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Three species of Lagenidiales isolated from the eggs and zoeae of the marine crab *Portunus pelagicus*

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Three species of Lagenidiales were isolated from the eggs and zoeae of the marine crab *Portunus pelagicus*. One of them, *Lagenidium callinectes*, is reported for the first time in Japan, with fungal infection in the eggs. *Haliphthoros milfordensis* was discovered from a zoea of the crab. *Atkinsiella okinawaensis* was also isolated from a zoea of the crab and described as a new species. Growth temperature range and optimum temperature of the fungi were examined. All of the isolates grew at various concentrations of NaCl or KCl, and optimum growth was observed on PYGS agar containing seawater. Pathogenicity to zoeae of the swimming crab *Portunus trituberculatus* was demonstrated by using a zoospore suspension of each fungus at 25°C.

Key Words——*Atkinsiella okinawaensis*; fungal infection; *Haliphthoros milfordensis*; *Lagenidium callinectes*; *Portunus pelagicus*.

Introduction

Difficulties in the seed production of marine crustaceans have sometimes been associated with fungal infections by species of Lagenidiales (Sparks, 1985). Among the fungi, *Lagenidium callinectes* Couch and *Haliphthoros milfordensis* Vishniac have been known as the most serious pathogens toward marine animals. In Japan, *L. callinectes* had not hitherto been reported in marine crustacean cultures. *H. milfordensis* was observed in the juvenile kuruma prawn *Penaeus japonicus* Bate (Hatai et al., 1992) and the abalone *Haliotis sieboldii* Reeve (Hatai, 1982).

In 1994, fungal diseases occurred in the eggs and zoeae of the crab *Portunus pelagicus* Linnaeus at Okinawa Prefectural Sea Farming Center, Okinawa Prefecture, Japan. The eggs with the fungal growth appeared as brown masses distinguishable from normal eggs. It was obvious that the causative agent was a species of *Lagenidium* because vesicles were observed on the eggs. The zoeae became whitish in color as they were filled with numerous non-septate hyphae. In the zoeae, zoospores were released from the orifices of the discharge tubes without vesicles. In this paper, the morphological and physiological characteristics of the three isolates were studied, and their pathogenicities for the zoeae of the swimming crab *Portunus trituberculatus* Miers were demonstrated by use of zoospore suspensions.

Materials and Methods

Isolation and identification Both eggs and zoeae of the crab *P. pelagicus* were collected from Okinawa Prefectural Sea Farming Center on 9 June 1994. The zoeae were reared in a tank at a water temperature of 25–28°C and fed with the rotifer *Brachionus plicatilis* Müller and brine shrimp. Each egg or zoea with the fungal infection was inoculated on PYGS agar (peptone 1.25 g, yeast extract 1.25 g, glucose 3 g, agar 12 g in seawater 1 L). For reduction of bacterial contamination, 500 μg/ml each of ampicillin and streptomycin sulphate were added to the medium. The plates were incubated at 25°C. Each fungal colony was transferred onto new PYGS agar to make a pure culture. Three different isolates belonging to Lagenidiales (NJM 9433, NJM 9434 and NJM 9435) were obtained by random selection from all of the fungal colonies. NJM 9433 was isolated from an egg, and NJM 9434 and NJM 9435 were isolated from zoeae.

For morphological observation, the fungi were inoculated into PYGS broth and incubated at 25°C for 2–3 days. The small colonies in PYGS broth were transferred into artificial seawater (Aqua-Ocean®, Japan Pet Drugs Co., Tokyo) and incubated at 25°C to induce zoospore production. Zoospore germination was observed under a microscope when they were incubated in PYGS broth at 25°C. The three species of Lagenidiales were identified according to Karling (1981), Bian and Egusa (1980), Kitancharoen et al. (1994), and Nakamura and Hatai (1994).

Effect of temperature on growth Growth temperature range and optimum temperature were examined using mycelia of the fungi. The three isolates of Lagenidiales were inoculated on PYGS agar. Isolates NJM 9433 and NJM 9434 were incubated at 25°C for 10 days, and NJM 9435 at 25°C for 25 days, to make a giant colony. Inocula were taken from the edge of each giant colony with a No. 2 cork borer (5.5 mm diam) and inoculated on

PYGS agar plates. Each medium was prepared with 25 ml of PYGS agar per plastic Petri dish (8.25 cm diam). Plates were incubated at eight different temperatures (10, 15, 20, 25, 30, 35, 40 and 45°C). The growth rate was checked by measuring the colony diameter every 2 days for 10 days after inoculation for NJM 9433 and NJM 9434, and every 5 days for 25 days for NJM 9435.

Mineral requirements for growth The fungi were inoculated on PYG agar containing various concentrations of NaCl or KCl to determine whether those minerals were required for growth. PYG agar was prepared like PYGS agar using distilled water instead of seawater. PYG agar was mixed with NaCl or KCl at concentrations of 1, 2.5 or 5%. PYGS and PYG agars were used as control media. Inoculation and measurement of the colony diameter were as mentioned above.

Experimental infection The zoeae of the swimming crab *P. trituberculatus* were used to determine pathogenicities of the present isolates. The test was carried out at two different temperatures (20 and 25°C). The zoeae were first dipped in sterilized seawater containing 500 μg/ml each of ampicillin and streptomycin sulphate for reduction of bacterial contamination. Then 45 ml of seawater with 30 zoeae and 5 ml of a zoospore suspension were poured into a 100-ml beaker. As a control, 50 ml of seawater with 30 zoeae in a 100-ml beaker was prepared. During the course of experimental infection, zoeae were not fed and weak aeration was provided in each beaker. Zoospore suspensions used were 10^3 spores/ml of NJM 9433, 10^4 spores/ml of NJM 9434 and $<10^3$ spores/ml of NJM 9435. At every 24-h intervals after exposure to zoospores, 5 zoeae from each beaker were taken at random to check for fungal infection under a microscope. Each infected zoea was reisolated on PYGS agar.

Results

Incidence The zoeae of *P. pelagicus* appeared to change from normal transparency to whitish color after infection. Dying zoeae showed white spots on the dorsal carapace spine. From direct observation of the zoeae, it was seen that non-septate stout hyphae filled the body of the zoeae (Fig. 1) and discharge tubes were observed on the surface of the body producing biflagellate zoospores. The mortality rate was approximately 100%, and the number of dead zoeae reached about 10^7. The dead eggs remaining after the other eggs had hatched appeared as brown masses. Numerous non-septate hyphae occupied inside the eggs and vesicles were produced at the orifices of the discharge tubes when zoospore formation occurred (Fig. 2). In both the eggs and zoeae, the fungal infections were most noticeable by a change in color.

Isolation and identification Three isolates belonging to Lagenidiales were obtained from *P. pelagicus*. One of them, NJM 9433, was isolated from an egg and identified as a member of the genus *Lagenidium* because vesicles were produced at zoospore production. Two isolates (NJM 9434 and NJM 9435) were obtained from the zoeal stages. Isolate NJM 9434 was classified as a species of the genus *Haliphthoros* because of fragmentation during sporulation. NJM 9435 was placed in the genus *Atkinsiella* because it formed zoosporangia of the same size and shape as the subthalli, and one to several discharge tubes from each sporangium. The present isolates were maintained at 20°C and subcultured on PYGS agar at approximately monthly intervals. The morphological characteristics of the present isolates are given as follows.

Lagenidium callinectes Couch, J. Elisha Mitchell Sci. Soc. 58: 158, 1942. Fig. 3

Colonies on PYGS agar were whitish and reached 14-16 mm diam after 5 days at 25°C. The centers were damp.

Hyphae were non-septate, irregularly branched, stout, with numerous shiny rod-shaped granules, 6-32 μm width. Zoospore formation was observed about 12 h after the mycelia were transferred into seawater. Masses of protoplasm flowed into the tip of discharge tubes, where vesicles appeared. Each protoplasmic mass was connected in a chain with a protoplasmic thread. The volume of the vesicles increased with the continuous entrance of protoplasmic masses, division into initial zoospores and active movement of zoospores. After all of the protoplasm had entered into the vesicles, flagellae appeared around the protoplasm in the first 5 min, and individual zoospores not divided completely were recognized in 10 min, swam freely inside the vesicles in 25 min and were released in 40 min. This morphological process was almost always observed after these time lapses. Zoospore production was successively observed up to 5 days. Mature vesicles were gelatinous, globose to subglobose, 15-97.5 μm diam. The discharge tubes were $5\text{-}15 \times 23\text{-}250$ μm, usually broad at the orifice. The way of zoospore liberation varied: sometimes they were released simultaneously by rupture of the vesicle, sometimes singly through a hole in the vesicle wall. When zoospores were discharged singly, vesicles usually persisted for a few minutes. Zoospores were laterally biflagellate, isokont, reniform to elongate, monoplanetic and $6\text{-}12.5 \times 10\text{-}14$ μm, 10×12 μm on average. Usually they swam for several to 20 h and did not swim over 24 h. When encysted, they were globose to subglobose without flagellae, 8-12 μm diam, 9.5 μm on average. Germination was observed about 4 h after spores had encysted. Sexual reproduction was not observed.

Specimen examined: NJM 9433 isolated from an egg of *P. pelagicus* with a fungal infection, obtained from Okinawa Pref., Japan, 9 June 1994.

Haliphthoros milfordensis Vishniac, Mycologia, 50: 75, 1958. Fig. 4

Colonies on PYGS agar were whitish and reached 13-15 mm diam after 5 days at 25°C. The centers were damp.

Hyphae in PYGS broth were stout, non-septate, branched with numerous shiny spherical granules, and some-

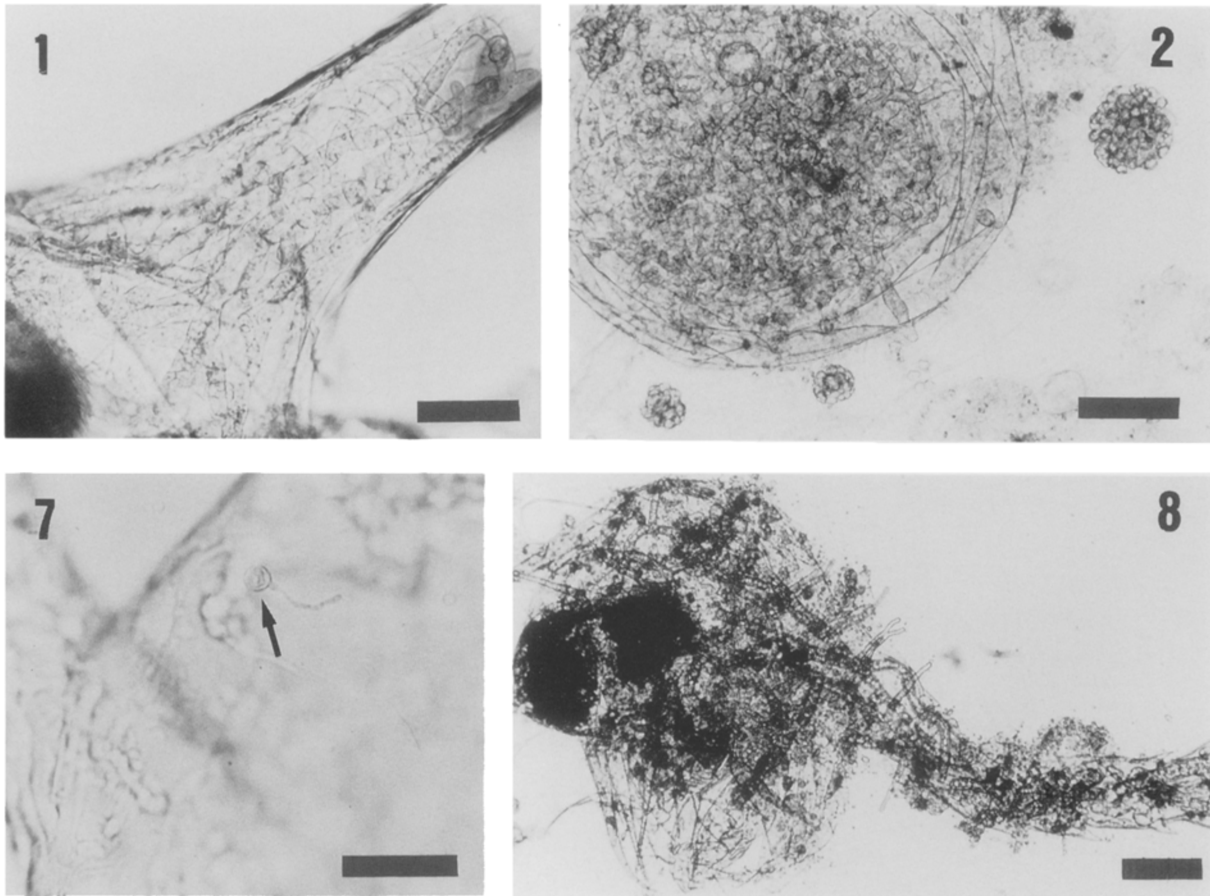

Fig. 1. A spontaneously infected zoea of *P. pelagicus*. Scale=50 μm.
Fig. 2. Vesicle formation on the egg of *P. pelagicus*, in spontaneous infection. Scale=100 μm.
Fig. 7. First invasion (arrow) of *L. callinectes* NJM 9433. Scale=50 μm.
Fig. 8. A zoea of the swimming crab *P. trituberculatus* experimentally infected with *H. milfordensis* NJM 9434. Scale=20 μm.

times protoplasm forming concentrated masses in the hyphae was observed. The width of the hyphae was 16-46 μm. In seawater, fragments were clearly constructed of concentrated masses of protoplasm in the hyphae. Fragments were tuberculate, saccate or irregular, and quite variable in size and shape. They changed into zoosporangia producing discharge tubes. Many vacuoles appeared in the zoosporangia and the extending discharge tubes, and were also observed in the active mycelia. Zoospore formation was observed about 10 h after the mycelia were transferred into seawater and continued for 5 days. One discharge tube was usually formed on the lateral side of each zoosporangium, and two tubes were rarely observed. The tubes were 5-9 μm diam and 14-820 μm length, and usually straight or slightly curved. Division of the protoplasm started in the sporangia and continued in the discharge tubes just before zoospore liberation. Zoospores were elongate, reniform and slipper-shaped, laterally biflagellate, isokont, monoplanetic, 4-7×5-9 μm, 4.8×7.1 μm on average. Encysted zoospores were globose or subglobose, 4-6 μm diam, 5.5 μm on average. Spores germinated with a hair-like filament measuring 30-380 μm length about 4 h after encystment. Sexual reproduction was not observed.

Specimen examined: NJM 9434 isolated from a zoea of *P. pelagicus* with a fungal infection, obtained from Okinawa Pref., Japan, 9 June 1994.

Atkinsiella okinawaensis sp. nov. Nakamura et Hatai Fig. 5

Thallus endobioticus holocarpus, crassus, ramosus, in maturitate septatus et divisus in subthallos. Subthalli cylindrici, lobati vel irregulares. Zoosporangium subthallum conforme, e latere vel apice tubulos emittentes singulos vel nonnullos formans. Tubulus raro ramosus, 6-10×40-510 μm, zoosporas bi- vel pluriseriatim faciens. Zoosporae pyriformes vel subglobosae, lateraliter biflagellatae, diplaneticae, 4.7(4-6.5)×6.3(5-8) μm. Cystosporae globosae vel subglobosae, 5.2(4-7) μm in diametro, in fibrarum 5-190 μm longarum germinantes. Reproductio sexualis ignota.

Holotypus: NJM 9435, colonia exsiccata e cultura ex zoeis *Portuni pelagici* Linnaeus, Okinawa Pref. in Japonia, 9 Iunius 1994, a K. Nakamura isolata et ea collectione culturae in Universitate Veterinarii et Scientificae Animalis

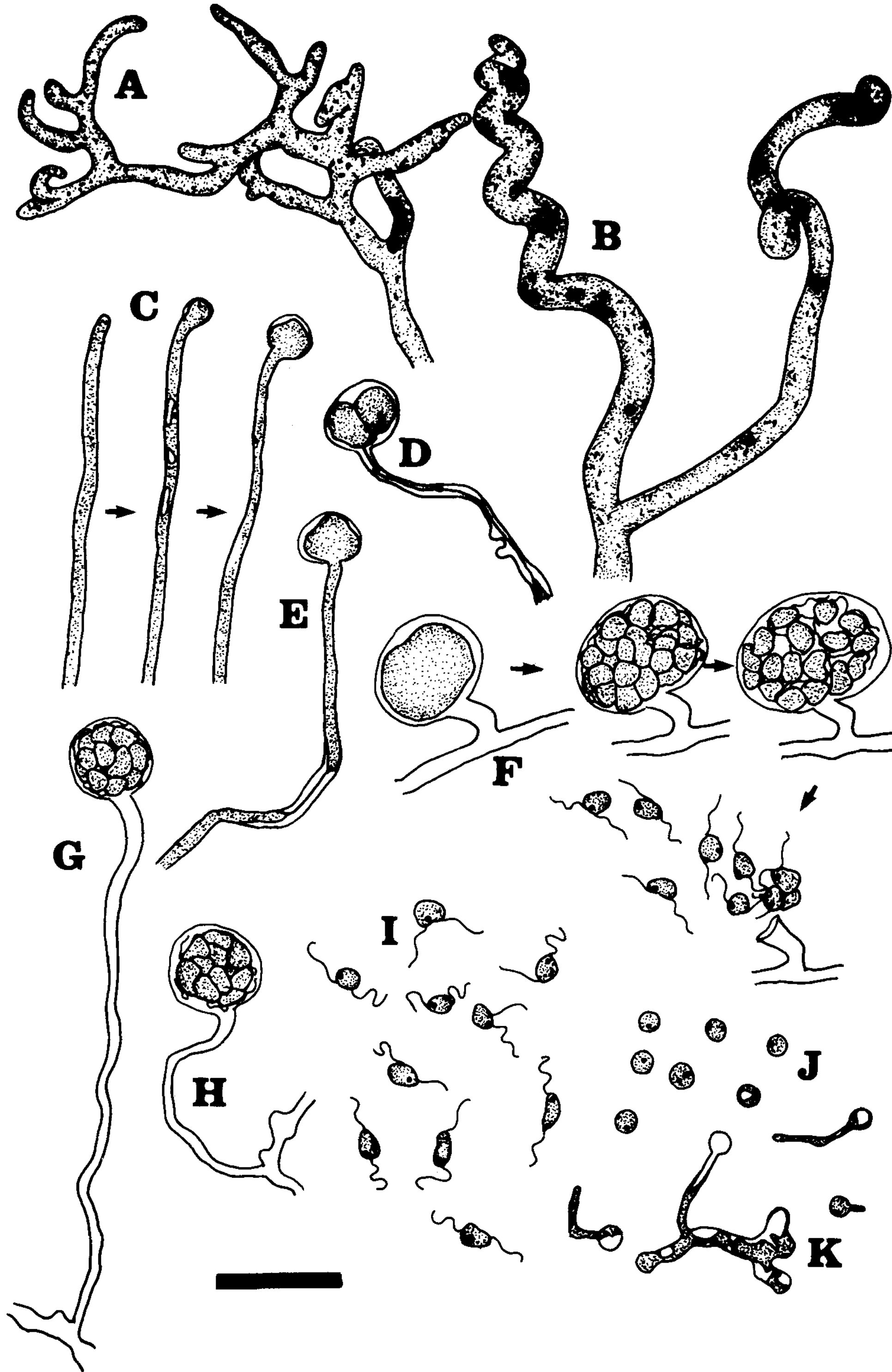

Fig. 3. Morphological characteristics of *L. callinectes* NJM 9433 isolated from an egg of *P. pelagicus*. Scale = 50 μm. A. Irregularly branched hyphae with numerous shiny rod granules; B. Coiled hyphae in PYGS broth; C. Vesicle formation; D, E. Protoplasmic masses flow into the vesicle with a protoplasmic thread; F. Division into initial zoospores and zoospore liberation; G, H. Mature vesicles; I. Zoospores; J. Encysted zoospores; K. Germination.

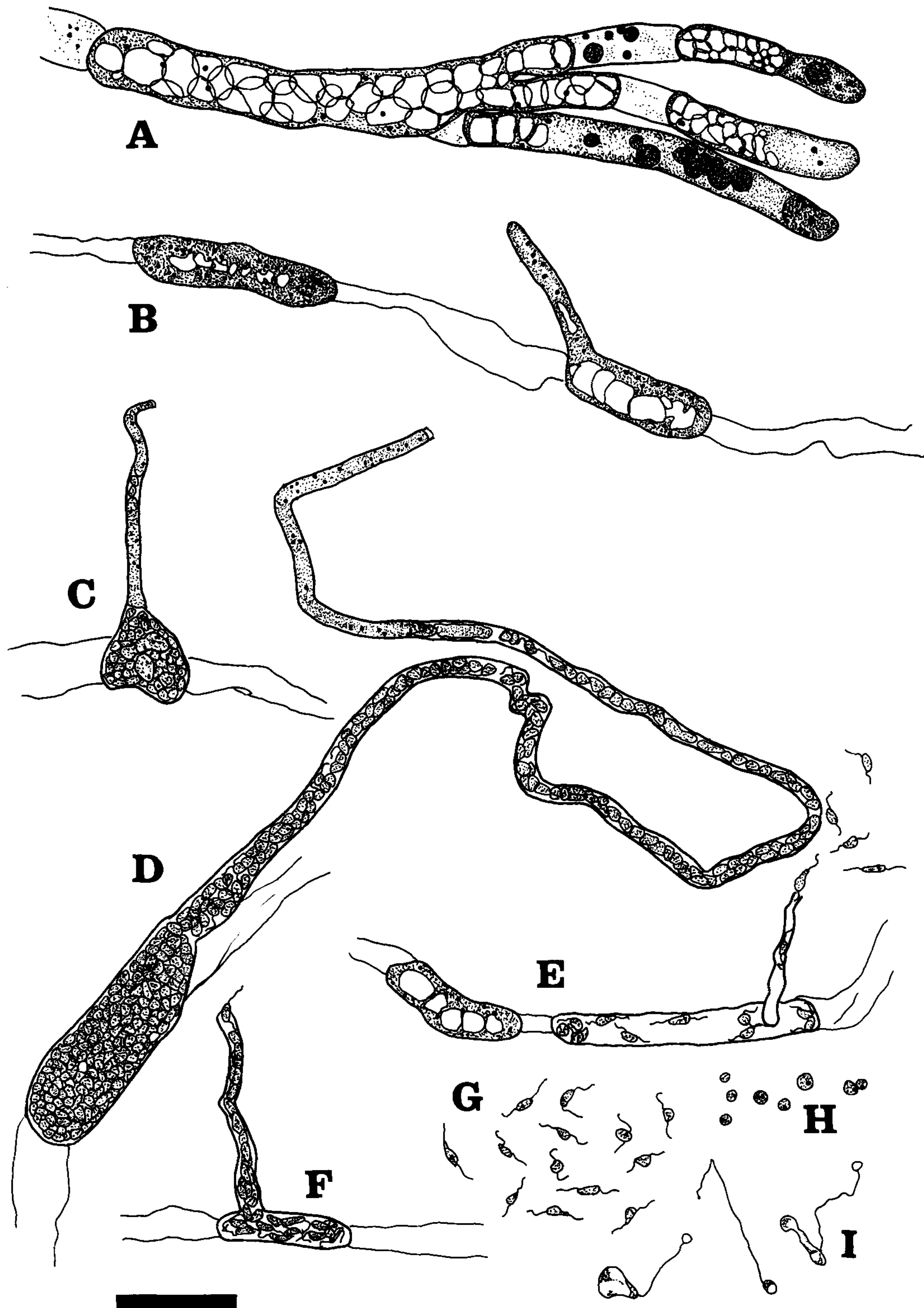

Fig. 4. Morphological characteristics of *H. milfordensis* NJM 9434 isolated from a zoea of *P. pelagicus*. Scale = 50 μm.
A. Hyphae in PYGS broth; B. Fragments. Discharge tube formation on the right fragment; C, D. Zoospore formation; E, F. Zoospore liberation; G. Zoospores; H. Encysted zoospores; I. Germination.

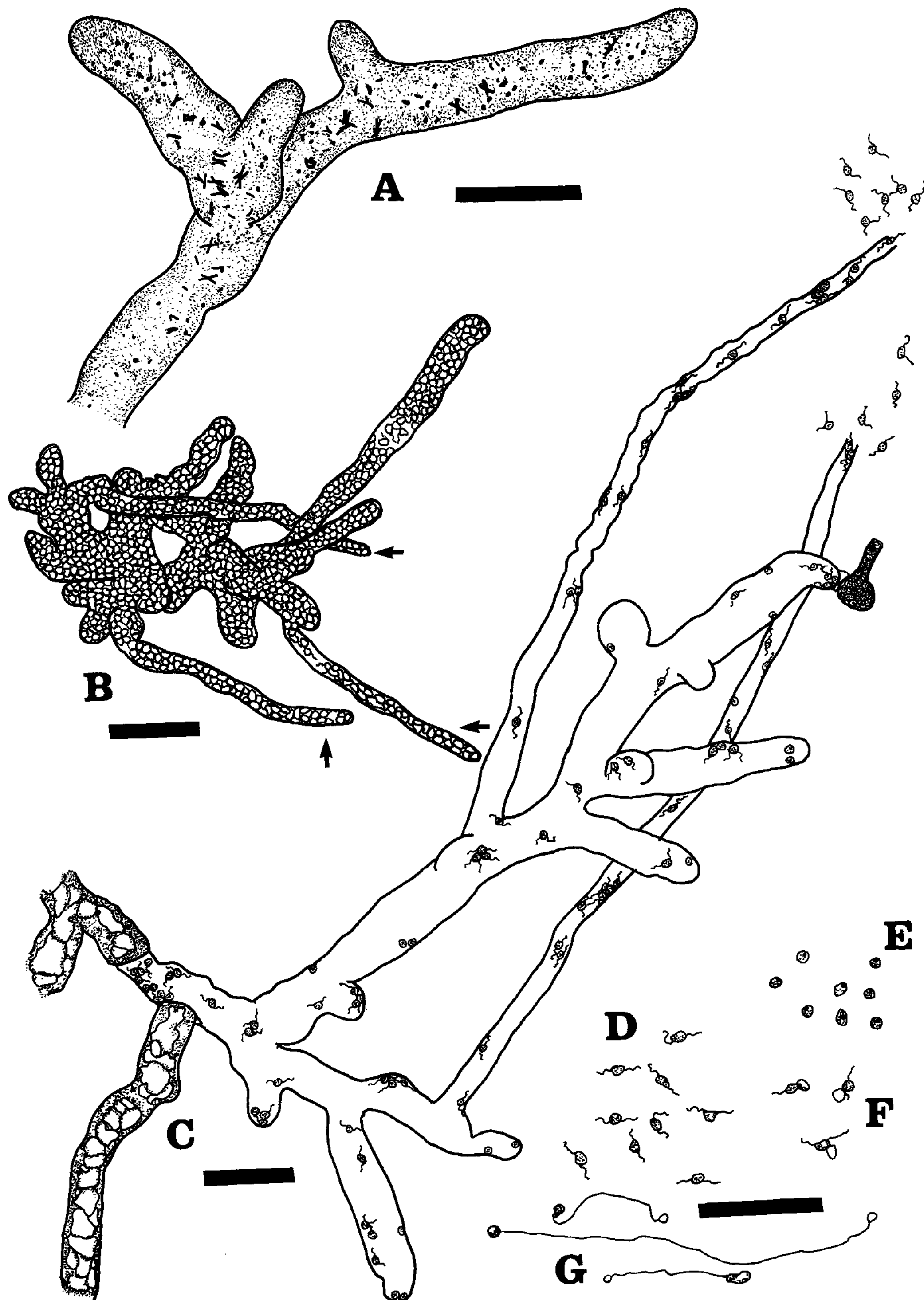

Fig. 5. Morphological characteristics of *A. okinawaensis* sp. nov. NJM 9435 isolated from a zoea of *P. pelagicus*. Scales = 50 μm. A. Hyphae in PYGS broth; B. A zoosporangium with three discharge tubes (arrows); C. Zoospores released from the orifices of two discharge tubes. Another zoosporangium with one discharge tube is on the right; D. Zoospores; E. Encysted zoospores; F. Secondary zoospores released from cysts; G. Germination.

Nipponensis (NJM) conservata.

Colonies on PYGS agar were cream to yellow with folds reaching only 2-3 mm diam at 5 days after incubation at 25°C.

Hyphae were stout, non-septate at first, irregularly branched with numerous shiny rod granules, 10-38 μm width. In seawater, hyphae were divided into subthalli with septa. Gemmae were present with thick walls, 22-190 μm width. Zoosporangia were the same size and shape as subthalli and gemmae. Discharge tubes were produced laterally or terminally from the sporangia, usually coiled or wavy. Each sporangium extended one to several discharge tubes. Zoospore formation was observed both in the sporangia and discharge tubes. In the discharge tubes, zoospores were lined more than two deep. The discharge tubes were 6-10 μm diam and 40-510 μm length, usually 8-9 μm diam and 200-300 μm length. Branches of the discharge tubes were rarely observed near the zoosporangia. Zoospore production occurred from about 18 h to 7 days after inoculation of the mycelia into seawater. Zoospores were laterally biflagellate, diplanetic, 4-6.5 × 5-8 μm, 4.7 × 6.3 μm on average. They were usually pyriform or subglobose. Encysted zoospores were globose or subglobose, 4-7 μm diam, 5.2 μm on average. Germination was observed about 3 h after spores had encysted, with a hair-like filament measuring 5-190 μm length. Sexual reproduction was not observed. Isolate NJM 9435 was obtained from a zoea of *P. pelagicus* with a fungal infection.

Effect of temperature on growth The results are displayed in Table 1. Isolate NJM 9433 could grow over a wide temperature range of 15-40°C with an optimum range of 30-35°C. NJM 9434 grew at 15-30°C and the optimum temperature was 30°C. NJM 9435 grew only at 20-35°C and the optimum temperature was 25°C. These results suggested that the organisms were adapted to tropical environments.

Mineral requirements for growth The results, shown in Table 2, revealed optimum growth on PYGS agar for all isolates. NJM 9433 could grow at every concentration except with 5% of KCl. NJM 9434 and NJM 9435 grew on PYG agar with 2.5% of NaCl or KCl, and did not grow on PYG agar without seawater. On all plates except for PYGS agar, NJM 9435 showed brown pigmentation around the inocula.

Experimental infection Experimental infection at 20°C succeeded only with NJM 9435 (Fig. 6). With NJM 9435, the accumulative mortality rate was only 40% with inoculation of $<10^3$ spores/ml. The other fungi infected the zoeae at levels of 2-5% at 20°C. The accumulative mortality rates of NJM 9434 and NJM 9435 were approximately 70% at 25°C. Even at 25°C, NJM 9433 infected very few zoeae during the experiments, and its pathogenicity to zoeae of the swimming crab seems to be low. Remarkable numbers of encysted zoospores germinating on the surface of the zoeae were observed one day after exposure to *Lagenidium* isolate NJM 9433 (Fig. 7). The affected zoeae were found at the bottom of the beaker, showing white coloration. They were often occupied by fungal mycelia (Fig. 8). New zoospores were released from two days after inoculation. The same species as the inocula were obtained from the zoeae with fungal infections. In control beakers, no fungal infection was observed during the experiments both at 20 and 25°C.

Table 1. Effect of temperature on growth of isolates NJM 9433, NJM 9434 and NJM 9435.

Temperature (°C)	Colony radius (mm) incubated for:		
	10 days		25 days
	NJM 9433	NJM 9434	NJM 9435
10	–*	–	–
15	1.7	8.2	–
20	12.2	12.9	5.6
25	31.8	30.0	14.3
30	>40.0	34.8	12.3
35	>40.0	–	0.6
40	30.3	–	–
45	–	–	–

* – No growth.

Discussion

Lagenidium callinectes Couch has been reported as a parasite of various marine crustaceans. The fungus was isolated from the eggs and larvae of the blue crab *Callinectes sapidus* Rathbun (Couch, 1942; Rogers-Talbert, 1948; Bland and Amerson, 1973), the eggs of the barnacle *Chelonibia patula* Ranzani (Johnson and Bonner, 1960), the larvae of the white shrimp *Penaeus setiferus* Linnaeus (Lightner and Fontaine, 1973), and the larvae of the Dungeness crab *Cancer magister* Dana (Armstrong et al., 1976). *L. callinectes* was also discovered on the surface of marine algae including *Chordaria* sp., *Cladophora* sp., *Ceramium* sp. and *Ectocarpus* sp. (Fuller et al., 1964). However, *L. callinectes* had not hitherto been reported in Japan.

L. callinectes was first reported in Japan when it was discovered on the eggs of *P. pelagicus*. However, the fungus was not thought to have been imported from abroad because *Lagenidium* infections at marine crustacean culture farms have been recognized in Japan, although the causative agents were not determined. It was considered that *L. callinectes* was involved in some of the infections. When the morphological characteristics of *L. callinectes* and the present isolate were compared, no significant differences were found.

In the present isolate, zoospores were released by rupturing of the vesicles or through a small hole in each vesicle. The former seems to be the main way of zoospore liberation for this fungus. The latter occurred when zoospores moved relatively slowly in the vesicles, and was also observed in *L. scyllae* Bian et al. (Bian et al., 1980). The growth temperature range and optimum temperature of the isolate NJM 9433 were similar to those of *L. scyllae*.

The second isolate, NJM 9434, was identified as

Table 2. Effect of NaCl or KCl on growth of isolates NJM 9433, NJM 9434 and NJM 9435.

Medium	Colony radius (mm) incubated for:		
	10 days		25 days
	NJM 9433	NJM 9434	NJM 9435
PYGS agar	31.3	33.9	13.5
PYG agar+1.0% NaCl	23.3	—*	4.2
PYG agar+2.5% NaCl	19.5	18.5	9.8
PYG agar+5.0% NaCl	8.8	11.6	—
PYG agar+1.0% KCl	5.3	—	—
PYG agar+2.5% KCl	5.9	14.3	9.2
PYG agar+5.0% KCl	—	8.7	—
PYG agar	4.8	—	—

* — No growth.

Haliphthoros milfordensis. *H. milfordensis* was first described on the eggs of the oyster drill *Urosalpinx cinerea* Say by Vishniac (1958). Later, it was isolated from various crustaceans (Fisher et al., 1975; Tharp and Bland, 1977; Hatai et al., 1992). It was also reported on the surface of marine algae (Fuller et al., 1964) and in the abalone *Haliotis sieboldii* Reeve (Hatai, 1982). Fragmentation was the most specific morphological feature of the genus *Haliphthoros* when sporulation occurred. Only two species were included in the genus *Haliphthoros*. Another strain, *H. philippinensis* Hatai et al., is known as a parasite of the larvae of the jumbo tiger prawn *Penaeus monodon* Fabricius with distinctive zoospore liberation (Hatai et al., 1980). *H. philippinensis* released zoospores from the orifice of the discharge tubes and also from the openings in the zoosporangia. Zoospores of the present isolate were always released from the discharge tubes, and thus it differed from *H. philippinensis*. However, its temperature range and optimum temperature for the growth were similar to those of *H. philippinensis*, while different from the those of *H. milfordensis* (Vishniac, 1958; Hatai, 1982). Gemmae were observed by Sparrow (1974), but were not present in the isolate.

NaCl was required for the growth of both species of the genus *Haliphthoros* (Vishniac, 1958; Hatai et al., 1980; Hatai, 1982). This agrees with the results of the present isolate. The two species of *Haliphthoros* could not be concluded to be obligately marine fungi.

The third isolate was identified as a member of the genus *Atkinsiella*. Species of the genus *Atkinsiella* have been reported from various aquatic animals. *A. dubia* (Atkins) Vishniac was observed on the eggs of crabs (Atkins, 1954; Sparrow and Gotelli 1969; Sparrow, 1973). *A. hamanaensis* Bian & Egusa was isolated from the eggs and larvae of the mangrove crab *Scylla serrata* Forsskål. *A. awabi* Kitancharoen et al. and *A. parasitica* Nakamura & Hatai were described on the abalone *Haliotis sieboldii* (Kitancharoen et al., 1994) and the rotifer *Brachionus plicatilis* (Nakamura and Hatai, 1994), respectively. Only *A. entomophaga* Martin was discovered in fresh water (Martin, 1977).

The present isolate NJM 9435 differed from these species in the following characteristics. Most distinctive was the discharge tubes, in which zoospores were produced more than two deep. This seems to have been observed in *A. dubia* judging by illustrations (Atkins, 1954; Sparrow, 1973), although a detailed description was not included in their reports. In other species of *Atkinsiella*, zoospores in the discharge tubes were formed in a row. Branched discharge tubes were observed in the present isolate, and also reported in two fungi, *A. entomophaga* and *A. parasitica*.

Dimensions of the isolate NJM 9435 were close to those of *A. parasitica* in regard to hyphae, gemmae, dis-

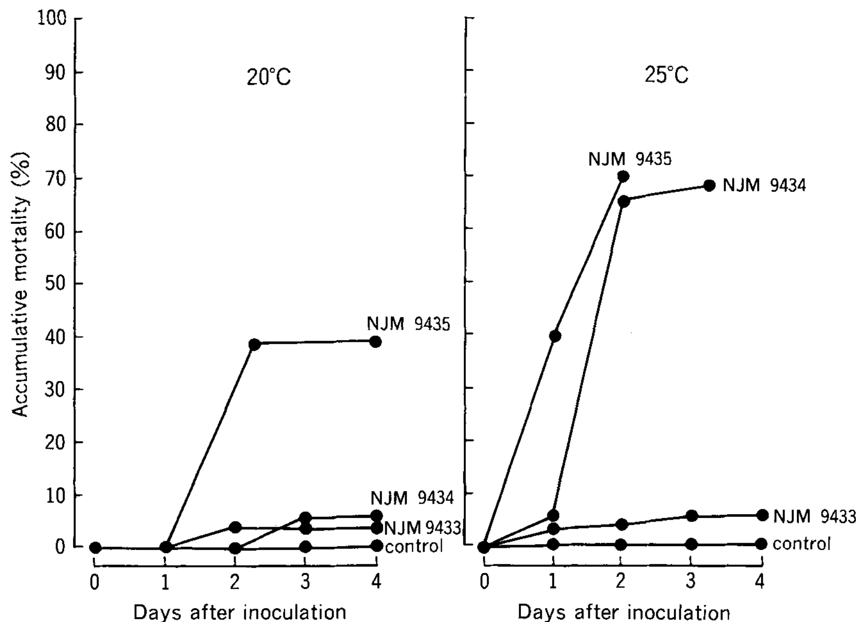

Fig. 6. Mortality rate of the zoea of the swimming crab *P. trituberculatus* experimentally infected with NJM 9433, NJM 9434 and NJM 9435 obtained from *P. pelagicus*.

charge tubes, zoospores and encysted zoospores. However, *A. parasitica* is monoplanetic. Other strains including the present fungus were observed to be diplanetic. There was no apparent difference between primary and secondary zoospores in the present isolate. As mentioned above, the present isolate is regarded as a new species of the genus *Atkinsiella, A. okinawaensis* sp. nov.

The optimum temperature and temperature range of the isolate NJM 9435 were 25°C and 20-35°C, respectively. The results were relatively similar to those of *A. parasitica* and *A. hamanaensis*, and different from *A. awabi*. The isolate NJM 9435 required NaCl at a concentration of 1-2.5%. The present isolate also differed from *A. parasitica* and *A. awabi* in this point. However, brown pigmentation was observed on the media containing any concentration of NaCl, while it did not occur on the medium containing normal seawater. This suggested that the presence of seawater stimulated active fungal growth.

From the results of experimental infection, *L. callinectes* isolated from an egg of *P. pelagicus* had the lowest pathogenicity toward the zoeae of the swimming crab. On the surface of the zoeae infected by *L. callinectes*, zoospore germination was discovered. This was easily recognized by the large zoospores and germ tubes, which were not present in the zoeae infected with the other two isolates. All isolates showed pathogenicity to the zoeae of the swimming crab although the mortality rates were different. Affected zoeae appeared whitish and sank to the bottom of the beaker. This clinical feature is similar to spontaneous infection. Isolate NJM 9435 was considered the most serious pathogen, giving a mortality rate of 70% with only $<10^3$ spores/ml. All of the dead animals were removed from the beaker during the experiments. In the marine crustacean cultures, it was difficult to separate the infected animals from normal ones in the same tank, the infection easily spread throughout the tank.

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