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Lagenidium infection in eggs and larvae of mangrove crab (*Scylla serrata*) produced in Indonesia

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A fungal infection occurred in the eggs and larvae of mangrove crab (*Scylla serrata*) in seed production in Bali, Indonesia. The causative fungus was classified as a member of the genus *Lagenidium* (Oomycetes, Lagenidiales). After comparison of its biological and physiological characteristics with those of *L. callinectes* ATCC 24973, a known parasite of various crustaceans, was concluded that the isolate is a new species of *Lagenidium, L. thermophilum*, because of its rapid and thermotolerant growth and unique discharge process. Fungal growth was observed on PYG agar containing 0-5.0% (w/v) NaCl and 0-2.5% (w/v) KCl. Similar pathogenicity toward the zoeae of swimming crab (*Portunus trituberculatus*) was demonstrated.

Key Words——*Lagenidium thermophilum*; mangrove crab; *Portunus trituberculatus*; *Scylla serrata*; seed production.

In seed production of crustaceans, gravid females are usually taken from the wild and reared in different tanks until zoeae hatch. Recently, seed production of mangrove crab, *Scylla serrata* Forsskål, has been attempted in Indonesia, but little success has been achieved because of non-septate, holocarpic fungal infections. Although many such infections occur worldwide, little etiological information on them is available. Thus far, both *L. scyllae* Bian et al. (Bian et al., 1979) and *Atkinsiella hamanaensis* Bian & Egusa (Bian and Egusa, 1980) have been reported from the eggs and larvae of cultivated mangrove crab. Unidentified species belonging to the genera *Lagenidium*, *Atkinsiella* and *Haliphthoros* have also been reported from mangrove crab (Kaji et al., 1991; Hamasaki and Hatai, 1993).

In 1993, a fungal infection occurred in the eggs and zoeae of mangrove crab produced at the Research Station for Coastal Aquaculture, Bali, Indonesia. The characteristic symptom of the disease was a change of color to white. Direct observation revealed many propagating thick non-septate hyphae inside the crab. Hyphae grew throughout the animals, and zoospore production was also observed. One species was obtained and classified as a member of *Lagenidium* (Oomycetes, Lagenidiales). This isolate is characterized by its whitish filamentous colony, unique discharge method (zoospore liberation occurred after the vesicles were separated from the discharge tubes), and rapid and thermotolerant growth. It differs from other *Lagenidium* species and is described here as a new species, *L. thermophilum*. This study included biological and physiological comparisons of the isolate and *L. callinectes* ATCC 24973. The isolate was also tested for pathogenicity on the zoeae of swimming crab, *Portunus trituberculatus* Miers.

Materials and Methods

Isolation and identification Eggs and zoeae of mangrove crab were taken from the plastic tank in which they were being raised and observed under a microscope to determine whether fungal elements were present. Each animal with a fungal infection was placed directly on a PYGS agar plate (peptone, 1.25 g; yeast-extract, 1.25 g; glucose, 3 g; and agar, 12 g; in seawater, 1 L) containing 500 μg/ml of streptomycin sulphate and ampicillin. The plates were kept at 25°C. Each fungus was purified by inoculation of the mycelium and maintained by monthly transfers onto a new PYGS agar plate at 25°C. A typical isolate (NJM 9338) was used in all experiments. For morphological observation, the isolate was cultured in PYGS broth at 25°C for two days. To observe zoospore production, mycelia were rinsed with sterilized artificial seawater (Aqua-Ocean, Japan Pet Drugs Co., Tokyo), then placed in a plastic Petri dish (8.25 cm in diam) containing about 30 ml of sterilized artificial seawater and incubated at 25°C. Zoospore suspension was inoculated into PYGS broth and incubated at 25°C to induce germination. The fungus was identified according to Sparrow (1960), Bian et al. (1979) and Karling (1981). *Lagenidium callinectes* ATCC 24973, a known parasite of various crustaceans, was used for biological and physiological comparisons.

Effect of temperature on growth The isolate and *L. cal-*

linectes ATCC 24973 were inoculated onto PYGS agar and incubated at 25°C for seven days and one month, respectively, to form a giant colony. PYGS agar discs with mycelia were cut out with a No. 2 cork borer (5.5 mm in diam) from the margin of the colonies and placed on the center of PYGS agar plates containing 25 ml of the medium. Mycelial growth was determined at eight different temperatures (10, 15, 20, 25, 30, 35, 40 and 45°C). The growth rate was checked daily for 10 days after inoculation by measuring the colony diameter. After the test, viability of the inocula was surveyed by incubation in PYGS broth at 25°C for 7 days.

Effect of NaCl or KCl concentration on growth To determine whether the isolate is a marine organism, it was inoculated onto plates of PYG agar (prepared like PYGS agar plates with the seawater replaced by distilled water) 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. Pre-culture, inoculation and growth determination were conducted as described above.

Experimental infection The pathogenicity of the isolate was estimated by using the zoea I stage of swimming crab. To reduce bacterial contaminants, the zoeae were dipped in sterilized seawater containing 200 μg/ml of oxytetracyclin. Thirty ml of sterilized seawater and 30 zoeae were put into plastic Petri dishes. Two PYGS agar discs cut with a No. 2 cork borer from the actively growing edge of a colony which had been incubated at 25°C for 5 days were also added. The zoeae were cultured with aeration at 25°C for 2 days. Infection rate was determined by direct microscopic examination of zoeae chosen at random every 24 h.

Results

Incidence Eggs and zoeae of mangrove crab were taken from the tank and observed under a microscope. Almost all of the dead eggs (about 10% of the total) had changed color to white and were filled with many aseptate hyphae. Zoospore production was also observed in more than 50% of the dead eggs. Hyphae were about 8-12 μm in width in vivo. Vesicles were produced on the orifices of the discharge tubes, which were shorter than those produced in vitro (Fig. 1). Zoospores were released all at once or in ones or twos. The latter liberation was usually observed on the smaller vesicles. Both hyphae and bacterial cells were found on the dead zoeae.

Lagenidium thermophilum K. Nakamura, M. Nakamura, Hatai & Zafran, sp. nov. Figs.2-7

Coloniae in agaro "peptone-yeast extract-glucose-seawater (PYGS)" expansae, albae, planae. Hyphae endobioticae, crassae, ramosae, aseptatae, 8-24(-40) μm diam. Sporangia per septatione hypharum formata. Tubulus ad sporangium singulus, 6-14×34-440 μm, apice dilatatus in vesiculam. Vesiculae sphaericae vel subsphaericae, 36-80 μm diam, ad maturitatem separatae ex tubulo ante liberationem zoosporae. Zoosporae pyriformes vel subglobosae, 10.3×14.4 μm, monoplanaticae, lateraliter biflagellatae. Cystosporae sphaericae, 6-16 μm diam. Reproductio sexualis ignota.

Ubiquinonum majus: Q-9.

Holotypus: NJM 9338, colonia exsiccata e cultura ex ova *Scylla serrata* Forsskål, Bali, in Indonesia, 2 Augustus 1993, a Zafran isolata et ea collectione cultureae in Universitate Veterinarii et Scientificae Animalis Nipponensis (NJM) conservata.

The present isolate, NJM 9338, on PYGS agar appeared as whitish, flat and filamentous colonies and attained a diameter of about 30 mm in one week at 25°C. Vegetative hyphae growing in PYGS broth were aseptate with numerous protoplasmic oil droplets, branched, and 8-24 μm in width (Fig. 2). Sometimes the ends of the hyphae were swollen to 40 μm thick. Zoospore formation occurred about 2 h after the mycelia were transferred into sterilized seawater, and this time-lapse tended to increase with repeated subculture. In the process of zoospore formation, protoplasmic masses with numerous oil droplets in the thallus moved into the vesicles formed at the orifices of the discharge tubes (Fig. 3). Each protoplasmic mass was connected with a protoplasmic thread. Masses of protoplasm occupied nearly all of the vesicles and divided into individual zoospores with two flagellae (Fig. 4). The envelopes of the vesicles were not apparent. Vesicles were spherical to subspherical, 36-80 μm in diam. Discharge tubes were straight or irregularly curved, 34-440 μm long and 6-14 μm in diam. Zoospore liberation occurred after the vesicles separated from the discharge tubes (Figs. 5A-C). The manner of zoospore discharge varied: either zoospores were all discharged simultaneously when the vesicles burst, or they were released in ones or twos through openings in the vesicles. Generally, the former was observed among the bigger vesicles and the latter among the smaller ones. Collapsed vesicles were not persistent. Zoospores were laterally biflagellate, pyriform to subglobose, 8-14 (av. 10.4)×10-16 (av. 13.3) μm, and monoplanetic (Fig. 6). They encysted after several minutes' to several hours' swimming. Encysted zoospores were generally spherical, and 6-16 (av. 10.5) μm in diam (Fig. 7). Germination was observed within 4 h in PYGS broth at 25°C after encystment. Occasionally, when discharge failed, zoospores swarmed within the discharge tubes and the vesicles, came to rest and germinated in situ.The fungus was holocarpic and endobiotic. Sexual reproduction was not present. The major ubiquinone type was coenzyme Q-9.

Effect of temperature on growth The results are summarized in Table 1. The isolate showed thermotolerant growth in the temperature range of 15-45°C, with an optimum of 30-40°C. It could not grow at 10°C, but the inoculum survived for more than 10 days. *Lagenidium callinectes* ATCC 24973 grew slowly in the temperature range of 10-30°C, with an optimum of 20-25°C. It did not tolerate 35°C.

Effect of NaCl or KCl concentration on growth As shown in Table 2, the isolate grew on PYG agar containing various concentrations of NaCl or KCl, and also on PYG agar without salts. Optimum growth was observed

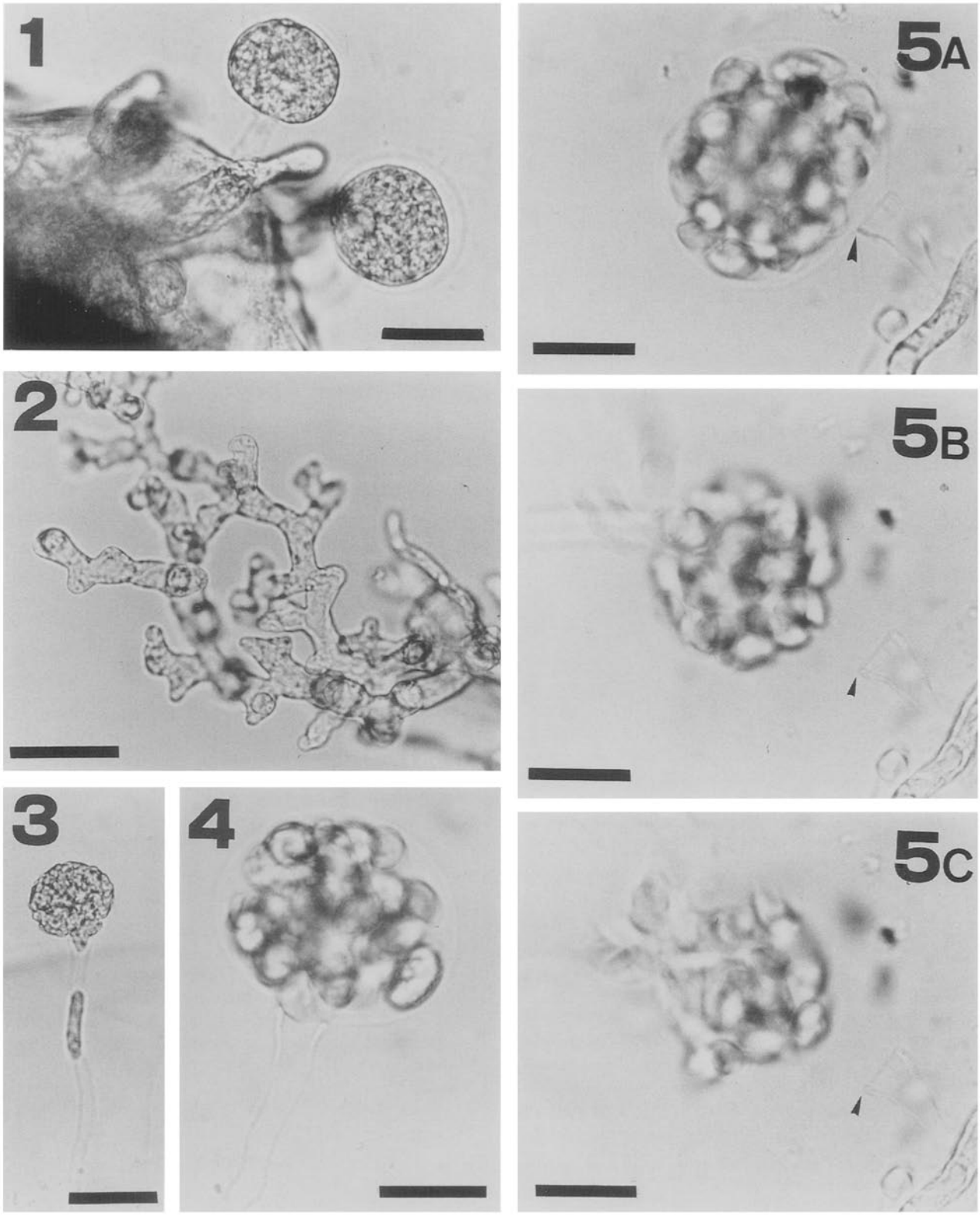
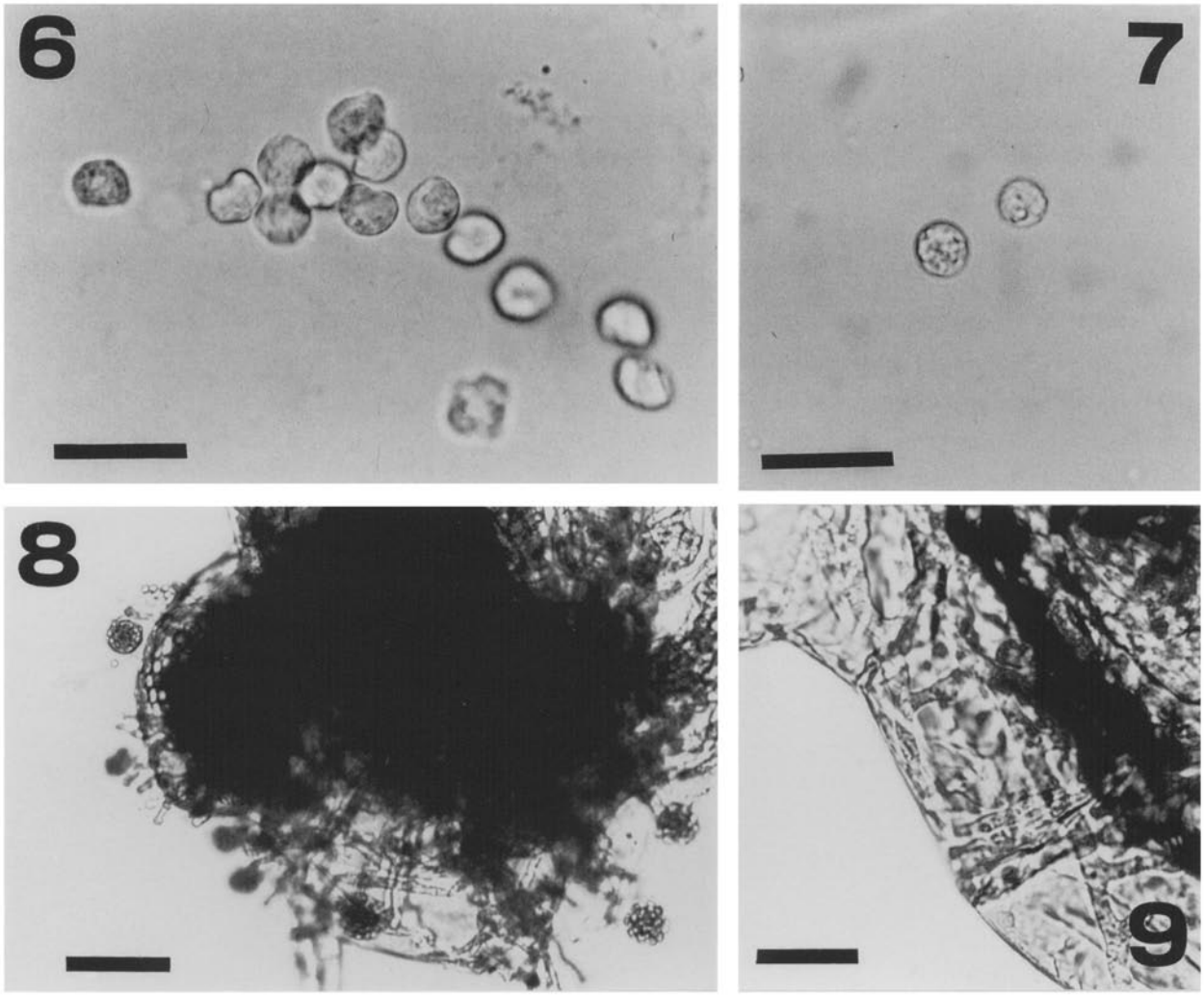


Fig. 1. A spontaneously infected zoea of mangrove crab, *Scylla serrata*. Vesicles formed on the orifices of the discharge tubes. Scale: 30 μm.

Figs. 2–5. Morphology of *Lagenidium thermophilum* NJM 9338.
2. Vegetative hyphae in PYGS broth. 3. Vesicle formation. 4. Mature vesicle. 5. Zoospore liberation. A. Mature vesicle. B. Vesicle separated from the discharge tube (arrow head). C. Zoospores released from a vesicle. Scales: 30 μm in Figs. 2, 4 and 5A–C; 50 μm in Fig. 3.



Figs. 6, 7. Morphology of *Lagenidium thermophilum* NJM 9338.
6. Swimming zoospores. 7. Encysted zoospores. Scales: 30 μm.
Figs. 8, 9. Zoeae of the swimming crab, *Portunus trituberculatus* experimentally infected with *Lagenidium thermophilum* NJM 9338. Scales: 100 μm in Fig. 8; 40 μm in Fig. 9.

on PYGS agar and PYG agar containing 1.0% (w/v) NaCl. On 5.0% (w/v) KCl medium, the fungus could not grow or survive. *Lagenidium callinectes* ATCC 24973 also showed optimum growth on PYGS agar and slight growth on PYG agar with 1.0-2.5% (w/v) NaCl. The inoculum on PYG agar containing 1.0% (w/v) KCl survived.

Experimental infection At 48 h after inoculation, hyphae growing inside the bodies of zoeae were observed by direct examination (Figs. 8, 9). Lesions with fungal infection were found on various parts of the bodies. Moreover, some larvae were not recognizable because their original shapes had been destroyed by the fungal elements and bacteria. Once bacteria invaded the zoeae, fungal growth was not observed. The cumulative mortality after 48 h was 20%. All zoeae in a control Petri dish survived except for a few victims of bacterial infection.

Table 1. Mycelial growth of *Lagenidium thermophilum* NJM 9338 and *L. callinectes* ATCC 24973 at different temperatures.

Strains	10°C	15°C	20°C	25°C	30°C	35°C	40°C	45°C
NJM 9338	0.0*(+)**	2.2	17.7	32.9	>40	>40	>40	1.2
ATCC 24973	0.8	2.6	4.8	4.6	0.4	0.0(−)	0.0(−)	0.0(−)

*Colony radius (mm/10 days).
**Viability of the inocula was checked by incubation in PYGS broth at 25 °C for 7 days.

Table 2. Mycelial growth of *Lagenidium thermophilum* NJM 9338 and *L. callinectes* ATCC 24973 on PYG agar with various concentrations of salts.

Strains	PYGS agar	PYG agar	% NaCl in PYG agar			% KCl in PYG agar		
			1.0	2.5	5.0	1.0	2.5	5.0
NJM 9338	39.3*	1.6	36.8	26.0	9.2	13.5	15.3	0.0(−)**
ATCC 24973	7.0	0.0(−)	1.3	3.5	0.0(−)	0.0(+)	0.0(−)	0.0(−)

*Colony radius (mm/10 days).
**Viability of the inocula was checked by incubation in PYGS broth at 25°C for 7 days.

Discussion

Based on the following morphological characteristics, isolate NJM 9338 was classified as a member of the genus *Lagenidium* (Oomycetes, Lagenidiales): the fungus was endobiotic and holocarpic, and laterally biflagellate zoospores were produced in the vesicles. Isolate NJM 9338 showed a unique discharge process (zoospore liberation occurred after the vesicles were separated from the discharge tubes), and it was proposed as a new species, *L. thermophilum*.

Three species of the genus *Lagenidium* have been reported from Decapoda (Clustacea). *Lagenidium callinectes* was first described by Couch (1942) on the eggs of blue crab, *Callinectes sapidus* Rathbun, and later discovered from the eggs and larvae of various crabs and shrimps (Crisp et al., 1989). *Lagenidium scyllae* is known as a parasite of the eggs and larvae of mangrove crab (Bian et al., 1979). *Lagenidium myophilum* Hatai & Lawhavinit has been reported from various stages of shrimps of the genus *Pandalus* (Hatai and Lawhavinit, 1988; Nakamura et al., 1994b). Isolate NJM 9338 was close to those of *L. callinectes* and *L. scyllae* in the dimensions of hyphae, vesicles, discharge tubes and zoospores (Bian et al., 1979; Crisp et al., 1989). *Lagenidium scyllae* was similar to *L. callinectes* except for its thermotolerant growth and the method of zoospore liberation (Couch, 1942; Bian et al., 1979). Zoospores of *L. scyllae* were released one by one from openings on the vesicles, or simultaneously by rapid deliquescence of the vesicles (Bian et al., 1979). However, we reported both modes of liberation in *L. callinectes* isolated from the egg of the crab *Portunus pelagicus* Linnaeus (Nakamura and Hatai, 1995). In *L. thermophilum*, these were also observed, but the vesicles left the discharge tubes before the zoospores were released. Zoospores of *L. thermophilum* and *L. scyllae* (Bian et al., 1979) were produced from about 2 h after transfer into seawater. In *L. myophilum*, zoospore production occurred about 12–24 h after transfer into seawater (Hatai and Lawhavinit, 1988; Nakamura et al., 1994b). However, these data should not be employed for classification because they change with repeated subculture. Incubation temperature also affects zoospore production (Nakamura et al., 1994a).

Thermotolerant growth was observed in *L. scyllae* (Bian et al., 1979), *L. callinectes* isolated from *P. pelagicus* (Nakamura and Hatai, 1995) and the present isolate. However, *L. myophilum* from *Pandalus* could grow at 5°C and could not grow at 30°C (Nakamura et al., 1994b). Growth temperature seems to be consistent with their hosts' habitat.

Isolate NJM 9338 differed from *L. callinectes* ATCC 24973 in its salt requirements. As *L. callinectes* ATCC 24973 grew on media containing seawater or 1-2.5 %(w/v) NaCl, it seems to be a marine fungus. However, as the present strain also grew on media without seawater, it is obvious that it is not exclusively marine, like *L. scyllae* and *L. myophilum* (Bian et al., 1979; Hatai and Lawhavinit, 1988; Nakamura et al., 1994b).

The eggs and larvae of mangrove crab were naturally infected by this fungus and had a high mortality rate. Zoospores were produced in one or two days in infected animals, allowing the infection to spread. However, the mortality rate among experimentally infected specimens was low. The difference in the mortality rates seems to depend on the host sensitivity and the concentration of the zoospore suspension. Also pathogenicity might decrease as a result of repeated subculture before the experiment.

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