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FULL PAPER

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Haliphthoros milfordensis isolated from black tiger prawn larvae (*Penaeus monodon*) in Vietnam

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Abstract A marine fungus was isolated from the black tiger prawn *Penaeus monodon* at Nha Trang, Vietnam, on March 20, 2001 and named isolate NJM 0131. The fungus was identified as *Haliphthoros milfordensis* from the characteristics of asexual reproduction, and its physiological characteristics were investigated. Although the optimum temperature for growth of the isolate was 25°–30°C, the fungus grew at a wide range of temperatures (15°–40°C). *H. milfordensis* grew well in 50%–100% seawater, but poorly in PYG agar containing 1.0%–5.0% NaCl and KCl. The fungus grew at a wide range of pH (4.0–11.0) with the optimum pH value of 7.0–9.0. The isolate also showed pathogenicity to swimming crab larvae (*Portunus trituberculatus*) by artificial infection, but mortality was not high. This is the first report of disease in the black tiger prawn *P. monodon* in Vietnam caused by *H. milfordensis*.

Key words *Haliphthoros milfordensis* · Marine fungus · Pathogenicity · *Penaeus monodon* · *Portunus trituberculatus*

Introduction

Production of the black tiger prawn *Penaeus monodon* Frabicius is increasing every year in Vietnam. Fungal infection, however, has been found in the larvae, and it has been known that larvae sometimes die of the infection at hatcheries. However, study of the classification of the causative agent has never been attempted in Vietnam.

In March 2001, a fungal infection occurred in larvae of the black tiger prawn *P. monodon* reared at a spawning tank in an experimental hatchery of Nha Trang University of Fisheries, Nha Trang, Vietnam. The causative agent was classified in the genus *Haliphthoros* and identified as *H. milfordensis* Vishnac from the morphological characteristics of the fungus. This is the first report of fungal disease caused by *H. milfordensis* in Vietnam.

The fungus *Haliphthoros milfordensis* was first described on the eggs of the oyster drill *Urosalpinx cinerea* Say (Vishniac 1958). Later, the fungus was reported on the surface of the alga *Enteromorpha* sp. (Fuller et al. 1964), juveniles of the American lobster *Homarus americanus* Edwards (Fisher et al. 1975), the mantle, foot, and epipode of abalone, *Haliotis sieboldii* Reeve (Hatai 1982), zoeae of the mangrove crab *Scylla serrata* Forsskal (Hatai et al. 2000), and zoeae of a marine crab, *Portunus pelagicus* Linnaeus (Nakamura and Hatai 1995). *Haliphthoros milfordensis* has been also reported from some prawns, the white shrimp *Penaeus setiferus* Linnaeus (Tharp and Bland 1977) and the kuruma prawn *Penaeus japonicus* Bate (Hatai et al. 1992). Another species in the genus, *Haliphthoros phillipinensis* Hatai, Bian, Baticados et Egusa, was described as a pathogen isolated from the jumbo tiger prawn *Penaeus monodon* (Hatai et al. 1980). In previous studies, *H. milfordensis* has been reported as an obligatory marine fungus (Vishniac 1958; Fisher et al. 1975; Hatai 1982; Nakamura and Hatai 1995).

This article describes the morphological and physiological characteristics of *H. milfordensis* isolated from larvae of the black tiger prawn *Penaeus monodon* in Vietnam.

Materials and methods

Isolation and identification

Zoeae of the black tiger prawn *P. monodon* were collected from a tank in an experimental hatchery of Nha Trang

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University of Fisheries, Nha Trang, Vietnam, on March 20, 2001. Dying zoeae, with fungal infection, were sampled and inoculated on PYGS agar (0.125% bacto-peptone, 0.125% yeast extract, 0.3% glucose, 1.2% agar, and 3.76% artificial seawater powder; Aqua-Ocean, Japan Pet Drugs, Tokyo, Japan). To reduce bacterial contamination, 500 μg/ml each ampicillin and streptomycin sulfate were added to the medium. The plates were incubated at 25°C. After fungus growth on the medium, a pure culture of the fungus was made by the single spore culture method.

For morphological observation, the fungus was inoculated into PYGS broth and incubated at 25°C for 4–5 days. Inoculated young mycelia were washed three times in sterilized artificial seawater in advance. The fungus was identified according to Sparrow (1960), Fuller et al. (1964), Tharp and Bland (1977), and Karling (1981).

Effect of temperature on growth

Temperature range and optimum temperature for growth were determined using the mycelia of the fungus. The advancing edges of colonies, cultured at 25°C for 3 days on PYGS agar, were cut with a no. 2 cork borer (5.5 mm in diameter) and placed onto the center of Petri dishes (90 mm diameter × 20 mm depth) containing 25 ml PYGS agar, then incubated at 5°, 10°, 15°, 20°, 25°, 30°, 35°, and 40°C. The radial growth of the colonies was measured every 2 days, and the mean of two perpendicular radii was calculated. All experiments were performed in duplicate.

Effect of various concentrations of seawater on growth

The advancing edges of colonies, cultured at 25°C for 3 days on PYGS agar, were inoculated onto plates of PYG agar (prepared in the same manner as PYGS agar plates, but with distilled water replaced by seawater) containing various concentrations [100%, 75%, 50%, 25%, and 0% (w/v)] of artificial seawater. The plates were incubated at 25°C. The radial growth of the colonies was measured every 2 days, and the mean of two perpendicular radii was calculated. All experiments were performed in duplicate.

Effect of NaCl or KCl concentration on growth

To determine whether the isolate was a marine organism, it was inoculated onto plates of PYG agar containing various concentrations (0%, 1.0%, 2.5%, and 5.0%, w/v) of NaCl or KCl and incubated at 25°C. PYGS agar was prepared as a control medium. Preculture, inoculation, and growth determination were conducted as already described.

Effect of pH on fungal growth

The PYGS broth was adjusted to pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0 by adding 1N HCl or 1N NaOH. PYGS agar blocks containing mycelia were cut out, with a no. 2 cork borer, from the margin of the colonies and put into test tubes containing 10 ml PYGS broth. Each test medium was incubated at 25°C, and the growth of hyphae was observed by the naked eye every 2 days for 2 weeks. After 2 weeks, the agar discs without fungal growth were inoculated onto PYGS agar to reconfirm hyphal survival. All experiments were performed in duplicate.

Experimental infection

The pathogenicity of the isolate was estimated by using the zoea stage of the swimming crab *Portunus trituberculatus* Miers, obtained from the Hyogo Prefectural Fisheries Station, Japan. Thirty milliliters of sterilized seawater (approximate salinity, 3.76%) and 30 zoeae were placed into plastic Petri dishes. To reduce bacterial contaminants, the zoeae were washed three to four times using sterilized seawater. The larvae were challenged with 1.0×10^4, 1.0×10^3, and 1.0×10^2 zoospores/ml of *H. milfordensis* NJM 0131. Larvae groups without zoospores were maintained as controls. Dying or dead larvae were removed from the Petri dishes every day and immediately examined under an inverted microscope for fungal infection. Infected larvae were inoculated on PYGS agar for re-isolation. The experiments were continued for 3 days at 25°C, and all experiments were performed in duplicate.

Results

Isolation and identification

A fungus belonging to the order Lagenidiales was isolated from infected zoeae of the black tiger prawn *Penaeus monodon*. One isolate, NJM 0131, was randomly selected from all the fungal colonies and examined in detail. The isolate was maintained at 25°C and subcultured onto PYGS agar once a month.

Fragmentation of hyphae occurred in the isolate NJM 0131 when the mycelia were transferred into sterilized artificial seawater. Each fragment changed into a sporangium, then developed a discharge tube. Zoospores formed within the sporangium were liberated into the seawater through the top of a discharge tube. Based on these observations, the isolate NJM 0131 was identified as a fungus of the genus *Haliphthoros*, in Sirolpidiaceae, Lagenidiales, Oomycetes, in reference to Sparrow (1976), from the characteristics of fragment formation (Fig. 1) and the mode of zoospore discharge (Fig. 2). The hyphae were 10–50 μm in width and the fragments were variable in shape, 25–95 μm in width and 30–375 μm in length.

The release of zoospores through a discharge tube was observed 12–24 h after the mycelia were transferred into sterilized artificial seawater. A discharge tube was usually formed from each zoosporangium: 4–10 μm in diameter, 40–600 μm in length, and usually straight or slightly curved. The release of zoospores continued for 3–23 min. Encysted zoospores were globose, (4–) 6 (–12) μm in diameter. Sexual

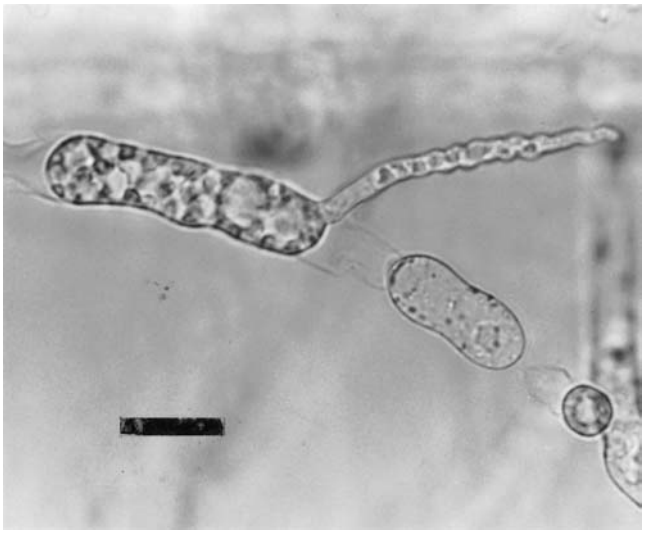

Fig. 1. Fragment formation of *Haliphthoros milfordensis* NJM 0131. *Bar* 30 μm

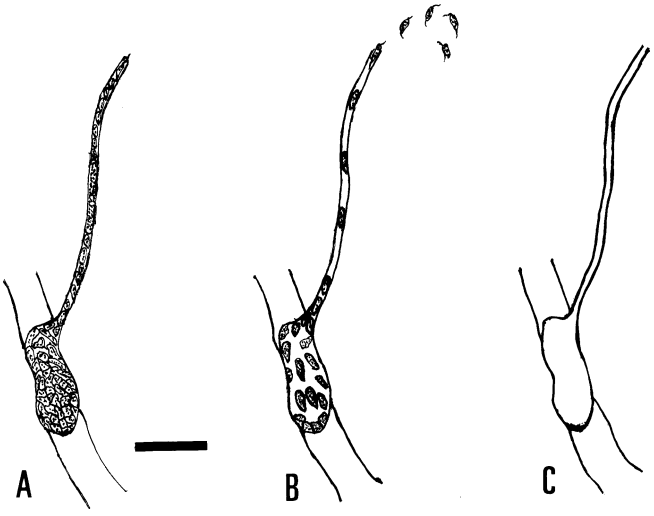

Fig. 2. Zoospore discharge of *H. milfordensis* NJM 0131. **A** Fragment with discharge tube. **B** Zoospore releasing. **C** Fragment and empty discharge tube. *Bar* 50 μm

reproduction was not observed. The isolate NJM 0131 was identified as *H. milfordensis* Vishniac based on the characteristics of asexual reproduction with reference to the description of Sparrow (1960), Fuller et al. (1964), Tharp and Bland (1977), and Karling (1981).

Specimen examined: NJM 0131 isolated from a zoea of the black tiger prawn *Penaeus monodon*, with fungal infection, obtained from hatchery at Nha Trang University of Fisheries, Vietnam, on March 20, 2001.

Effect of temperature on growth

The fungal isolate grew at 15°–40°C, but exhibited optimal growth at 25°–30°C (Table 1). No growth was observed at 5°–10°C.

Table 1. Effect of various temperatures on fungal growth

Temperature (°C)	Days after incubation						
	2	4	6	8	10	12	14
5	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0
15	0	0	0	1.0	1.0	2.1	2.8
20	0.8[a]	5.5	11.8	20.0	26.5	32.0	37.5
25	3.0	10.8	17.0	26.8	36.5	37.5	37.5
30	3.5	10.0	19.0	28.8	36.0	37.5	37.5
35	0.3	2.8	7.0	11.0	20.0	26.5	28.5
40	0.3	2.5	3.0	3.5	5.0	7.5	10.5

[a] Data represent the mean of radius (mm) of colony; radius of the plate was 37.5 mm

Table 2. Effect of various dilutions of seawater on fungal growth

Seawater (%)	Days after inoculation						
	2	4	6	8	10	12	14
100	3.5[a]	10.3	19.0	28.3	36.0	37.5	37.5
75	4.8	11.5	18.8	28.0	36.5	37.5	37.5
50	6.0	14.0	22.5	31.1	36.5	37.5	37.5
25	1.5	4.1	6.5	9.8	14.3	17.8	20.3
0	0	0	0	0	0	0	0

[a] Data represent the mean of radius of colony; radius of the plate was 37.5 mm

Table 3. Effect of minerals on fungal growth

Media	Days after inoculation						
	2	4	6	8	10	12	14
PYG + 1.0% NaCl	0	3.3	6.8	11.0	15.8	18.0	23.5
PYG + 2.5% NaCl	3.0[a]	7.0	11.0	15.5	20.0	25.5	28.5
PYG + 5.0% NaCl	0	0	0.5	2.5	2.5	2.5	2.5
PYG + 1.0% KCl	0	0	1.0	2.0	2.5	4.5	6.5
PYG + 2.5% KCl	0	0.5	2.0	3.3	4.0	5.0	6.8
PYG + 5.0% KCl	0	0	0.5	0.5	0.5	1.3	2.0
PYG	0	0	0	0	0	0	0
PYGS	3.0	10.8	17.0	26.8	36.5	37.5	37.5

[a] Data represent the mean of radius of colony; radius of the plate was 37.5 mm

Effect of seawater concentration on growth

The isolate grew well in 50%–100% seawater and poorly in 25% seawater (Table 2). Growth was not observed on PYG agar without seawater. It was expected that seawater was necessary for the isolate growth.

Effect of NaCl or KCl concentration on growth

The isolate grew on PYGS agar and PYG agar with various concentrations of NaCl and KCl, but did not grow on PYG agar (Table 3).

Effect of pH on fungal growth

The fungus in PYGS broth grew at a pH range of 4.0–11.0, with optimal growth observed at pH 7.0–9.0 (Table 4).

Table 4. Effect of pH on fungal growth

pH	Days after inoculation into PYGS broth			
	2	6	10	14
4.0	−	−	−	+
5.0	−	+	+	+
6.0	−	+	+	++
7.0	−	++	++	+++
8.0	−	++	++	+++
9.0	−	++	++	+++
10.0	−	+	+	++
11.0	−	+	+	+

−, no growth; +, ++, +++, slight to excellent growth

Table 5. Pathogenicity of *Haliphthoros milfordensis* isolate NJM 0131 to swimming crab zoeae

No. of zoospores challenged (spores/ml)[a]	Days after challenge		
	1	2	3
1.0×10^4	13	26	46
1.0×10^3	13	13	13
1.0×10^2	13	16	16
0	0	0	0

[a] Thirty swimming crab zoeae, *Protunus trituberculatus*, were used in each experiment; Data represent cumulative mortality of swimming crab larvae infected with the fungus

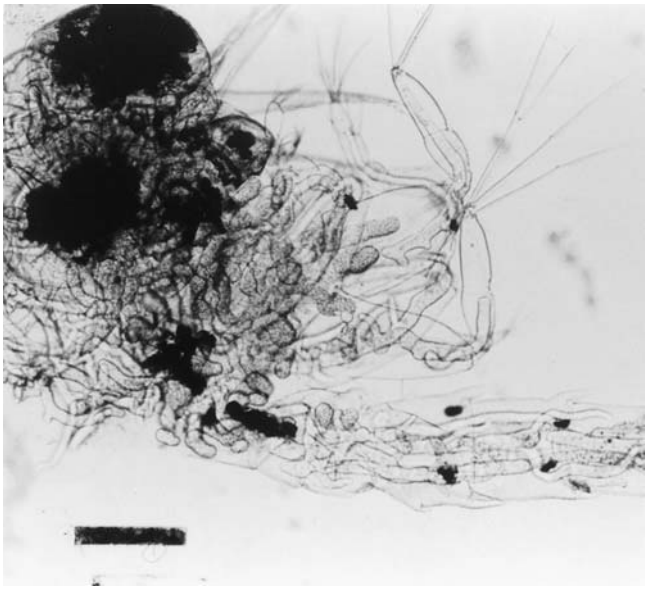

Fig. 3. Zoea of the swimming crab *Portunus trituberculatus* experimentally infected with *H. milfordensis* NJM 0131. *Bar* 100 μm

Experimental infection

The affected zoeae showed slow movement, lethargy, lying down at the bottom of the Petri dish, and eventual death. The infected larvae contained many fungal hyphae (Fig. 3). After 3 days, the cumulative mortality of the swimming crab larvae was 46%, 13%, and 16% when the larvae were challenged with 1.0×10^4, 1.0×10^3, and 1.0×10^2 fungal spores/ml, respectively (Table 5).

Discussion

The present isolate released zoospores only from discharge tubes, thus differing form *H. philippinensis*, which releases zoospores both from the orifices of the discharge tubes and from openings in the zoosporangia (Hatai et al. 1980).

The range of optimal temperature for hyphal growth of *H. milfordensis* NJM 0131 was 25°–30°C, which was similar to that of *H. milfordensis* as previously reported by Vishniac (1958), Hatai (1982), Nakamura and Hatai (1995), and Hatai et al. (2000). The isolate NJM 0131, however, grew even at 40°C and an isolate of *H. philippinensis* reported by Hatai et al. (1980) also grew at 36.3°C. It was suggested that the isolates were adapted to the environment in the tropics.

The isolate NJM 0131 grew on PYG agar containing 25%–100% artificial seawater but not without seawater. The results showed that the fungus could survive in the sea from low to high salinity.

The present fungus could not grow on PYG agar, but grew rapidly on PYG agar with artificial seawater and poorly on PYG agar with 5.0% NaCl and 1.0%–5.0% KCl. The results showed that the fungus was a marine fungus but not an obligate marine fungus. The present isolate differed from previous reports, because *H. milfordensis* has been reported as an obligate marine fungus (Vishniac 1958; Fisher et al. 1975; Hatai 1982; Nakamura and Hatai 1995).

The isolate NJM 0131 grew at a wide range of pH (4.0–11.0). These results suggest that the fungal growth is not strongly affected by the pH of seawater.

Haliphthoros milfordensis has been reported as a parasite of various marine crustaceans (Nakamura and Hatai 1995; Hatai et al. 2000). The fungus isolated from the black tiger prawn *Penaeus monodon* with fungal infection in this study showed low pathogenicity to the larvae of the swimming crab *Portunus trituberculatus* in Japan. Although the pathogenicity of the isolate NJM 0131 to *Penaeus monodon* was not examined in this study, *H. milfordensis* might be a pathogen of *P. monodon* larvae, because the infection in larvae of *P. monodon* has sometimes been observed at the hatchery. The species was also known to be a pathogen of larvae of the mangrove crab *Scylla serrata* (Roza and Hatai 1999), *Artemia salina* (Overton and Bland 1981), juvenile American lobsters *Homarus americanus* (Fisher et al. 1975), and larvae of the swimming crab *Portunus trituberculatus* (Nakamura and Hatai 1995).

This is the first report of *Haliphthoros milfordensis* in Vietnam, and the species is probably distributed widely in countries of Southeast Asia.

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