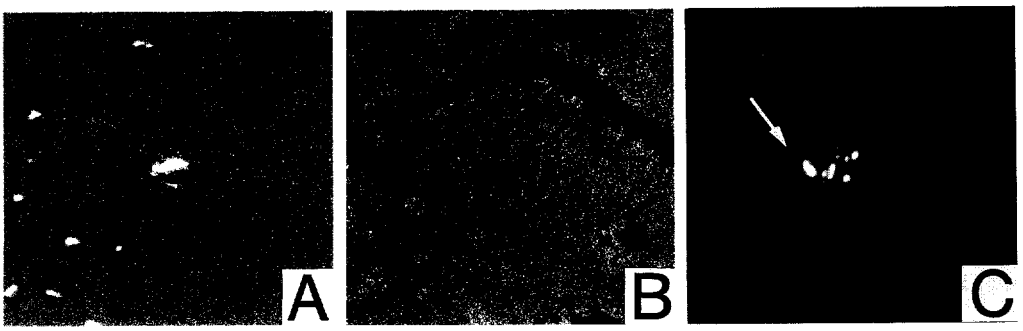

Fig. 1 A zoospore of the clonal fungus, Pjmg3-A9C10. A. A zoospore with two oppositely directed flagella (arrows) laterally attached to the zoospore body. Differential interference. ×1000; B. A tinsel-like flagellum of a zoospore. Arrows indicate mastigonemes. Shadowed with Au-Pd. ×10000; C. A zoospore losing its flagellum. The left flagellum had already shortened and disappeared into a bead (arrow). Phase contrast. ×1980.

Swimming zoospores in a glass slide attached to the glass surface in 10 to 20 min and became sluggish in movement. After a few minutes, they ceased to move, lost the flagella and became small spherical cells with a diameter of about 3 μm. When the zoospore was losing the flagella, beads appeared at the base of each flagellum (Fig. 1C). The flagella steadily shortened and finally disappeared into the beads which then were detached from the cell body.

The small spherical cell gradually increases in diameter, resulting in various sizes of spherical vegetative cells in the culture of Pjmg3-A9C10 (Fig. 2A). The diameters ranged between about 3 and 20 μm, though large spherical cells with a diameter of about 40 μm sometimes also appeared (Fig. 2A). Most of the vegetative cells possessed rhyzoids for 2 or 3 days after subculture (Fig. 2B), but later they disappeared (Fig. 2A).

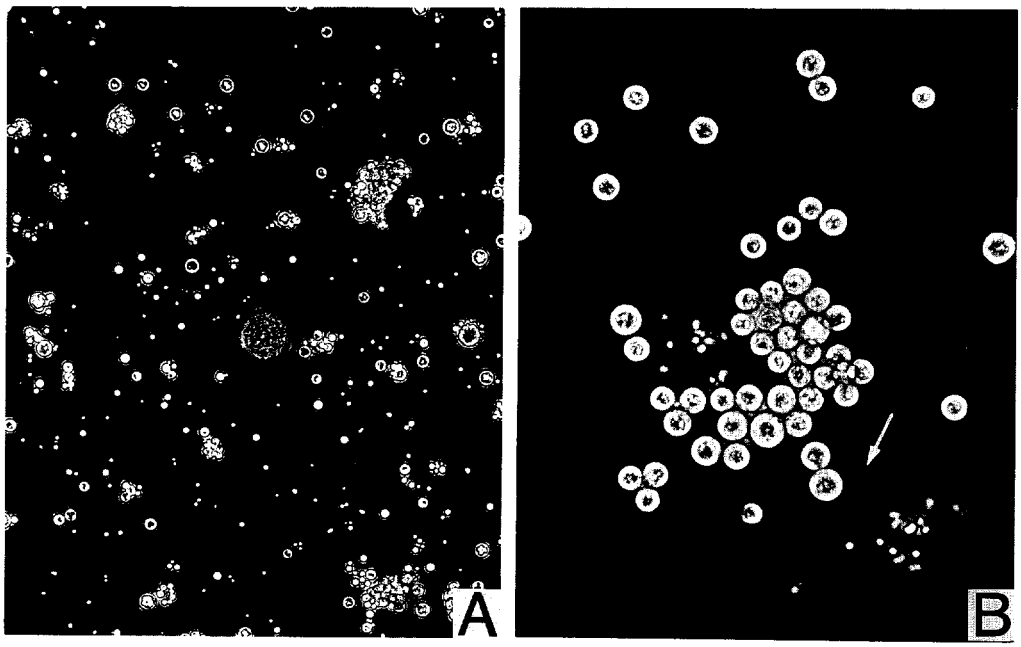

Fig. 2 Vegetative cells of the clonal fungus, Pjmg3-A9C10. A. Eight days after subculture. Various types of cells, including an especially large one. Phase contrast. ×188; B. Two days after subculture. Cells with rhyzoids (arrow) around them. Phase contrast. ×376.

An electron micrograph of a vegetative cell, 13 μm in diameter, shows the cell to be a coenocyte (Fig. 3). Many mitochondria with tubular cristae, cocentric circular rough endoplasmic reticula, Golgi bodies and two types of granules were observed in the cytoplasm (Fig. 3). Nuclei stained weakly compared to usual animal cells.

Division of the enlarged vegetative cell into several small cells was often observed in microcinematography (Fig. 4). The small cells were approximately the same diameter as that of zoospores (about 3 μm), but were not zoospores since they did not have flagella. The ultrastructure of the dividing cell was similar to that of the coenocyte described above. The irregular shape of the dividing cell

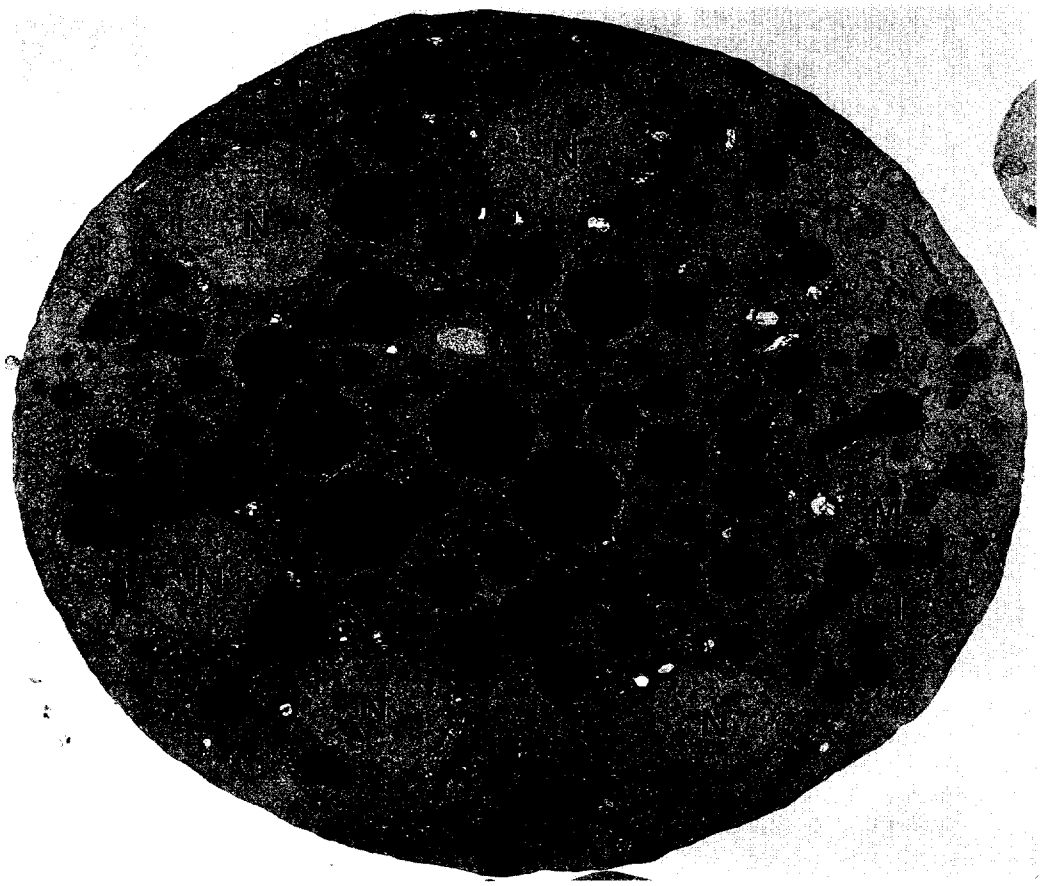

Fig. 3 Ultrastructure of a coenocyte. Two types of granules, electron dense (G1) and moderately dense (G2), were seen. N, nucleus; M, mitochondria with tubular cristae; G, Golgi body; ER, rough endoplasmic reticula. ×9600.

with several solid nuclei (Fig. 5) indicated that cytokinesis occurred in the coenocyte.

Discussion

The fungal zoospores possess two laterally attached flagella, one of them was of the tinsel type. This indicates that Pjmg3-A9C10 should be classified as Oomycetes (Mastigomycotina) according to conventional taxonomy (Sparrow 1973). Whether the zoospores are dimorphic or not, which is an important character for further identification of the fungus (Sparrow 1973), remains uncertain. The large spherical cell with a diameter of 40 μm could represent a zoosporangium, but this has not yet been clarified.

Phase contrast micrographs of Pjmg3-A9C10 and other clones were similar with one another. All of the clones seem to belong to the same or closely related species. The original fungus culture be-

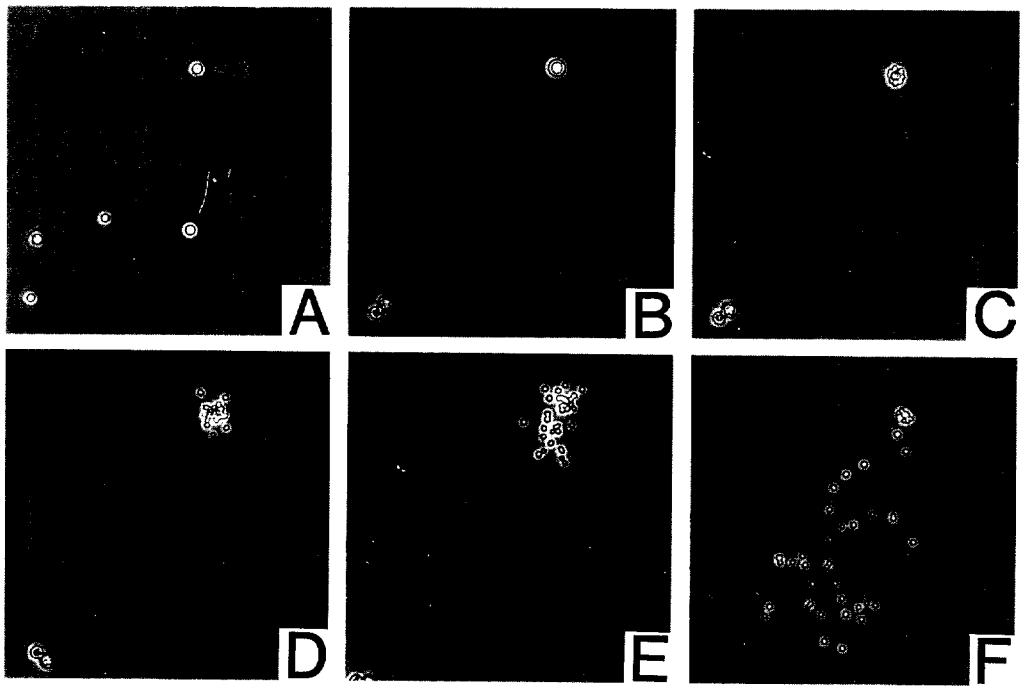

Fig. 4 Cell division of a coenocyte revealed through 16 mm cinematography with a phase contrast microscope. The lapse of time sequence indicated alphabetically. A. Small spherical cells obtained from zoospore suspension. Note a cell with an arrow head.; B. 20 h later. The cell has increased its diameter.; C. 10 min after B. The cell becomes irregular in shape.; D. 20 min after B. Cell division occurred.; E. 40 min after B. The small cells spreading.; F. 2 h after B. The small cells spread out. ×157.

fore cloning may represent a pure culture of a fungus after many times of subculture.

Ellis *et al.* (1985) cultured the larval tissue of *Mytilus californianus* (Mollusca) and observed vigorous proliferation of spherical cells. They concluded from isozyme analyses that the sperical cells did not originate from *M. californianus* and might be thraustochytrids. Phase contrast micrographs and electron micrographs of their contaminants closely resemble those of Pjmg3-A9C10.

Thraustochytrids were formerly situated as one of the families of the order Saplolegniales (Oomycetes) (Dick 1973). However, ultrastructural studies of thraustochytrids have contributed information of considerable taxonomic significance which separates them from the zoosporic fungi and supports their close phyletic affinity with labyrinthulids. Thraustochytrids are presently classified as the order Thraustochytriales which containes 7 genera with 31 species. The orders Thraustochytriales and Labyrinthulales are regarded as one group, but they have no class to be assigned to. Their taxonomic affinities to higher taxa also remain unresolved (Moss 1986).

Thraustochyrids are characterized by monocentric thalli with rhyzoid-like extensions of the thallus, the ectoplasmic net. Their zoospores have two laterally attached flagella, one of which is of the tinsel type, and encyst to form daughter thalli. A second type of asexual reproductive propagule, the aplan-

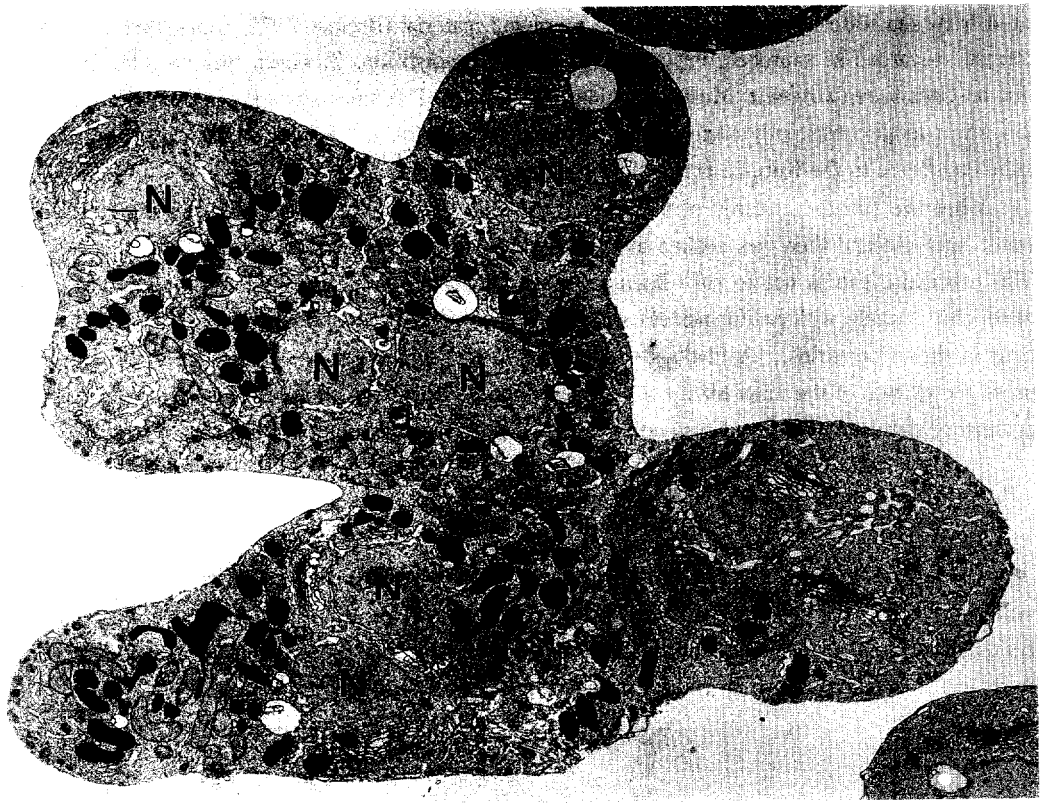

Fig. 5 Ultrastructure of the dividing coenocyte. N, nucleus. ×9600.

ospore, has been recorded for species from all genera (Moss 1986). These are characters similar to those found for Pjmg3-A9C10. The small cells formed from the coenocyte in Pjmg3-A9C10 could represent aplanospores.

Ultrastructural studies of thraustochytrids revealed the presence of a structure associated with the origin of the ectoplasmic net from the thallus, termed the sagenogenetosome, and the presence of a cell wall composed of compact layers of scales in the vegetative thallus. These two ultrastructures are considered diagnostic for the order Thraustochytriales together with the ectoplasmic net (Moss 1986). In the vegetative cells of Pjmg3-A9C10, the sagenogenetosome could not be confirmed. However, the sagenogenetosome is known to be associated with lamellae of rough endoplasmic reticula (Moss 1986) and the cocentric circular endoplasmic reticula observed in Pjmg3-A9C10 could represent the structure related to the sagenogenetosome. The cell wall composed of scales was not conspicuous. Although the relation between thraustochytrids and the fungi appearing in our culture is still uncertain, they seem to have several characters in common.

The ecology of the thraustochytrids may offer some suggestion about the route of contamination of the primary cultures. Thraustochytrids occur consistently in the marine environment and can be iso-

lated from sea water, sediments, soils and moribund material (Bremer 1976, Moss 1986). They are normally regarded as saprobes, but a species of *Thraustochytrium, T. inglei,* was reported to be parasitic on *Crassostrea virginica* (Mollusca) (Alderman 1976). The fungi appearing in our culture are probably lower fungi consistently occurring in the marine environment. It is also possible to suppose them to be endobiotic to the Kuruma prawn, but we have no concrete data.

When we initiated cultures of the midgut gland, the prawn was kept under running sterilized sea water until use and then was soaked in sodium hypochlorite solution prior to use (Machii *et al.* 1988). This procedure seems not to have been adequate for ensuring sterility. More careful sterilization of materials (possibly with antifungicides) will be necessary to avoid the contamination by the zoosporic fungi or thraustochytrids. Antibiotics or antifungal drugs added to the medium were ineffective to suppress the growth of the fungi (data not shown). Future studies on antifungicides effective against lower fungi or thraustochytrids will also be necessary for succeeding in primary cultures.

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クルマエビ中腸腺初代培養に混入した鞭毛菌類の特徴

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クルマエビ中腸腺初代培養に混入し，継代培養されてきた鞭毛菌類をクローニングした。得られた1クローンの遊走子は体側面にほぼ等長の2本の鞭毛を有し，その1本は羽型であった。この形質から，分離されたクローンは卵菌綱に属することが示された。遊走子は培養液中で遊泳した後，鞭毛を失い直径約3 μmの球状の栄養体となり，次第に径を増し直径15から20 μmの多核体となった。多核体の細胞質内には管状クリステを有するミトコンドリア，ゴルジ体，2種の顆粒や粗面小胞体が観察された。また多核体が運動性の小さい小栄養体に分裂することが顕微鏡映画および電子顕微鏡像で確認された。これらの性状から本菌は thraustochytrids に属する菌と考えられた。本菌の初代培養への混入経路について考察した。