Mycoscience 41: 565–572, 2000

Identification of lower fungi isolated from larvae of mangrove crab, *Scylla serrata*, in Indonesia

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Accepted for publication 4 September 2000

Since 1992, seed production of mangrove crab, *Scylla serrata*, has been attempted at the Gondol Research Station for Coastal Fisheries, Bali, Indonesia. During the production process, almost all of the larvae have died due to fungal infection. Fungi isolated from the larvae with fungal infection were classified into three species in the order Lagenidiales: *Lagenidium callinectes*, *Haliphthoros milfordensis* and *Halocrusticida baliensis* sp. nov. based on detailed morphological characteristics. The effects of temperature, pH and mineral content of the water on their growth were also examined.

Key Words——fungal diseases; *Haliphthoros milfordensis*; *Halocrusticida baliensis*; *Lagenidium callinectes*; *Scylla serrata*.

Seed production of the mangrove crab, *Scylla serrata* Forsskal, is an important industry in Indonesia. A serious problem facing this industry is fungal diseases, due to fungi belonging to the order Lagenidiales, which cause high mortality in the eggs and zoeal larvae. Fungal diseases in crabs caused by the fungi of the order Lagenidiales have been previously reported. In the genus *Lagenidium*, *L. callinectes* Couch has been reported from blue crab, *Callinectes sapidus* Rathbun (Couch, 1942; Bahnweg and Gotelli, 1980), and swimming crab, *Portunus pelagicus* (Nakamura and Hatai, 1995a). *L. scyllae* Bian et al. (Bian et. al., 1979) and *L. thermophilum* Nakamura et al. (Nakamura et al., 1995) have been isolated from mangrove crab, *Scylla serrata*. In the genus *Haliphthoros*, *Hali. milfordensis* has been reported from swimming crab, *Portunus pelagicus* (Nakamura and Hatai, 1995a). In the genus *Halocrusticida*, *Halo. hamanaensis* (Bian & Egusa) Nakamura & Hatai (Bian and Egusa, 1980) and *Halo. okinawaensis* (Nakamura & Hatai) Nakamura & Hatai (Nakamura and Hatai, 1995a) have been reported from mangrove crab, *Scylla serrata*, and swimming crab, *Portunus pelagicus* Linnaeus, respectively. *Halocrusticida hamanaensis* and *Halo. okinawaensis* were formerly identified as fungi in the genus *Atkinsiella*. *Atkinsiella dubia* was also reported from the mantle of abalone, *Haliotis sieboldii* Reeve (Nakamura and Hatai, 1995a).

In 1997, fungal diseases occurred in the eggs and zoeae of the mangrove crab *S. serrata* at the Gondol Research Station. The mortality rate reached almost 100% in the larvae. The infected larvae were whitish in color and filled with numerous aseptate hyphae.

In this paper, the morphological and physiological characteristics of three strains of fungi isolated from the larvae of the mangrove crab with fungal infections were studied.

Material and Methods

Isolation and identification Zoeae of the mangrove crab, *Scylla serrata* Forsskal, which were produced at the hatchery of the Gondol Research Station for Coastal Fisheries, Bali, Indonesia, were reared at 25–27°C in a tank and fed with rotifers, *Branchionus plicatilis* Müller, and brine shrimp, *Artemia salina* Linnaeus. The infection occurred in the zoeae in July 1997. Zoeae from the tank were observed under the microscope. If fungal elements were present, we attempted to isolate them using PYGS (1.25 g of Bacto peptone, 1.25 g of Bacto yeast extract, 3 g of glucose, and 12 g of Bacto agar and 1,000 ml of seawater) containing streptomycin sulphate and ampicillin, each at a concentration of 500 μg/ml, to retard bacterial growth. The agar plates were incubated at 25°C for 5–7 d. After fungal colonies developed on the agar plates, each one was transferred onto fresh PYGS agar to make a pure culture. The fungi were maintained at 25°C and subcultured on PYGS agar at approximately monthly intervals. Three different isolates (GSM 9701, GSM 9703 and GSM 9706) belonging to Lagenidiales were obtained by random selection from all of the fungal colonies. They were used for all experiments.

For morphological observations, the isolates were inoculated into PYGS broth and incubated at 25°C for 3–5 d. Small colonies in PYGS broth were rinsed twice with sterilized artificial seawater (Aqua-Ocean®, Japan Pet Drug Co., Tokyo) and transferred into Petri dishes (9.0 cm diam) containing 25 ml of sterilized artificial sea-

water, and then incubated at 25°C to induce zoospore production. Three isolates belonging to the Lagenidiales were identified according to Bian and Egusa (1980), Couch (1942), Nakamura and Hatai (1995a; 1995b), Nakamura et al. (1995) and Vishniac (1958).

Effect of temperature on growth The growth temperature range and optimum temperature were examined using mycelia of the fungi. The three isolates of the Lagenidiales were inoculated onto PYGS agar. Each isolates was incubated at 25°C for 7–15 d 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 onto PYGS agar plates containing 25 m/ of PYGS agar per plastic Petri dish (8.25 cm diam). Plates were

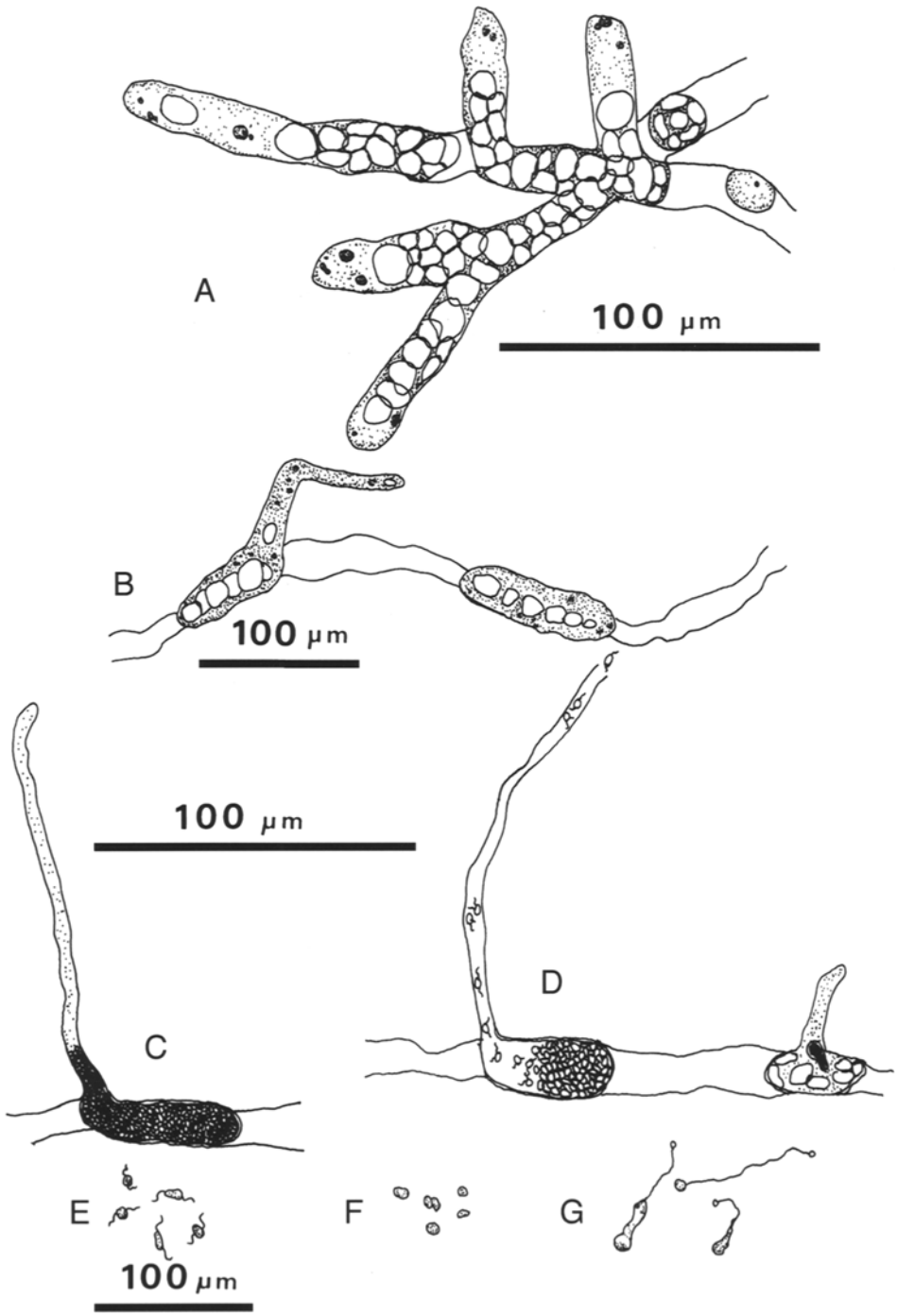

Fig. 1. Morphological characteristics of *Haliphthoros milfordensis* GSM 9701 isolated from a zoea of *S. serrata*.
A. Hyphae in PYGS broth; B. Fragments. Discharge tube formation on the left fragment; C. Zoospore formation; D. Zoospore liberation; E. Zoospores; F. Encysted zoospores; G.Germination.

incubated at six different temperatures (5, 10, 15, 20, 30 and 35°C). The growth rate was checked by measuring the colony diameter every day for 14 d after inoculation.

Effect of temperature on zoospore production Zoospores were produced as described above. Similar amounts of mycelia were then put into small Petri dishes (50 × 15 mm) containing 10 m/ of sterilized artificial seawater and incubated at 15, 20, 25 and 30°C. The number of motile zoospores was determined with a Neubauer counting chamber (Erma^R) every day for 1 wk.

Effect of pH on mycelial growth Zoospore suspensions of each isolate were adjusted to 1.0×10^4 spores/m/. A 100-μ/ portion of zoospore suspension of each fungal isolate was inoculated into a small Petri dish (50 mm diam) containing 10 m/ of PYGS broth adjusted to pH 3, 5, 7, 9 and 11. The inoculated plates were incubated at 25°C for 7 d to observe mycelial growth.

Mineral requirements for growth The fungal isolates were inoculated onto PYG agar containing various concentrations of NaCl or KCl to determine whether these minerals were required for growth. PYG agar was prepared in a similar way to PYGS agar using distilled water instead of seawater. PYG agar was mixed with NaCl or KCl at concentrations of 1, 2.5, and 5% (w/v). PYGS and PYG agars were used as control media. The cultures were inoculated and the colony diameters were measured as described above.

Results

Incidence Spawners of the mangrove crab, *Scylla serrata*, 200–300 g (av. 250 g) in body weight, were kept in a 16-ton concrete tank at 25–28°C (av. 27°C) at a private hatchery in Probolinggo, East Java, Indonesia and brought to the Gondol Research Station for Coastal Fisheries in July 1997. The eggs first hatched on July 2, 1997. Fungal infection was first found in the zoeal larvae, and the mortality rate reached almost 100%. In some trials of seed production, they always died due to the same fungal infection. The zoeae appeared to change from normal transparency to a whitish color when they became infected. The bodies of all the dead zoeae were filled with aseptate stout hyphae.

Isolation and identification Three fungi belonging to Lagenidiales were isolated from diseased and dead larvae of the mangrove crab. One of the isolates, GSM 9701, was identified as a member of the genus *Haliphthoros*, because of fragmentation during sporulation. Isolate GSM 9703 was placed in the genus *Halocrusticida*, because it formed zoosporangia of the same size and shape as the subthalii, and one to several discharge tubes from each sporangium. Isolate GSM 9706 was classified as a species of the genus *Lagenidium*, because vesicles were produced on the tip of the discharge tube when zoospore production occurred. The morphological characteristics of the fungi isolated from the mangrove crab larvae are as follows.

Haliphthoros milfordensis Vishniac, Mycologia, 50: 75, 1958. Fig. 1

Colonies on PYGS agar were whitish and reached a diameter of 20–25 mm after 5 d at 25°C. The centers were damp.

Hyphae in PYGS broth were stout, aseptate, branched with numerous shiny spherical granules, and sometimes concentrated masses of protoplasm were observed in the hyphae. The width of the hyphae was 7.5–30 μm. In artificial seawater, fungal fragments were clearly observed to be 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 sporangia and extending discharge tubes, and were also observed in the active mycelia. Zoospore formation was observed about 8–12 h after the mycelia were transferred into sterilized artificial seawater and continued for one week. One discharge tube was usually formed on the lateral side of each zoosporangium. The tubes were 5–10 μm in diam and 15–300 μm in 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, 6–7.5 × 7–12 μm, 6.5 × 8.5 μm on average. Encysted zoospores were globose or subglobose, 3–7 μm in diam, 5 μm on average. Spores germinated with a hair-like filament measuring 15–150 μm in length about 4–5 h after encystment. Sexual reproduction was not observed.

Specimen examined: GSM 9701 isolated from a zoea of the mangrove crab, *S. serrata* with a fungal infection, obtained from Gondol Research Station for Coastal Fisheries, Bali, Indonesia, 2 July 1997.

Halocrusticida baliensis Hatai, Roza et Nakayama, sp. nov. Figs. 2, 3

Coloniae in agaro "peptone-yeast extract-glucose-seawater (PYGS)" luteolae, post 1 mensem ad 25°C 17 mm diam attingentes, ex hyphis crassis irregulariter ramosis aseptatis saccato-labulatis 15–40 μm latis constants. Thallus endobioticus holocarpus, crassus, ramosus, in maturitate septatus et divisus in subthallus; subthalli cylindrici, lobati vel irregulares, 15–40 μm diam. Gemmae intra zoosporangia in aquamarina observata, 20–60 μm diam. Zoosporangia subthallum conformia, e latere vel apice tubulos emittentes singulos vel nonnulos formantia. Tubuli raro ramosi, 7.6–25 μm lati, 30–450 μm longi, zoosporas bi- vel pluriseriatim facientes. Zoosporae pyriformes vel subglobosae, lateraliter biflagellatae, diplaneticae, 6.5 × 8.5 μm. Cytosporae globosae vel subglobosae, 5.0 μm diam, cum fibra 7.5–210 μm longa germinantes. Reproductio sexualis ignota.

Holotypus: Colonia exsiccata ex cultura e zoeae *Scylla serrata* Forsskal in "Gondol Research Station for Coastal Fisheries (GSM)," Bali, Indonesia, 5 Jul. 1997 a D. Roza isolata et ea Herbario GSM concervata (GSM 9703).

Colonies on PYGS agar were yellowish and 17 mm in

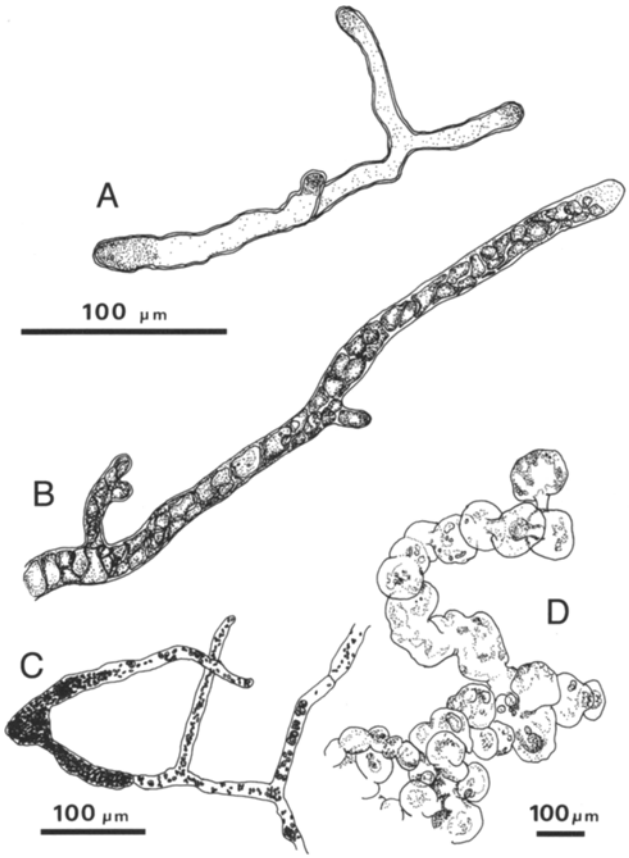

Fig. 2. Morphological characteristics of *Halocrusticida baliensis* GSM 9703 isolated from a zoea of *S. serrata*.
A, B. Hyphae in PYGS broth; C. Zoosporangia; D.Gemma.

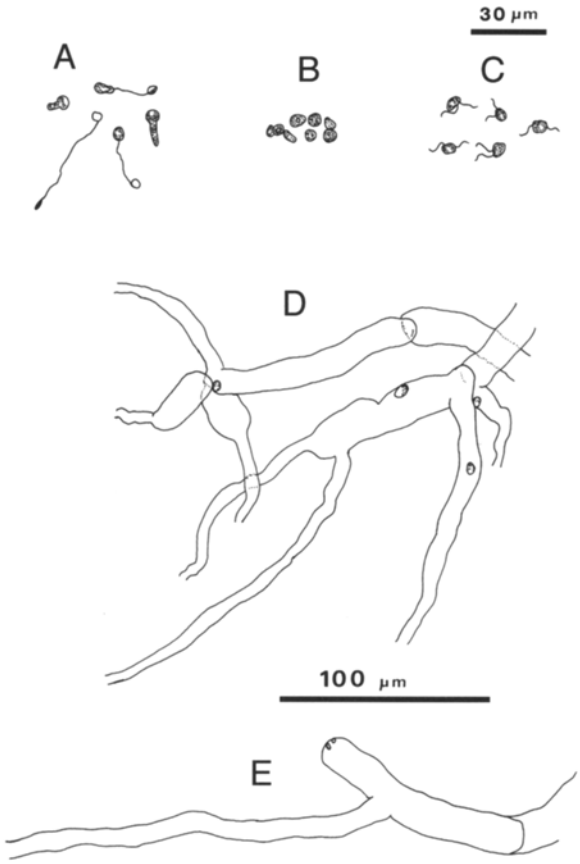

Fig. 3. Morphological characteristics of *Halocrusticida baliensis* GSM 9703 isolated from a zoea of *S. serrata*.
A. Germination; B. Encysted zoospores; C. Zoosopres; D, E. Empty zoosporangia with discharge tubes.

diam at one month after incubation at 25°C. Hyphae in PYGS broth were stout, irregularly branched, aseptate, saccate-lobed, and 15–40 μm in width. In PYGS broth, the thallus was aseptate at first, and then became septate as it divided into subthalli. Vacuoles were observed during the process of zoosporogenesis with numerous shiny granules. Gemmae present, saccate-lobed, thick-walled with shiny globules, 20–60 μm diam, developed in zoosporangia in seawater. Subthalli cylindrical, saccate, irregular, tuberculate, variable in size and shape. Zoosporangia were the same size and shape as subthalli. Each sporangium extended one to several discharge tubes. In the discharge tubes, zoospores were lined more than two deep. Discharge tubes were produced laterally or terminally from the sporangia, straight or wavy, usually with a broad cone-shaped base, tapering or equal, measuring 7.6–25 μm in width and 30–450 μm in length. Branches of the discharge tubes were rarely observed near the zoosporangia. Zoospore formation was observed 22–24 h after the vegetative hyphae were transferred into sterilized artificial seawater and incubated at 25°C, and it continued for 10 d. Zoospores were laterally biflagellate, monoplanetic, 7.2(5.6–8.5) × 6.1(4.9–7.4) μm size, pyriform, slipper-shaped, oblong, and spherical. In zoosporangia with several discharge tubes, zoospores were usually released from one of them, but sometimes all the zoospores were released at the same time. The encysted spores were 5.9(5.3–6.8)μm in diam, spherical to subglobose, with or without oil droplets. The encysted spores in sterilized artificial seawater developed a hair-like filament, 7.5–210 μm in length. The tip of the filament enlarged and developed in 10–12 h into a hyphal bud, 12.2 × 50 μm, after the zoospores became encysted. Sexual reproduction was not observed.

Specimen examined: GSM 9703 isolated from zoea of the mangrove crab, *S. serrata* Forsskal with a fungal infection, obtained from Gondol Research Station for Coastal Fisheries, Bali, Indonesia, 5 July 1997.

Lagenidium callinectes Couch, J. Elisha Mitchell Sci. Soc. 58: 158. 1942. Fig. 4

Colonies on PYGS agar were whitish and were 35–40 mm in diam after 5 d at 25°C. The centers were damp.

Hyphae were aseptate, irregularly branched, stout, with numerous shiny rod-shaped granules, 5–30 μm in width. Zoospore formation was observed about 12 h after the mycelia were transferred into sterilized artificial seawater. Masses of protoplasm flowed into the tips of

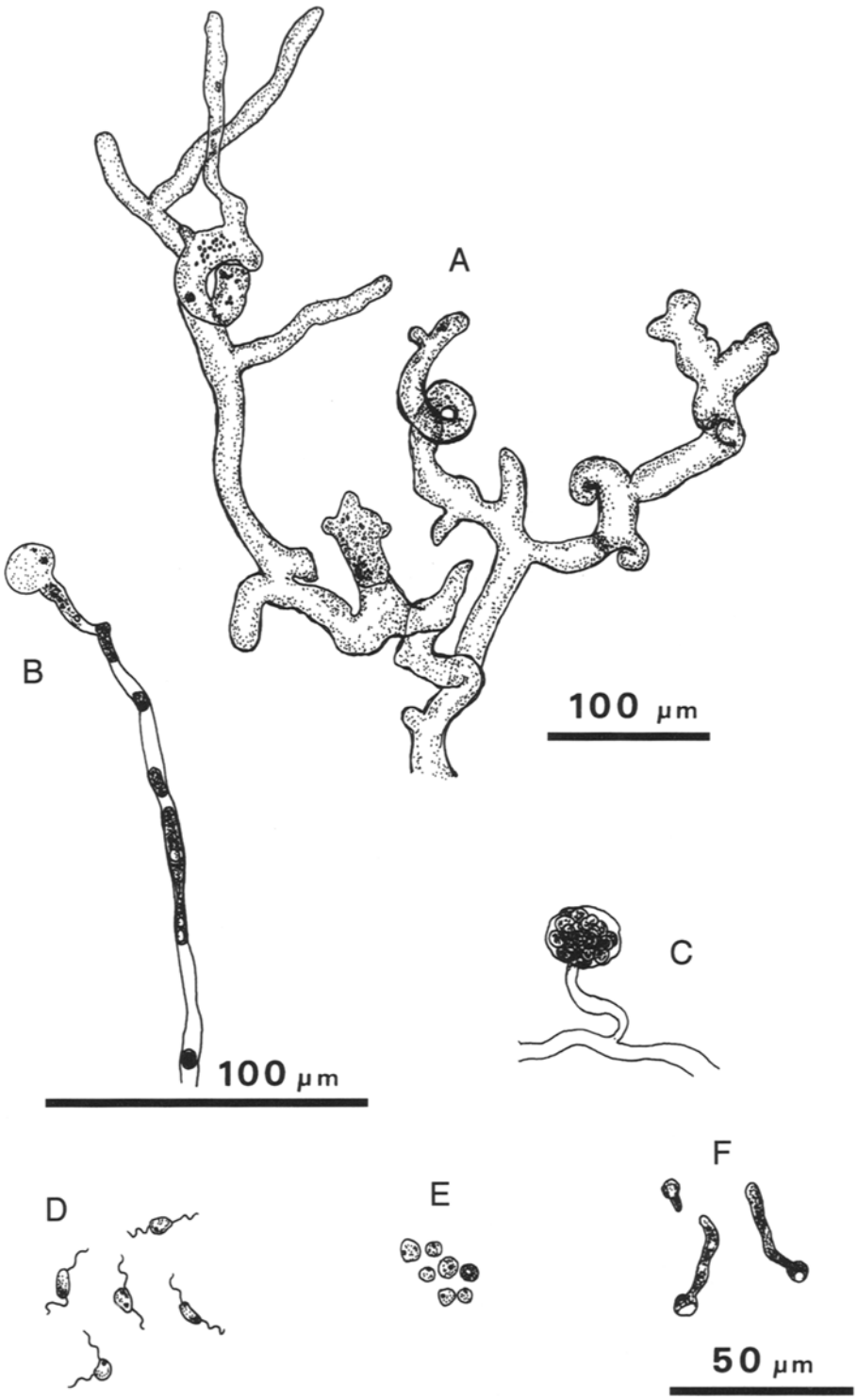

Fig. 4. Morphological characteristics of *Lagenidium callinectes* GSM 9706 isolated from a zoea of *S. serrata*. A. Hyphae in PYGS broth; B. Vesicle formation; C. Zoospores formation in the vesicle; D. Zoospores; E. Encysted zoospores; F. Germination.

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 vesicles, flagellae appeared around the protoplasm within 5 min. By 10 min, individual zoospores that had not divid-

Table 1. Effect of temperature on growth of isolates GSM 9701, GSM 9703 and GSM 9706.

Temperature (°C)	Colony radius (mm) after incubation for 14 d		
	GSM 9701	GSM 9703	GSM 9706
5	−[1]	−	−
10	−	−	−
15	15.1	−	17.2
20	31.5	8.2	29.5
25	55.3	14.5	62.5
30	−	3.1	37.0
35	−	−	3.0
40	−	−	−

[1] No growth.

ed completely were observed. These zoospores were observed to swim freely inside the vesicles at 25 min and were released by 40 min. This morphological process was almost always observed at these times. Zoospore production was successively observed up to 7 d. Mature vesicles were gelatinous, globose to subglobose, 10×80 μm in diam. The discharge tubes were $5–17 \times 18–200$ μm, usually broad at the orifice. The way in which the zoospores were liberated varied: sometimes they were released simultaneously by rupture of the vesicles, and sometimes they were released 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 $5–15 \times 8–15$ μm, 9×10.5 μm on average. Usually they swam for several hours to 20 h and did not swim past 24 h. When encysted, they were globose to subglobose without flagellae, 6–13 μm in diam, 8.6 μm on average. Germination was observed about 4 h after spores had encysted. Sexual reproduction was not observed.

Specimen examined: GSM 9706 isolated from zoea of the mangrove crab, *S. serrata* Forsskal with a fungal infection, obtained from Gondol Research Station for Coastal Fisheries, Bali, Indonesia, 29 July 1997.

The holotype specimens of each isolate described above are deposited in the Herbarium, Division of Fish Diseases, Nippon Veterinary and Animal Science University, Tokyo, Japan. Cultures derived from the holotypes have been maintained.

Table 2. Effect of temperature on zoospore production of isolates GSM 9701, GSM 9703 and GSM 9706.

Temperature (°C)	Number of zoospores*		
	GSM 9701	GSM 9703	GSM 9706
15	−[1]	−	−
20	+[2]	+	+
25	+++[3]	+++	+++
30	−	−	++[4]

* The number of zoospores was counted every day for 1 wk.
[1] No zoospore production, [2] $1–5 \times 10^3$ zoospores/ml, [3] $> 1 \times 10^4$ zoospores/ml, [4] $6 \times 10^3–1 \times 10^4$ zoospores/ml.

Table 3. Effect of pH on mycelial growth of isolates GSM 9701, GSM 9703 and GSM 9706.

pH	Growth of each isolate incubated for 7 d		
	GSM 9701	GSM 9703	GSM 9706
3	−	−	−
5	+	+	++
7	+++	+++	+++
9	+	+	+
11	−	−	−

Symbols: −, indicates no growth; +, ++, +++, increasing amounts of growth from slight to abundant.

Effect of temperature on growth The results are displayed in Table 1. Isolate GSM 9701 could grow only in the temperature range of 15–25°C. The optimum temperature for growth was 25°C. Isolate GSM 9703 grew at 20–30°C and the optimum temperature was 25°C. However, isolate GSM 9706 could grow over a wide temperature range of 15–35°C and the optimum temperature was 25°C. None of the isolates could grow at temperatures as low as 5 and 10°C, or as high as 40°C. These results showed that the organisms were adapted to tropical environments.

Effect of temperature on zoospore production The ability to produce zoospores seemed to depend on the temperature, as shown in Table 2, and many zoospores were produced at 25°C. The number of zoospores reached the maximum at 2–3 d after the mycelia were transferred to sterilized artificial seawater. Isolates GSM 9701 and GSM 9706 were observed to continually produce zoospores for 7 d after the mycelia were transferred into the seawater, and GSM 9703 was observed to continually produce zoospores for 10 d.

Effect of pH on mycelial growth All isolates could grow at pH 5–9 with an optimum at pH 7 (Table 3).

Mineral requirements for growth As shown in Table 4, each isolate grew best on PYGS agar. Isolate GSM 9701 could grow on PYG agar with 2.5% NaCl, but not

Table 4. Mineral requirements for growth of isolates GSM 9701, GSM 9703 and GSM

Media	Colony radius (mm) incubated for 14 d		
	GSM 9701	GSM 9703	GSM 9706
PYG agar + 1% NaCl	−[1]	6.0	51.0
PYG agar + 2.5% NaCl	40.1	12.0	45.5
PYG agar + 5% NaCl	−	−	34.0
PYG agar + 1% KCl	−	−	20.5
PYG agar + 2.5% KCl	−	8.5	9.0
PYG agar + 5% KCl	−	−	−
PYGS agar	58.3	15.6	73.5
PYG agar	−	−	−

[1] No growth.

on PYG agar without seawater. Isolate GSM 9703 grew on PYG agar containing 1 and 2.5% NaCl, and 2.5% KCl, but did not grow on the medium without seawater. Isolate GSM 9706, however, could grow on PYG agar containing various amounts of NaCl and KCl except 5% KCl, but not on PYG agar.

Discussion

Haliphthoros milfordensis has been reported as a parasite of various marine crustaceans (Nakamura and Hatai, 1995a). Isolate GSM 9701 showed fragmentation when the mycelia were transferred into sterilized artificial seawater. Each fragment then developed a discharge tube and changed into a sporangium. Zoospores formed within the sporangium were liberated into the water through the top of the discharge tube. Isolate GSM 9701 was identified as a fungus of the genus *Haliphthoros* in Sirolpidiaceae, Lagenidiales, Oomycetes (Sparrow, 1976) based on its morphological characteristics. The present isolate released zoospores only from the discharge tubes, and differed from *Hali. philippinensis*, which released zoospores from the orifices of both the discharge tubes and openings in the zoosporangia (Hatai et al., 1980).

For *Hali. milfordensis* GSM 9701, the range of temperature for hyphal growth was 15–25°C and that for zoospore production was 20–25°C. The optimum temperature for both growth and zoospore production was 25°C. These results showed that the present isolate was identical to that of Vishniac (1958) and Hatai (1982). The present fungus could not grow on PYG agar without artificial seawater, PYG with 1% NaCl or PYG with 5% NaCl, but it rapidly grew on PYG agar containing artificial seawater and PYG with 2.5% NaCl. This finding confirms that GSM 9701 is an obligatory marine fungus as previously reported (Hatai, 1982; Nakamura and Hatai, 1995a).

Fungi of the genus *Halocrusticida* have been reported as pathogens to various hosts. Such species include *Halo. entemophaga* Martin isolated from various midges and caddis flies (Martin, 1977), *Halo. hamanaensis* isolated from the mangrove crab (Bian and Egusa, 1980), *Halo. parasitica* (Nakamura & Hatai) Nakamura & Hatai isolated from rotifers (Nakamura and Hatai, 1994), *Halo. awabi* (Kitancharoen et al.) Nakamura & Hatai isolated from the abalone (Kitancharoen et al., 1994), and *Halo. okinawaensis* isolated from swimming crabs (Nakamura and Hatai, 1995a). However, as the morphological characteristics of the present isolate differed from those of the above fungi, the isolate GSM 9703 was identified as a new species in the genus *Halocrusticida.*

The present isolate and *Halo. hamanaensis* (Bian and Egusa, 1980) formed yellowish pigmentation on PYGS agar, but gemmae were not produced in *Halo. hamanaensis*. Gemmae have also been reported from other species of *Halocrusticida*, but the other species do not produce any pigmentation. Some characters of the present isolate were identical with those of *Halo. parasitica* (Nakamura and Hatai, 1994). However, the zoosporogenesis of this isolate was similar to that of *Halo. hamanaensis* (Bian and Egusa, 1980; Hatai, 1989). The effect of pH on the growth of the present isolate was also similar to those of *Halo. hamanaensis*. Its growth on various media suggested that isolate GSM 9703 was an obligatory marine fungus.

Lagenidium callinectes, *L. scyllae* and *L. myophilum* Hatai & Lawhavinit have been reported as parasites in eggs and larvae of the blue crab, *Callinectes sapidus* (Couch, 1942), the mangrove crab, *S. serrata* and the northern shrimp, *Pandalus borealis* Kroyer (Hatai and Lawhavinit, 1988). Recently, Nakamura et al. (1995) isolated another species of *Lagenidium*, *L. thermophilum*, from eggs and larvae of *S. serrata*. The zoospores of the isolate GSM 9706 were mainly released by rupturing of the vesicles, and rarely through a small hole in each vesicle. The present isolate grew slightly at 35°C, but *L. thermophilum* grew at 30–40°C, even though both species were isolated from the same station in Indonesia. Thus, the characteristics of the present isolate closely resembled *L. callinectes* (Couch, 1942; Bahnweg and Gotelli, 1980; Bahnweg and Bland, 1980; Hatai, 1989). Isolate GSM 9706 was thought to be a marine fungus, but not an obligatory one.

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