Mycoscience 40: 235–240, 1999

Atkinsiella dubia infection in the larvae of Japanese mitten crab, *Eriocheir japonicus*

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Accepted for publication 22 February 1999

Heavy mortalities reaching 100% among larvae of the Japanese mitten crab, *Eriocheir japonicus*, occurred in Yamaguchi Prefectural Naikai Fisheries Experimental Station, Yamaguchi, Japan. Under the microscope, infected zoeal larvae were seen to be filled with numerous aseptate hyphae. An investigation was carried out to identify the pathogenic fungus and determine its pathogenicity under laboratory conditions. The pathogenic fungus was identified as *Atkinsiella dubia*. Its optimum growth temperature was 25°C, and it grew only at 2.5% NaCl. Under laboratory conditions, it showed pathogenicity to the larvae of the swimming crab, *Portunus trituberculatus*. This is the first report of mass mortality in crustaceans due to *A. dubia* infection in Japan.

Key Words——*Atkinsiella dubia*; *Eriocheir japonicus*; fungal infection; pathogenicity.

The main difficulty in the seed production of marine crustaceans is fungal infection by species belonging to Lagenidiales (Sparks, 1985). Among Lagenidiales, *Lagenidium*, *Halocrusticida* and *Haliphthoros* are considered as serious pathogens of the larvae of the mud crab, *Scylla serrata* Forsskal (Hamasaki and Hatai, 1993), and the swimming crab, *Portunus pelagicus* Linnaeus (Nakamura and Hatai, 1995a). *Atkinsiella dubia* (Atkins) Vishniac was originally described from the eggs of the pea crab, *Pinnotherus pissum* Pennant, and *Gonoplax angulata* Pennant in England by Atkins (1954), and subsequently isolated from marine algae, *Chordaria* sp. and *Cladospora* sp. (Fuller et al., 1964). Sparrow (1973) reported the species as a parasite of eggs of various crabs, such as *Hyas* sp. and *Oregonia* sp. Other reported members of the genus *Atkinsiella* include *A. hamanaensis* Bian & Egusa isolated from *S. serrata* (Bian and Egusa, 1980), *A. parasitica* Nakamura & Hatai from the rotifer, *Brachionus plicatilis* Muller (Nakamura and Hatai, 1994), and *A. awabi* Kitancharoen, Nakamura, Wada & Hatai from the abalone, *Haliotis sieboldii* Reeve (Kitancharoen et al., 1994). Subsequently, *A. dubia* was isolated from the mantle of the abalone, *H. sieboldii*, and from the gills of the adult swimming crab, *Portunus trituberculatus* Miers (Nakamura and Hatai, 1995b). Species of the genus *Atkinsiella* other than *A. dubia* were transferred into the genus *Halocrusticida* by Nakamura and Hatai (1995b).

Seed production of Japanese mitten crab, *Eriocheir japonicus* de Haan, is now under trial at the Yamaguchi Prefectural Naikai Fisheries Experimental Station in Yamaguchi Prefecture, Japan. Recently, however, mortalities have occurred caused by fungal infection in the zoeal stages. This paper reports the identification of the fungus based on its morphological and biological characteristics, and some data on its pathogenicity to the swimming crab, *Portunus trituberculatus* Miers.

Materials and Methods

Isolation and identification Zoea of the Japanese mitten crab with fungal infection were sent from Yamaguchi Prefectural Naikai Fisheries Experimental Station to our laboratory on 20 May 1998. Infected larvae were taken onto a slide glass and examined under a microscope. Zoea, randomly selected from diseased and dead samples, with fungal infection as indicated by the presence of hyphae, were inoculated onto plates of peptone-yeast extract-glucose-seawater (PYGS) agar (composed of 1.25 g of Bacto peptone, 1.25 g of Bacto yeast extract, 3 g of glucose, 37.6 g of artificial seawater (Aqua-Ocean®, Japan Pets Drugs Co.), and 12 g of Bacto agar in 1000 ml distilled water) containing 500 μg/ml each of streptomycin sulphate and ampicillin to retard bacterial contamination. Cultures were incubated at 25°C for 7–10 d. A small agar block with fungal mycelium was cut out and inoculated onto a fresh PYGS agar plate to make a pure culture. As described below, two isolates, NJM 9802 and NJM 9808, randomly chosen were used for all experiments. For morphological observation and identification, the fungus was inoculated into PYGS broth and incubated at 25°C for 3 d. The small colonies in PYGS broth were transferred into sterilized artificial seawater and incubated at 25°C to induce zoospore production. Germination of zoospores was observed under the microscope when they were incubated in PYGS broth at

25°C. The isolates were identified according to Atkins (1954), Fuller et al. (1964), Sparrow (1973), Martin (1977) and Nakamura and Hatai (1995b).

Effect of temperature on vegetative growth Range and optimum temperature for growth were examined using mycelia. Each isolate was inoculated on PYGS agar and incubated at 25°C for 10 d to produce 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 in a plastic Petri dish (8.25 cm diam). Plates were incubated at six different temperatures (5, 10, 15, 20, 25 and 30°C). The growth rate was observed by measuring the colony diameter at days 1, 3, 5, 7, 10 and 15 and compared with those of two isolates of *Atkinsiella dubia*: ATCC 200323, isolated from the mantle of the abalone, *Haliotis sieboldii*; and NJM 9531, isolated from gills of the swimming crab, *Portunus trituberculatus* (Nakamura and Hatai, 1995b).

Mineral requirements for vegetative growth The two isolates were inoculated on PYG agar containing various concentrations of NaCl or KCl to determine whether these minerals were required for growth. PYG agar was prepared using distilled water instead of artificial seawater and supplemented with NaCl or KCl at concentrations of 1, 2.5 and 5%. PYGS and PYG agars were used as control media. Inoculation and measurement of the colony diameter followed the method for estimation of the effect of temperature on growth, and *A. dubia* ATCC 200323 and *A. dubia* NJM 9531 were again used for comparison.

Artificial infection Larvae of the swimming crab, *P. trituberculatus*, in the zoea 1 stage were obtained from the hatchery of Yamaguchi Prefectural Naikai Fisheries Experimental Station, Yamaguchi Prefecture. *P. trituberculatus* larvae were chosen because they have previously been used for infection with *A. dubia*, are readily available, and easy to rear in laboratory conditions. Zoea were reared in tank with weak aeration and fed with rotifer, *Brachionus plicatilis*. To determine the pathogenicity of the two isolates from Japanese mitten crab, NJM 9802 and NJM 9808, the larvae were challenged with different numbers of zoospores. Ten larvae were put into a 100-ml beaker with 50 ml of artificial seawater. A zoospore suspension of 2.7×10^5 zoospores/ml was prepared, 10 ml or 1 ml of the suspension was inoculated into the beaker to give a final concentration of 5.4×10^4 zoospores/ml or 5.4×10^3 zoospores/ml, respectively. As a control, 50 ml of artificial seawater with 10 larvae in a 100-ml beaker was also prepared. Larvae were observed 24 h and 48 h after the inoculation. The dead larvae were counted and examined for infection with the fungus under the microscope. Infected larvae were inoculated on PYGS agar for reisolation.

Results

Incidence Seven spawners of the Japanese mitten crab, *Eriocheir japonicus*, ranging from 100 g to 134 g in body weight were introduced into Yamaguchi Prefectural Naikai Fisheries Experimental Station, Yamaguchi Prefecture, Japan, on 16 April 1998 and kept in a 0.5-ton FRP tank at 15–17.2°C (mean 16°C). After hatching out, larvae first exhibited fungal infection in the zoeal stages on 17 May 1998, and all of them died within several days. Body color in the zoea appeared to change from transparenct to whitish after the infection. Dying zoea sometimes showed white spots on the spine of the dorsal carapace. The zoea were filled with aseptate stout hyphae (Fig. 1).

Isolation and identification Fungi belonging to Lagenidiales were isolated from dying larvae of Japanese mitten crab. They were identified as members of the genus *Atkinsiella*, because they formed zoospores encysted in the zoosporangia following the first motile stage (Nakamura and Hatai, 1995b). Two isolates, NJM 9802 and NJM 9808, were randomly selected for further experiments. They were maintained at 25°C and subcultured on PYGS agar at approximately monthly intervals. Their morphological characteristics were as follows.

Atkinsiella dubia (Atkins) Vishniac, J. Mar. Biol. Ass. U.K. 33: 731, 1954. Figs. 2–5

Colonies on PYGS agar attaining a diameter of about 25 mm in 15 d at 25°C, crystalline, tuberculate, and moist; moderately heaped at the center. Mycelia in the broth aseptate, radially branched, stout, swollen up to 150 μm in diam, with clusters of shiny spherical granules, without oil droplets and vacuoles. Granular clusters evenly distributed inside mycelia, generally consisting of several decades of granules. Mycelia in seawater developing narrow branches (discharge tubes), followed by zoospore production. Gemmae present. Zoospores in the first motile stage produced after 30 h at 25°C. Protoplasmic masses due to gathering of granular clusters on zoosporogenesis, supported at the center of zoosporangia by several protoplasmic threads; differentiated into loose networks of zoospores, then into free individual zoospores in the first motile stage. Zoosporangia the same in size and shape as the mycelia, with several discharge tubes extending from each zoosporangium. Zoospores in the first motile stage swimming dully and encysting within zoosporangia and discharge tubes, biflagellate, subglobose to globose, 3–6 μm in size. Zoospores in the second motile stage releasing one by one from encysted zoospores within zoosporangia and discharge tubes, swimming freely for a long time; laterally biflagellate, pyriform, slipper-shaped, isokont, 2–7 μm. Zoospores dimorphic and diplanetic. Encysted spores globose to subglobose, 3–7 μm in the first motile stage and 3.5–6 μm in the second motile stage. Discharge tubes unbranched or occasionally branched, straight or tapering with flared openings, rarely with a central swelling, 4–9 μm in width, 5–16 μm in length. Germination produced after 6–8 h after spores transferred to broth with a slender germ tube.

Specimen examined: NJM 9802 and NJM 9808 isolated from zoea 5 of the Japanese mitten crab, *Eriocheir japonicus*, with fungal infection, obtained from Yamaguchi Prefectural Naikai Fisheries Experimental Station,

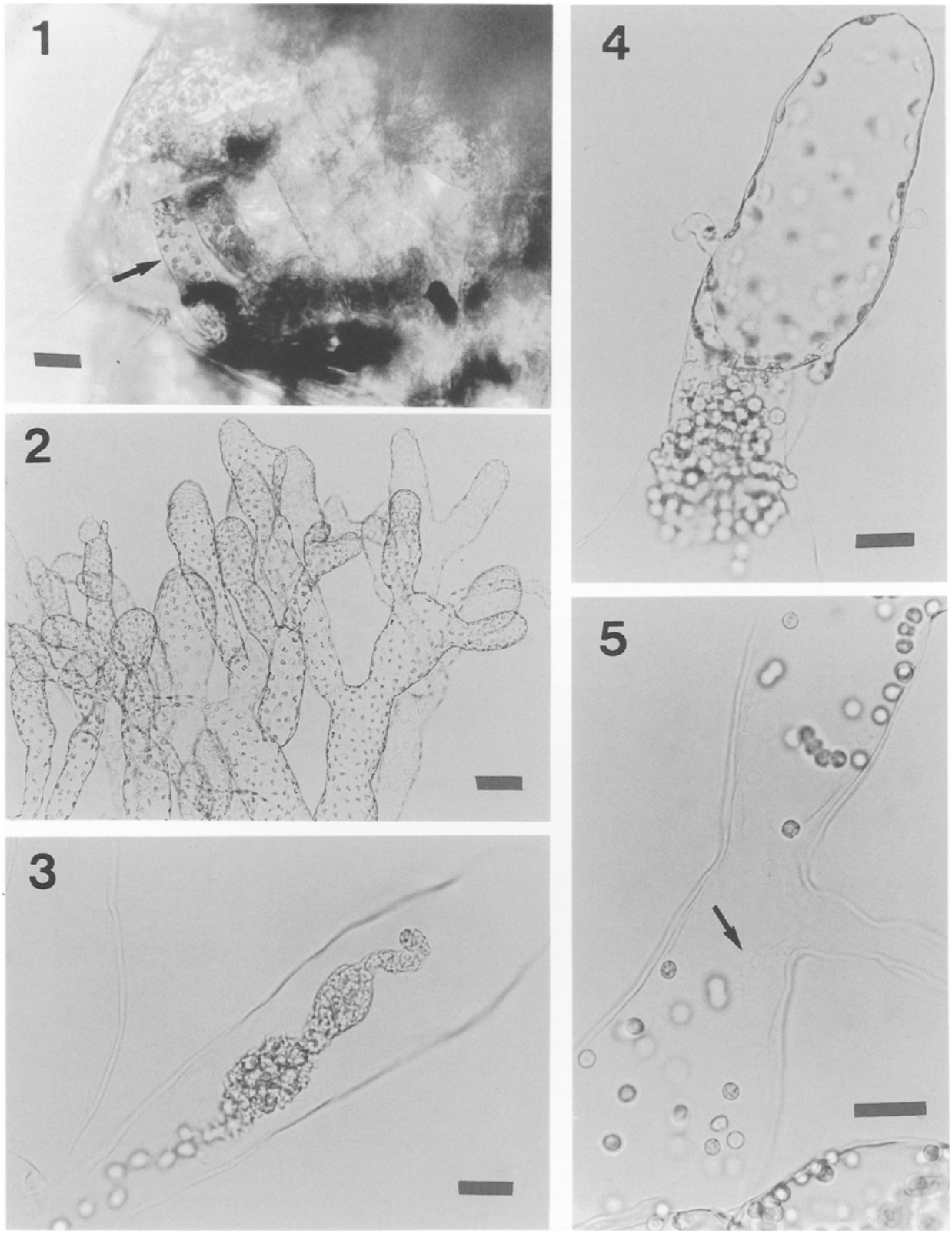


Fig. 1. Hyphae (arrow) in larvae of the Japanese mitten crab naturally infected with *Atkinsiella dubia*. Scale: 100 μm.
Fig. 2. Mycelia with granular clusters. Scale: 100 μm.
Fig. 3. A protoplasmic mass formed in zoosporangium. Scale: 100 μm.
Fig. 4. Vegetative hyphae and a primary zoospores which encysted in zoosporangium. Scale: 100 μm.
Fig. 5. Empty encysted zoospores (arrow) in zoosporangium. Scale: 100 μm.

Yamaguchi Prefecture, Japan, 20 May 1998.

Effect of temperature on vegetative growth As shown in Table 1, the isolates grew at 10–25°C with optimum growth at 25°C but did not grow at 5 and 30°C. The results were almost the same as those of *Atkinsiella dubia* ATCC 200323 isolated from the abalone, *Haliotis sieboldii*, and *A. dubia* NJM 9531 isolated from the swimming crab, *Portunus trituberculatus* by Nakamura and Hatai (1995b).

Effect of mineral requirements for vegetative growth As shown in Table 2, each isolate used in this study grew only on PYG agar with 2.5% NaCl and PYGS agar.

Artificial infection As shown in Table 3, the accumulative mortalities of larvae challenged by 5.4×10^4 zoo-

Table 1. Effect of temperature on vegetative growth of NJM 9802 and NJM 9808 isolated from larvae of the Japanese mitten crab, *Eriocheir japonicus*.

Temperature (°C)	Colony radius (mm) at day 15			
	NJM 9802	NJM 9808	ATCC 200323[a]	NJM 9531[b]
5	—[c]	—	—	—
10	9.0	9.5	8.9	9.3
15	12.7	13.5	10.0	13.0
20	19.0	19.0	12.0	19.8
25	23.7	24.5	13.5	20.5
30	—	—	—	—

a) *Atkinsiella dubia* isolated from the abalone, *Haliotis sieboldii* (Nakamura and Hatai, 1995b).
b) *A. dubia* isolated from the swimming crab, *Portunus trituberculatus* (Nakamura and Hatai, 1995b).
c) —: No growth.

Table 2. Effect of NaCl or KCl on vegetative growth of NJM 9802 and NJM 9808 isolated from larvae of the Japanese mitten crab, *Eriocheir japonicus*.

Medium	Colony radius (mm) at day 15			
	NJM 9802	NJM 9808	ATCC 200323[a]	NJM 9531[b]
PYG agar + 1% NaCl	—[c]	—	—	—
PYG agar + 2.5% NaCl	17.5	15.7	11.0	12.8
PYG agar + 5% NaCl	—	—	—	—
PYG agar + 1% KCl	—	—	—	—
PYG agar + 2.5% KCl	—	—	—	—
PYG agar + 5% KCl	—	—	—	—
PYGS agar	23.7	24.5	13.5	20.5
PYG agar	—	—	—	—

a) *Atkinsiella dubia* isolated from the abalone, *Haliotis sieboldii* (Nakamura and Hatai, 1995b).
b) *A. dubia* isolated from the swimming crab, *Portunus trituberculatus* (Nakamura and Hatai, 1995b).
c) —: No growth.

Table 3. Pathogenicity to the larvae of the swimming crab, *Portunus trituberculatus*, of two isolates of *Atkinsiella dubia* from larvae of the Japanese mitten crab, *Eriocheir japonicus*.

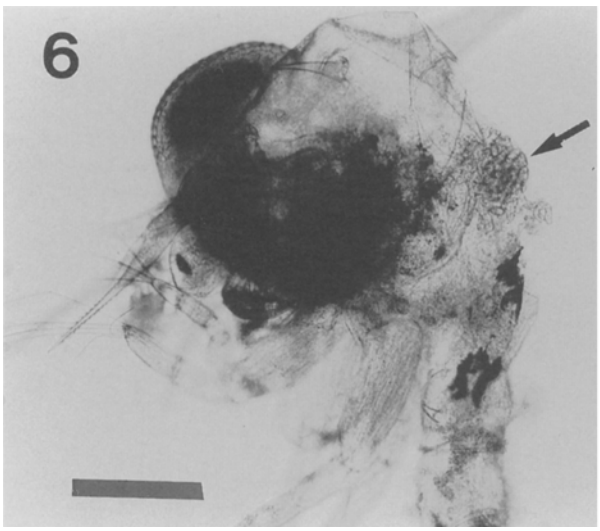
Isolates	Mortalities (%) of larvae[a] challenged with different no. of zoospores (zoosp./ml) after			
	24 h		48 h	
	5.4×10^4	5.4×10^3	5.4×10^4	5.4×10^3
NJM 9802	100	30	—	100
NJM 9808	100	40	—	100
Control[b]	10	10	10	10

a) 10 larvae were used in each test.
b) without zoospore.

spores/ml reached 100% after 24 h. In case of larvae challenged by 5.4×10^3 zoospores/ml the mortalities were 30% and 40% for NJM 9802 and NJM 9808, respectively, after 24 h and reached 100% after 48 h. The same fungus was reisolated from all dead larvae (Fig. 6–7).

Discussion

Atkinsiella dubia is charactrized by thallus endobiotics, holocarpic, saccate-lobed, bulbous irregular, or consisting of stout swollen hyphae (Atkins, 1954; Fuller et al., 1964; Sparrow and Gotelli, 1969; Sparrow, 1973; Hatai, 1989). Its vegetative cells are characterized by granular clusters without oil droplets and vacuoles. The central



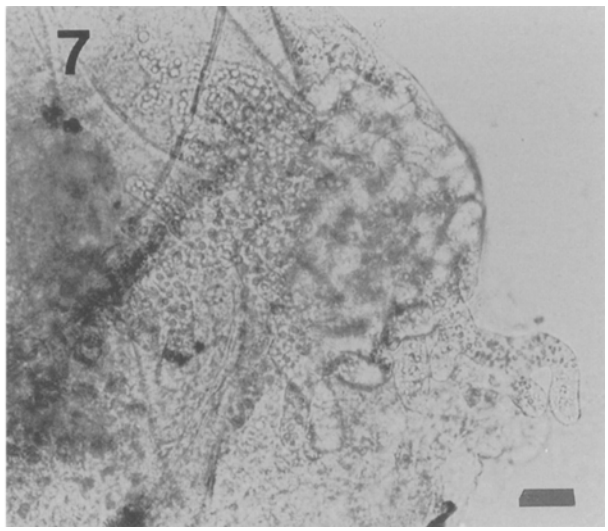


Fig. 6. Mycelia (arrow) in larvae of the swimming crab artificially infected with *Atkinsiella dubia*. Scale: 100 μm.
Fig. 7. High magnification of the lesion shown in Fig. 6. Scale: 100 μm.

Table 4. Comparison of morphological characteristics of the isolates NJM 9802 and NJM 9808 with previous descriptions of *Atkinsiella dubia*.

	Present isolates		Descriptions by				
	NJM 9802	NJM 9808	Atkins (1954)	Fuller et al. (1964)	Sparrow (1973)	Martin (1977)	Nakamura and Hatai (1995b)
Sources	Japanese mitten crab	Japanese mitten crab	eggs of pea crab	eggs of various crabs	marine algae	eggs of insect	mantle of abalone, gills of adult swimming crab
Rhizoids	absent	absent	—[a]	—	present	—	absent
Discharge tubes (μm)[b]	w. 4–9 l. 5–16	w. 5–7 l. 2–11	w. 10 l. 50–400	short tubes	w. 4–6 l. 9–10	l. 50–100	w. 8–50 l. 8–470
Zoospore production (h)[c]	about 30	about 30	—	about 12	—	—	about 35
Proliferation	not observed	not observed	observed	—	—	—	not observed
Zoospores (μm)[d]	f. 3–6 s. 2–7	f. 2–6.2 s. 2–5.8	f. 10 s. 11–12	f. 6×8.2 s. 4.8×8.7	4–6×6–8	f. 8.5–16 s. 5.5–12.5	f. 8–11 s. 4–7×8–12
Encysted zoospores (μm)[d]	f. 3–7 s. 3.5–6	f. 5–8 s. 3–7.5	f. 7–8 s. 6–7	f. 7.4 s. 6.8	7–9	10.5 (diam)	7–9
Germination (μm)	with a slender germ tube	with a slender germ tube	—	with a small tube, about 1.7 (diam)	with a long slender germ tube	with a hyphal germ tube	with a slender germ tube
Gemmae	present	present	present	—	absent	absent	absent
Sexual reproduction	absent	absent	absent	absent	absent	absent	absent

a) —: not described.
b) w. or l.: width or length of the discharge tube.
c) Zoospore production: time lapse between transferring hyphae to seawater and beginning of zoospore production.
d) f. or s.: zoospores in the first or second motile stage.

protoplasmic mass is supported by several protoplasmic threads in the process of zoospore production, and the most important character is that zoospores encysted within the sporangium and discharge tubes following the first motile stage (Martin, 1977; Nakamura and Hatai, 1995b). The first description of the fungus in Japan was from the mantle of the abalone, *Haliotis sieboldii*, and the gills of the adult swimming crab, *Portunus trituberculatus*, obtained from Chiba Prefecture (Nakamura and Hatai, 1995b). The fungus was not then thought to be a pathogen of the aquatic animals, because it was isolated from materials from marine animals that showed no clinical signs.

As described in this paper, however, *A. dubia* was isolated from infected larvae of the Japanese mitten crab and showed the pathogenicity to the larvae of the swimming crab. From these results, the fungus was thought to be a pathogen in marine crustaceans. The morphological characteristics of *A. dubia* previously reported and those of the present isolates were compared in the Table 4, but no significant differences were found.

The growth temperature range and optimum temperature of the present isolates, NJM 9802 and NJM 9808, were similar to those of *A. dubia* ATCC 200323 reported by Nakamura and Hatai (1995b).

The present isolates were considered to be an obligate marine fungus, because their growth was observed only on PYGS agar and 2.5% NaCl PYG agar. The results were the same as those reported by Nakamura and Hatai (1995b).

The finding that the present isolates were pathogenic to the larvae of *P. trituberculatus* as a result of artificial infection suggests that *A. dubia* is a pathogen of not only the Japanese mitten crab but also other crustaceans.

Acknowledgements——The authors wish to thank to Yamaguchi Prefectural Naikai Fisheries Experimental Station, Yamaguchi Prefecture, Japan for kindly sending specimens and supplying the zoea of the swimming crab.

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