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A marine oomycete *Atkinsiella panulirata* sp. nov. from philozoma of spiny lobster, *Panulirus japonicus*

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A marine oomycete fungus was isolated from philozoma of the spiny lobster, *Panulirus japonicus*. The fungus exhibited slow growth, occasionally submerged, with a creamy white, raised moist colony. Hyphae were stout, arranged in radiating pattern, irregularly branched, 10–22 μm diam, occasionally separated by cross walls into subthalli. Thalli occasionally consisted of swollen features. Sporangia formed from the subthalli had 1–3 wavy or partly coiled discharge tubes at the terminal or subterminal area. Zoospores were pyriform or reniform, biflagellate, isokont and diplanetic. Encysted spores germinated as a hairlike filament with a globular enlarged tip in sterilized synthetic seawater, and directly as stout initial hypha in PYGS broth. This fungus was identified as belonging to the genus *Atkinsiella* and was designated *Atkinsiella panulirata* sp. nov. The optimal temperature for the fungus was 25°C. Hyphal growth occurred in both sterilized synthetic seawater and NaCl in the range of 1.5–6.0% and 2.0–4.0%, respectively. The fungus could utilize all carbohydrates and derivatives tested as the carbon sources. From in vitro tests of five fungicides against the fungus malachite green was found to be the most effective.

Key Words——*Atkinsiella panulirata*; identification; nutritional study; physiology; spiny lobster.

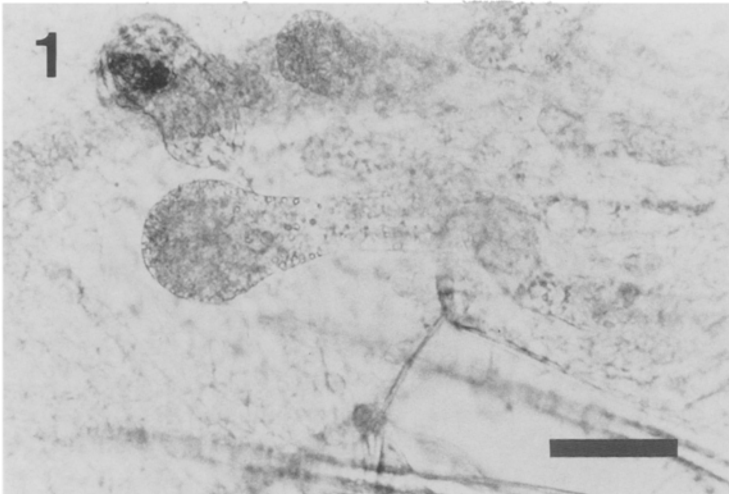
There have been no scientific reports of fungal infection in the spiny lobster *Panulirus japonicus* (von Siebold), unlike the American lobster *Homarus americanus* and the European lobster *Homarus gammarus*, which have been sporadically reported as having infestations by pathogenic marine oomycetes of the genera *Lagenidium* and *Haliphthoros* or imperfect fungi of the genus *Fusarium* (Fisher et al., 1975; Lightner and Fontaine, 1975; Nilson et al., 1976; Fisher et al., 1978). This study revealed the presence of an endobiotic fungus in the philozoma of spiny lobster cultured at the Japan Sea Farming Association, Minami Izu Station, Shizuoka Prefecture. This holocarpic oomycete was identified as belonging to the genus *Atkinsiella*, and was designated *A. panulirata* sp. nov.. The genus *Atkinsiella* has not hitherto been reported as an infection in cultured lobster. It has been found as a parasite of eggs and larval stages of various marine crustaceans. *A. dubia* (Atkins) Vishniac, was primarily found in pea-crab, *Pinnotherus pisum* Pennant, *Gonoplax angulata* (Pennant), etc. by Atkins (1954), and subsequently as a parasite in eggs of various crabs such as *Hyas* sp., *Oregonia* sp., etc. (Sparrow, 1973). Fuller et al. (1964) also isolated *A. dubia* from marine algae, *Chordaria* sp. and *Cladospora* sp. Martin (1977) reported a new member of this genus, designated *A. entomophaga*, infecting eggs of various midges and caddis flies. Another member of the genus *Atkinsiella* was isolated from mangrove crab, *Scylla serrata* (Forsskål) and identified as *A. hamanaensis* Bian & Egusa by Bian and Egusa (1980). Two new species belonging to this genus have been recently reported from rotifer, *Brachionus plicatilis* Muller, designated *A. parasitica* Nakamura & Hatai by Nakamura and Hatai (1994) and from abalone, *Haliotis sieboldii* Reeve, designated *A. awabi* Kitancharoen, Nakamura, Wada & Hatai by Kitancharoen et al. (1994).

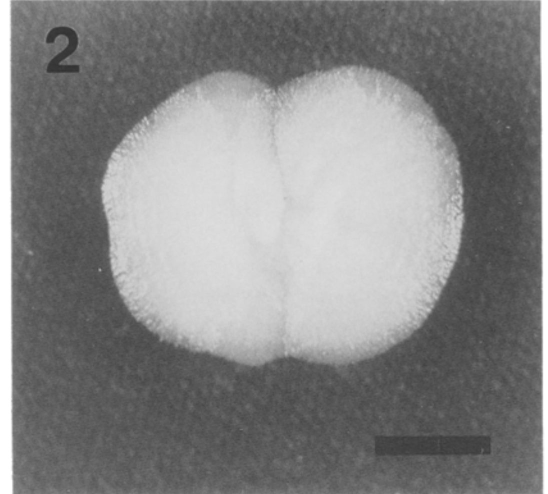
In this study, the fungus from philozoma of the spiny lobster was isolated into pure culture. Its morphological characteristics and asexual reproduction were elucidated and compared with those of other members of the genus *Atkinsiella*. Its physiological characteristics, nutritional profile and susceptibility to chemicals were also investigated.

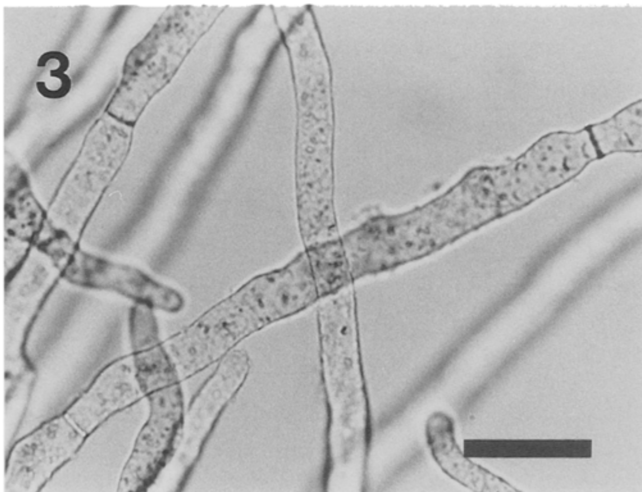
Materials and Methods

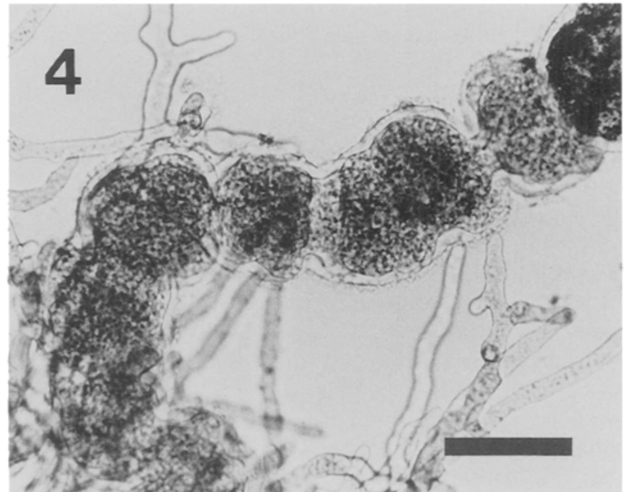
Identification Philozoma of the spiny lobster *Panulirus japonicus* infected with the fungus, were obtained from the Japan Sea Farming Association, Minami Izu Station, Shizuoka Prefecture, where the problem of philozoma loss occurred. Microscopic examination disclosed fungal hyphae in the body of philozoma (Fig. 1). The fungus was isolated by placing excised parts of philozoma with fungal hyphae onto peptone-yeast extract-glucose-seawater (PYGS) agar (composed of 1.25 g of peptone, 1.25 g of yeast extract, 3 g of glucose, 37.6 g of synthetic seawater in 1 l of distilled water) containing 500 μg/ml

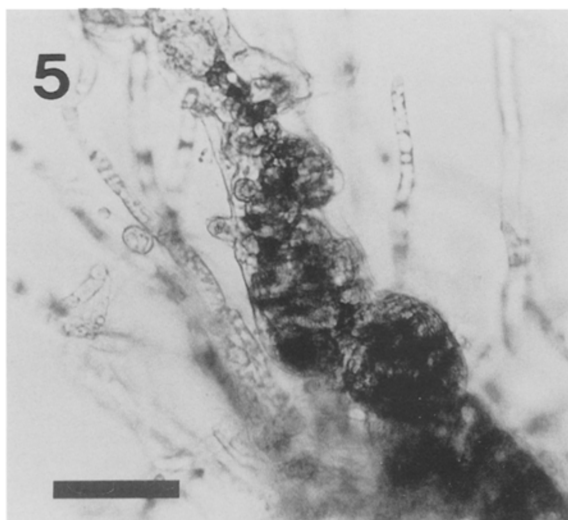
each of ampicillin and streptomycin sulfate to retard contamination by bacteria, and incubating them at 25°C. The appearance of fungal hyphae was observed at one-week intervals. The fungus thus isolated was propagated on PYGS agar without antibiotics and retained at 15°C as stock culture. Subcultures were made at two-month intervals and another stock culture was maintained at 25°C for use in all experiments. For morpho-

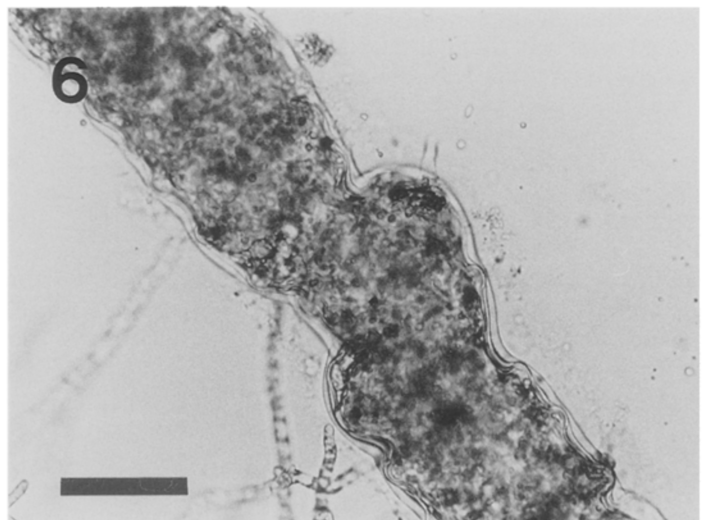












logical observation and identification, an active part of the colony was removed with a no. 2 cork borer (5.5 mm diam), put into 30 ml of PYGS broth, then incubated at 25°C for 3 days. Some mycelia were transferred into 30 ml of synthetic seawater and incubated at 25°C to induce zoospore formation. To promote spore germination, zoospores were placed into 30 ml of PYGS broth or synthetic seawater. The fungus was identified according to Sparrow (1960), Karling (1981), Martin (1977), Bian and Egusa (1980), Nakamura et al. (1994) and Kitancharoen et al. (1994).

Temperature range for growth A sample of actively growing colony on PYGS agar was removed with a no. 2 cork borer and placed on the center of 20-ml PYGS agar plates (8.25 cm diam), then incubated at eight different temperatures: 5, 10, 15, 20, 25, 30, 35 and 40°C. Mycelial growth was determined by measuring the colony diameter with vernier callipers every week for five weeks.

Salt requirements for growth PYG agar was prepared as PYGS agar but supplemented with synthetic seawater or NaCl at various concentrations: 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0%. PYG agar without either compound was also examined. An active part of the fungal colony was removed with a no. 2 cork borer and placed onto the center of 20 ml of each medium. Mycelial growth was verified after incubation at 25°C for four weeks.

Nutritional study Samples of three-day cultures on PYS agar (formula as PYGS, with the usual base replaced by yeast extract nitrogen base) were removed with a no. 2 cork borer and inoculated onto 20 ml of PYS agar containing 0.5% (w/v) of individual carbohydrates and derivatives as described in Table 2. The colony diameters were measured after incubation at 25°C for 14 days.

Effects of five potential fungicides Potential fungicides were tested against both hyphae and zoospores in PYGS broth using the two-fold dilution method, with ranges as follows: formalin 3.2-100 ppm, malachite green 0.0025-2 μg/ml, tachigaren 8-1000 μg/ml, metaraxyl 8-1000 μg/ml and polyphenone 8-1000 μg/ml. Two sets of each chemical were prepared. Zoospores were inoculated into one set at approximately 2×10^2 spores/ml, and the free small three-day-old colonies were washed twice with synthetic seawater and placed into another set. All were incubated at 25°C for 7 days. Germination of zoospores and viability of hyphae were investigated under a light microscope. For concentrations at which hyphal growth could not be clearly distinguished, the colony was recultured on PYGS agar at 25°C for 7 days to verify the viability.

Results

Identification The growth of this fungus was slow, occasionally submerged, with creamy white, raised moist colonies with smooth margins on PYGS agar at 25°C (Fig. 2). The three-day-old culture of the fungus in PYGS broth at 25°C appeared as small dense globular colonies about 0.5-2.5 mm diam. Thalli were stout, radially arranged, irregularly branched, diameter usually 10 to 22 μm. Cross walls were regularly observed (Figs. 3, 12A). Thalli swollen up to 64 μm were occasionally found. The appearance of the protoplasm varied with age, at first shiny tubular granules, later often with round vacuoles. Gemmae spontaneously occurred in three-day-old culture in PYGS broth at 25°C. They were characterized by saccate-lobed-chained, thick-walled, dense cytoplasmic and non-vacuolate features, widths of 179-270 μm and various lengths up to 18.0 mm. (Figs. 4, 12B). Gemmae not only developed new thalli on PYGS agar or in PYGS broth (Fig. 5), but also displayed as zoosporangia in synthetic seawater (Fig. 6).

After colonies had been transferred into synthetic seawater, the thalli became vacuolate and occupied the position of rungs on a ladder (Fig. 7). Sporangia with 1-3 discharge tubes originating at terminal or subterminal sites developed within the thallus by formation of the cross walls. The discharge tubes were slightly wavy or partly coiled structures, usually of 4-6 μm in diam at the orifice and 5-9 μm at the base, and 44-456 μm in length (Figs. 12C, D, E). Zoospores formed and cleaved within the sporangium and then in the discharge tube. After moving slightly for a short time in the sporangium (Fig. 8), zoospores squeezed in an amoeboid fashion through the discharge tube, emerging as free-swimming spores. They were laterally biflagellate, isokont, diplanetic, pyriform or reniform of 7-10 × 4-5 μm in size (Fig. 12F). The step of zoospore discharge took approximately 20-30 h after the transfer into synthetic seawater. Occasionally, some zoospores remained and encysted within the sporangium (Fig. 9). After a swarming period of about 5-24 h, the zoospores encysted. The encysted spores were spherical or subglobose, usually about 5-7 μm in diam (Figs. 10, 12G). Cysts left empty after the secondary zoospores had swum away were frequently found. Germination of the encysted spores commenced after about 15-24 h both in synthetic seawater and PYGS broth. In synthetic seawater the encysted spores germinated with the production of a hairlike filament, 14-253 μm long. The tip of the filament enlarged and germinated a globular incipient hyphal bud with a thin-walled empty spore at the end that was originally the encysted spore (Figs. 11, 12H). In PYGS broth, the encysted spores exhibited direct germination with stout initial thalli.

Fig. 1. Mycelium of *A. panulirata* sp. nov. penetrating the appendage of philozoma of spiny lobster, *P. japonicus*. (Scale bar = 50 μm.)
Fig. 2. *A. panulirata* sp. nov. colony after growth on PYGS agar at 25°C for 14 days. (Scale bar = 5 mm.)
Fig. 3. Three-day hyphae in PYGS broth at 25°C with cross walls. (Scale bar = 50 μm.)
Fig. 4. Gemmae demonstrated in PYGS broth after incubation at 25°C for three days. (Scale bar = 100 μm.)
Fig. 5. Gemmae germinating by slender hyphae in PYGS broth. (Scale bar = 100 μm.)
Fig. 6. Gemmae functioning as zoosporangia in synthetic seawater. (Scale bar = 100 μm.)

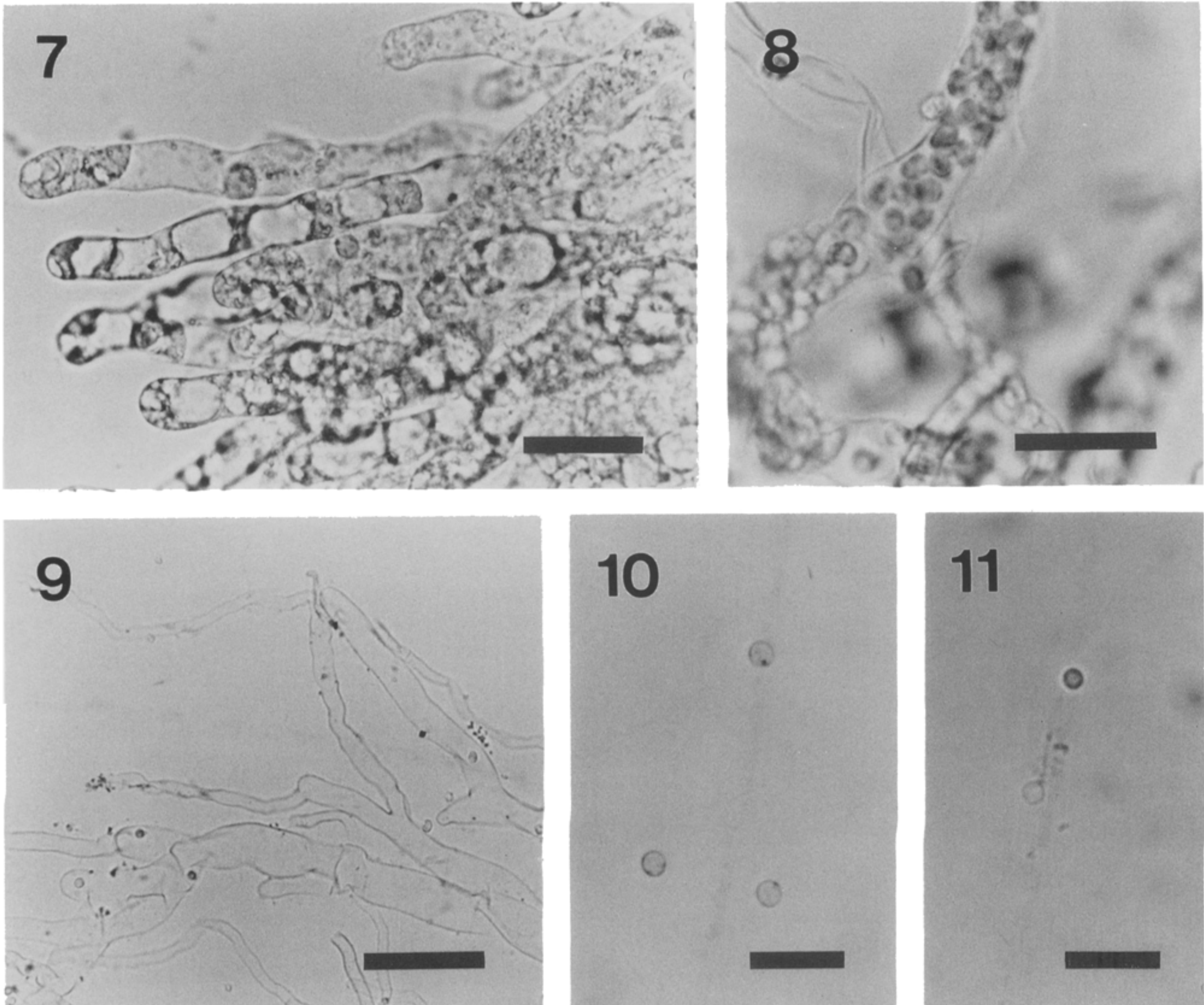


Fig. 7. Hyphae became vacuolate after transfer into synthetic seawater. (Scale bar=50 μm.)
Fig. 8. Primary zoospores formed within zoosporangium. (Scale bar=30 μm.)
Fig. 9. Some zoospores remained and encysted within the zoosporangia. (Scale bar=100 μm.)
Fig. 10. Encysted spores in synthetic seawater. (Scale bar=20 μm.)
Fig. 11. Spore germinating as a hairlike filament with globular incipient hypha. (Scale bar=20 μm.)

Type material—The present fungus has been preserved in the culture collection of Nippon Veterinary and Animal Science University as NJM 9439. The type material was collected from philozoma of the spiny lobster *Panulirus japonicus* (von Siebold) at the Japan Sea Farming Association, Minami Izu Station, Shizuoka Prefecture, Japan.

Diagnosis—Parasite in philozoma of spiny lobster. Thallus endobiotic, holocarpic, stout, branched with cross walls, 10-22 μm diam, occasionally swollen up to 64 μm diam. Thallus converted directly into a zoosporangium with 1-3 wavy or partly coiled discharge tubes of 44-456 μm in length. Zoospores pyriform or reniform, diplanetic, biflagellate, 7-10×4-5 μm in size. Encysted spores spherical or subglobose, 5-7 μm diam. Germinating to form a filament, 14-253 μm in length. Sexual reproduction not observed.

Atkinsiella panulirata Kitancharoen et Hatai sp. nov. Figs. 1-12

In philozomate *Panuliri japonici* parasitica. Thallus endobioticus, hololcarpicus, crassus, ramosus et septatus, 10-22 μm diam, aliquando ad 64 μm diam. Zoosporangium thallum conforme, ad vel prope apicem tubulos emittenes singulos vel tres formans, 44-456 μm. Zoosporae pyriformes vel reniformes, diplaneticae, biflagellatae, 7-10×4-5 μm. Cystosporae sphaericae vel subsphaericae, 5-7 μm diam, in fibrarum 14-253 μm longarum germinans. Reproductio sexualis non visa.

Holotypus: NJM 9439, colonia exsiccata e cultura ex philozomatibus *Panuliri japonici* (Sielbold), Japan Sea Farming Association, Minami Izu Station, Shizuoka Prefecture, in Japania, Aug. 1994. a Kitancharoen isolata

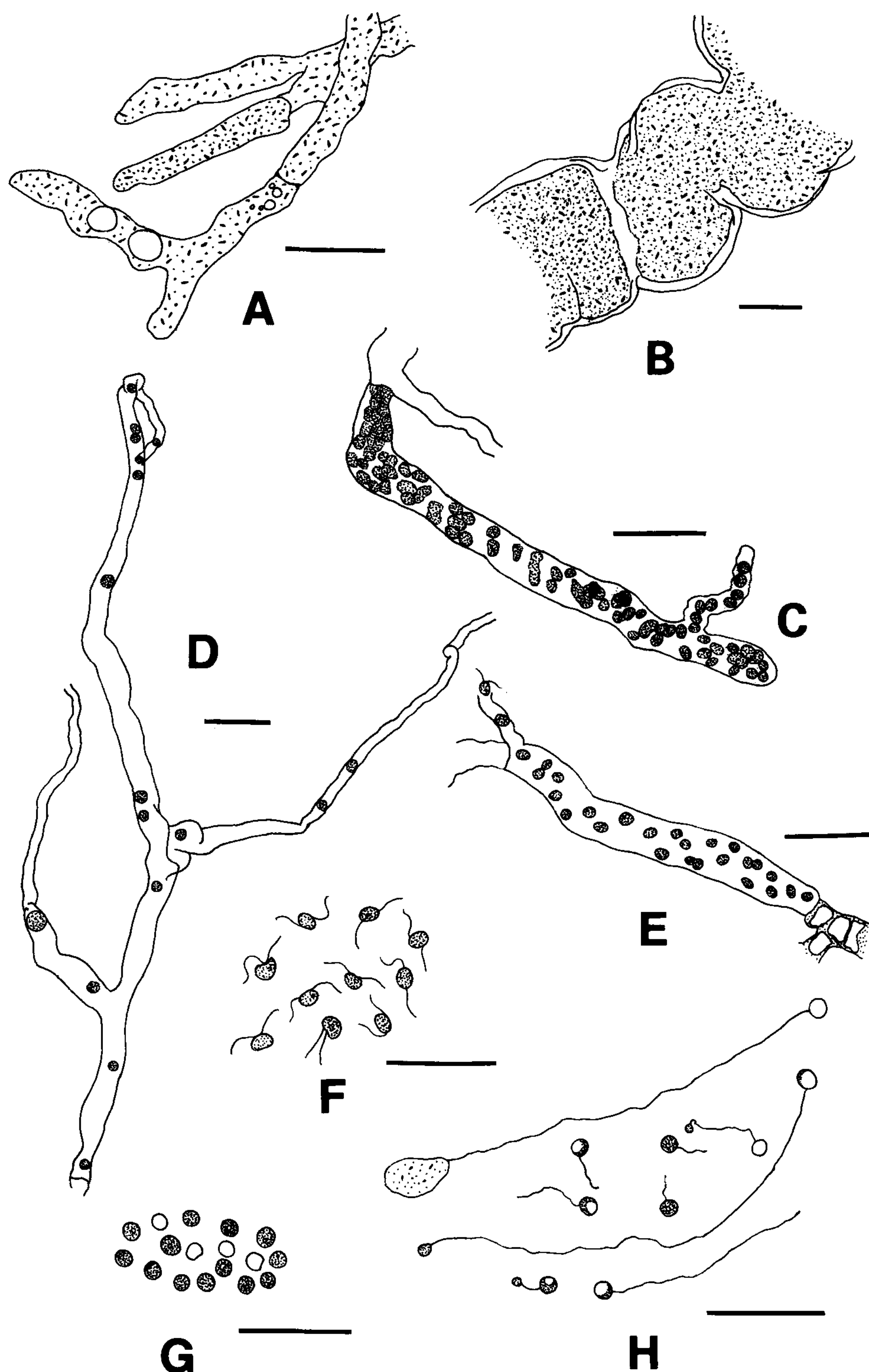


Fig. 12. *Atkinsiella panulirata* sp. nov. isolated from philozoma of spiny lobster. A. Vegetative hyphae with cross wall; B. Gemmae; C. Young sporangium with primary zoospores and differentiating protoplasm; D. Sporangium with three discharge tubes, some zoospores encysted inside; E. Primary zoospores liberated through the discharge tube; F. Swimming zoospore; G. Encysted spores and empty cysts from which secondary zoospores had squeezed out; H. spore germination. (Scale bars = 50 μm.)

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Temperature range for growth The consistent growth of *A. panulirata* sp. nov. appeared at 15–30°C on PYGS agar. At 15°C, the hyphal growth revealed after 7 days post-inoculation. The optimal temperature for growth of

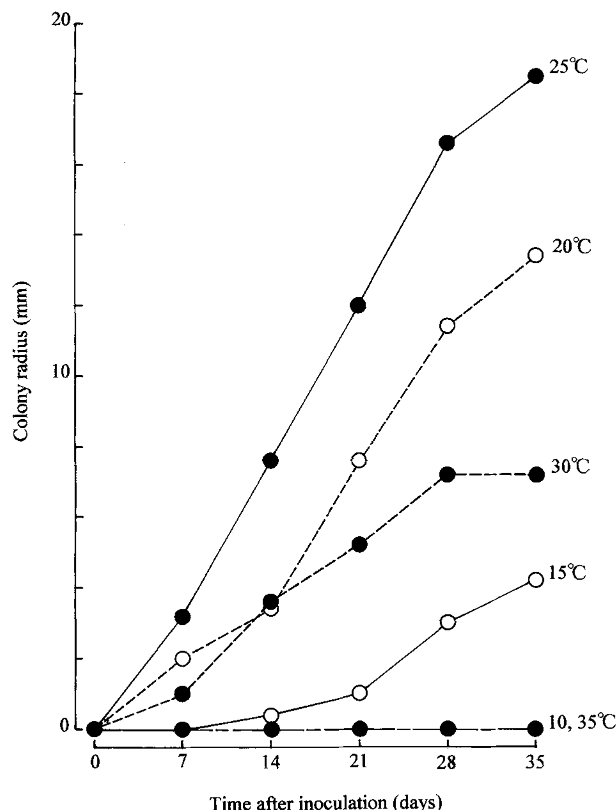

Fig. 13. Effects of various temperatures on growth of *A. panulirata*.

this isolate was 25°C. No growth appeared at 5, 10 and 35°C (Fig. 13).

Salt requirements for growth *A. panulirata* sp. nov. could grow in either synthetic seawater or NaCl, but not in media without these compounds. The effects on growth of synthetic seawater or NaCl are shown in Table 1. Hyphal growth was observed in 1.5-6.0% synthetic seawater and 2.0-4.0% NaCl. Growth in 1.5% synthetic seawater and 2.0% NaCl was grossly abnormal, with part of the colony forming folds. Growth was optimum in the medium containing 3.0% synthetic seawater or 2.5% NaCl. The concentrations where hyphal growth could not be observed were mortal except for 5% NaCl, where hyphae were still viable.

Table 1. Effects of various concentrations of synthetic seawater and NaCl on growth of *A. panulirata* sp. nov.

Concentration of salts (%)	Growth potential in synthetic seawater	Growth potential in NaCl
0	−	−
0.5	−	−
1	−	−
1.5	++	−
2	++++	++
2.5	++++	++
3	++++	++
3.5	+++	+
4	+++	+
5	++	−*
6	+	−

* Growth occurred after reculture on PYGS agar.

Table 2. Utilization of carbohydrates and derivatives by *A. panulirata* sp. nov.

Carbohydrates and derivatives	Utilization activity*
Monosaccharides	
Arabinose	++
Galactose	+
Glucose	+++
Mannose	++
Rhamnose	++
Sorbose	++
Xylose	++
Disaccharides	
Lactose	+
Maltose	+++
Trehalose	++
Tri-Polysaccharides	
Dextrin	+++
Melezitose	+++
Raffinose	++
Salicin	+
Starch	+++
Sugar alcohols	
Ducitol	++
Erythriol	++
Inocitol	++
Mannitol	++
Sorbitol	+++
Xylitol	++

* Evaluated by colony radius after incubation at 25°C for 14 days.
+ Colony diameter < 5.0 mm.
++ 5.0 mm. ≦ Colony diameter > 10.0 mm.
+++ Colony diameter ≧ 10.0 mm.

Nutritional study Carbohydrates and derivatives capable of serving as sole carbon sources of *A. panulirata* sp. nov. are presented in Table 2. The isolate was able to utilize all carbohydrates and derivatives tested, including

Table 3. Effects of five chemicals on hyphal growth and zoospore germination of *A. panulirata* sp. nov.

Chemical	Effective dose against hyphae (μg or μl/ml)		Effective dose against zoospore germination (μg or μl/ml)
	Inhibitory	Fungicidal	
Formalin	100	≧100	50
Malachite green oxalate	0.5	1	0.125
Polyphenone	500	1000	62.5
Tachigaren	500	1000	250
Metaraxyl	1000	≧1000	500

salicin, galactose and lactose. Glucose and carbohydrates composed of glucose molecules, such as maltose, starch and dextrin, provided good support as carbon sources.

Effects of five potential fungicides The results of the in vitro test are summarized in Table 3. Malachite green was the most effective chemical inhibiting hyphal growth at 0.5 μg/ml, being fungicidal at 1.0 μg/ml, and suppressing zoospore germination at 0.125 μg/ml. Formalin was effective against hyphae at 100 μl/ml, suppressing growth during the 96-h observation period, but hyphae still lived and grew afterwards. Against zoospore germination, formalin was the second most effective chemical at a concentration of 50 μg/ml, while polyphenone was effective at 62.5 μl/ml, and tachigaren and metaraxyl were effective at 250 and 500 μg/ml, respectively.

Discussion

The present isolate, NJM 9354, is characterized as endobiotic; holocarpic; thallus with cross walls; zoosporangia converted from thalli; forming 1-3 discharge tubes during zoosporangiosis; diplanetic, biflagellate zoospores; germination by a hairlike filament. On the basis of these characteristics, the fungus has been included in the genus *Atkinsiella*.

This isolate obviously differs from *A. dubia* in the characteristics of the thallus, which in *A. dubia* are saccate-lobed, bulbous, or broadly tubular with tufts of rhizoids that are delimited from the thallus by cross walls (Sparrow and Gotelli, 1969). These characteristics were not exhibited in the isolate. The pigmentation reported in *A. hamanaensis* (Bian and Egusa, 1994) did not occur in *A. panulirata* sp. nov. There are fundamental differences between the present fungus and *A. awabi* in the colony characteristics on PYGS agar and in PYGS broth, *A. panulirata* sp. nov. formed a raised colony with smooth margin on PYGS agar and dense globular colonies in PYGS broth, whereas *A. awabi* exhibited a flattened colony with rhizoid margin on PYGS agar and small free irregular colonies in PYGS broth (Kitancharoen et al., 1994). Zoospores of *A. panulirata* sp. nov. occasionally encysted within the zoosporangia, similar to most species in the genus *Atkinsiella*, except for *A. entomophaga*, in which primary zoospores are typically released outside of the zoosporangium (Martin, 1977). Zoospore discharge follows the same processes in *A. entomophaga, A. hamanaensis*, and also *Haliphthoros milfordensis* Vishniac, the relative genus (Vishniac, 1958). *A. dubia* exhibits the pyriform, 8.2×6 μm primary zoospores and reniform, 8.7×4.8 μm secondary zoospores, and 6.8-7.4 μm encysted spores (Fuller et al., 1964), similar to the present fungus. Zoospores of *A. entomophaga* were described by Martin (1977), to measure about 11.6×6.9 μm, after a period of motility forming encysted spores of 10.5 μm diam, larger than the present fungus. Spore germination of *A. entomophaga* revealed a swelling along the germ tube. Although many characteristics of the present fungus, such as spore size and shape, and diplanetism, are identical to *A. hamanaensis*, the pigmentation reported in *A. hamanaensis* (Bian and Egusa, 1994) did not occur in the fungus. The present fungus approaches *A. parasitica* in some characteristics, but the former exhibited diplanetism and unbranched discharge tubes, whereas *A. parasitica* demonstrated monoplanetism and branched discharge tubes (Nakamura et al., 1994). The length of the discharge tubes and germ tubes are not thought to be crucial enough to distinguish between species, because variability might be caused by culture conditions.

The fungus grew at 15-30°C on PYGS agar, similar to *A. hamanaensis* (Bian and Egusa, 1980) and *A. parasitica* (Nakamura et al., 1994). However its optimum temperature of 25°C was lower than that of *A. hamanaensis* (Bian and Egusa, 1980) but higher than those of *A. parasitica* (Nakamura et al., 1994) and *A. awabi* (Kitancharoen et al., 1994).

This isolate could grow in medium containing NaCl, similar to *A. hamanaensis* but different from *A. parasitica*, which was capable of growth only in medium with sterilized synthetic seawater (Nakamura et al., 1994). This result does not conclusively erect this fungus as an obligatory marine fungus, but it can be considered to be euryhaline.

The present fungus could utilize all carbohydrates and derivatives tested as carbon sources, even salicin, galactose and lactose, albeit poorly. It might have enzymes that can utilize glucose and break the bonds between glucose molecules, as it showed rapid growth on glucose and carbohydrates composed of glucose molecules. Yuasa and Hatai (1994) reported that sorbose inhibited the growth of *Saprolegnia* spp., a freshwater oomycete; but it showed no effect on the growth of this fungus. The nutrition study might provide criteria to identify the fungal level in taxa. This hypothesis needs more research to elucidate the potential.

Malachite green, formalin and polyphenone were found to be effective against *A. panulirata* sp. nov., similar to *A. awabi*, of which the latter was more sensitive. Malachite green has been reported as an effective chemical to control fungal infestation. In the lobster culture system, malachite green was also recommended for use in fungal infection treatment (Fisher, 1975; Fisher et al., 1976; Abrahams and Brown, 1977). Malachite green has been endorsed for fungicidal activity against *Lagenidium* spp. and *Haliphthoros philippinensis* Hatai, Bian, Baticados & Egusa (Armstrong et al., 1976; Lio-Po et al., 1982, 1985). However, malachite green has remained unregistered because of its carcinogenic and terratogenic properties (Bailey, 1983). Formalin is extensively recommended for use in fish and shrimp culture systems.

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