Mycoscience 35: 265–270, 1994

Atkinsiella awabi sp. nov. isolated from stocked abalone, *Haliotis sieboldii*

Nilubol Kitancharoen, Kazuyo Nakamura, Shinpei Wada and Kishio Hatai

Division of Fish Diseases, Nippon Veterinary and Animal Science University, 1-7-1 Kyonan-cho, Musashino, Tokyo 180, Japan

Accepted for publication 29 July 1994

A fungal disease in the abalone, *Haliotis sieboldii*, stocked in Yamaguchi Prefecture, Japan, showed external signs of infection of tubercle-like swelling on the mantle and melanized lesions on the peduncle. The fungus responsible was isolated by inoculating materials taken from the lesions onto PYGS agar with streptomycin sulphate and ampicillin, and incubation at 20°C. For morphological observation and spore formation study, the fungus was transferred respectively into PYGS broth and sterilized artificial seawater and incubated at 20°C. Resulting, hyphae were stout, irregular, branched, 16–140 μm diam, sporadically consisting of dense cytoplasmic swollen hyphae. Sporangia were formed through the formation of septa and lateral or terminal discharge tubes which were wavy or coiled. Zoospores were pyriform, biflagellate and diplanetic. The encysted spore generally developed a hairlike filament with globular enlarged tip in PYGS broth. Direct germination without filament formation also occurred occasionally. This fungus was identified as belonging to the genus *Atkinsiella*, and was designated *Atkinsiella awabi* sp. nov. The fungus was exclusively a marine fungus and grew best in shrimp extract medium at 20°C. Five chemicals were tested for their effects against fungal zoospores.

Key Words——abalone; *Atkinsiella awabi*; fungal disease; morphological characteristics and histopathology.

There have been few reports of fungal infection in abalone. In Japan, infection of the abalone *Haliotis sieboldii* Reeve by the parasitic fungus *Haliphthoros milfordensis* Vishniac has been reported (Hatai, 1982). This study revealed another parasitic fungus of abalone, which were stocked in Yamaguchi Prefecture, Japan. The fungus was identified as a new species belonging to the genus *Atkinsiella*, holocarpic biflagellate fungus, namely *A. awabi* sp. nov. Fungi of the genus *Atkinsiella* have been found as parasites on many marine animals' eggs and young stages. *Atkinsiella dubia* (Atkins) Vishniac was primarily found in pea-crab, (*Pinnotherus pisum* Pennant, *Gonoplax rhomboides*, etc.) by Atkins (1954), and but it has been proved to be parasitic in eggs of various crabs, including *Hyas* sp. and *Oregonia* sp. (Sparrow, 1973). Fuller et al. (1964) also isolated *A. dubia* from marine algae (*Chordaria* sp. and *Cladophora* sp.). Martin (1977) reported a holocarpic parasitic fungus, *A. entomophaga* Martin in eggs of various midges and caddis flies. Another species of this genus was isolated from mangrove crab, *Scylla serrata* (Forsskål) and identified as *A. hamanaensis* Bian & Egusa by Bian and Egusa (1980).

In this study, an attempt was made to isolate the fungus in pure culture. Morphological characteristics, and asexual reproduction were observed and compared with those of other members of the genus *Atkinsiella*. Growth studies were also made on its optimum temperature, media, mineral requirements and effect of chemicals.

Materials and Methods

Isolation and identification Abalones, *Haliotis sieboldii*, with fungal infection were obtained from Yamaguchi Prefecture. The clinical signs were tubercle-like swelling on the mantle and melanized lesions on the peduncle (Fig. 1). Many fungal hyphae were discovered in the lesions, which were excised and subjected to an initial light microscopic examination (Fig. 2). Isolation of this fungus into culture was accomplished by placing small pieces of lesion material with hyphae onto peptone-yeast extract-glucose-seawater (PYGS) agar (composed of 1.25 g of Bacto peptone, 1.25 g of Bacto yeast extract, 3.0 g of glucose, 37.6 g of artificial seawater and 12.0 g of Bacto agar in 1 l of distilled water) containing a small amount of streptomycin sulfate and ampicillin to retard bacterial contamination. Cultures were incubated at 20°C for 2 weeks. Then a small block of agar with the fungal mycelium was cut out and propagated on PYGS agar plate to make a pure culture and used for all experiments. For morphological observation and identification, an actively growing part of the colony was cut with a no. 2 cork borer (5.5 mm diam) put into 30 ml of PYGS broth, then incubated at 15°C for 3 days for mycelial observation. Some mycelium was transferred into 30 ml of steriled artificial seawater and incubated at 15°C for 2

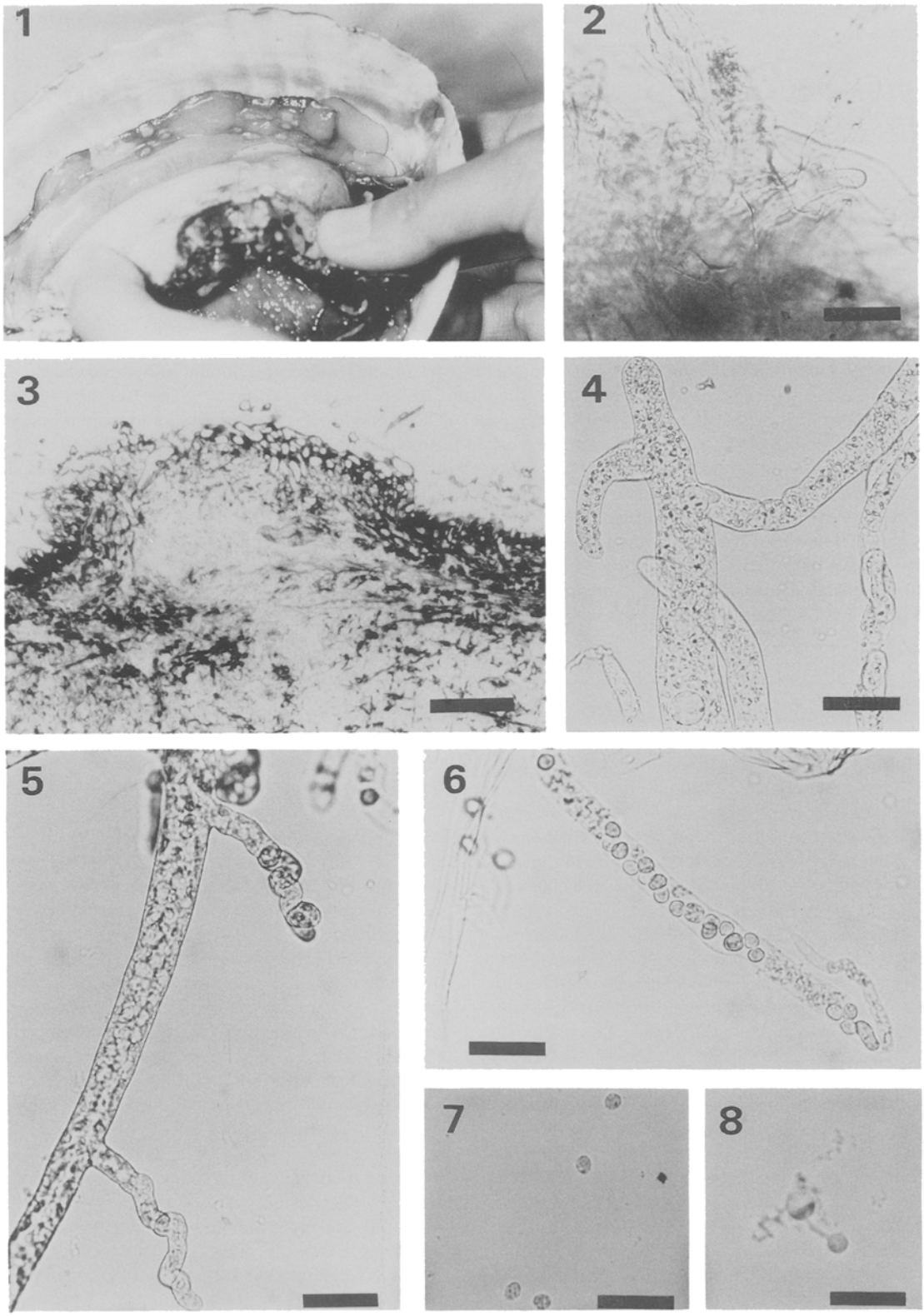

Fig. 1. External appearance of an abalone infected with the fungus, *A. awabi* sp. nov.. Note tubercle-like swelling on the mantle and melanized lesions on the peduncle.

Fig. 2. Hyphae of *A. awabi* sp. nov. taken from the lesions of abalone. (Scale bar=40 μm.)

Fig. 3. Histopathological section through a lesion of an infected abalone illustrating many fungal hyphae (Grocott-Giemsa). (Scale bar=600 μm.)

Fig. 4. Vegetative hyphae grown in PYGS broth after three days of incubation at 15°C. (Scale bar=40 μm.)

days to induce zoospore formation. To promote spore germination, zoospores were placed into 30 ml of PYGS broth. The fungus was identified according to Sparrow (1960), Karling (1981), Atkins (1954), Martin (1977) and Bian and Egusa (1980).

Histopathology After gross observation, necropsy was routinely performed. The tubercle-like swellings on the mantle and melanized lesions on the peduncle were cut and fixed in 10% phosphate-buffered formalin solution, embedded in paraffin, and sectioned at 4-5 μm. Sections were stained with methanamine-silver nitrate-Grocott's variation with Giemsa (Grocott-Giemsa) and periodic acid Schiff (PAS) reaction.

Effect of various temperatures on growth PYGS agar discs were cut from an actively growing part of the fungal colony 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. The mycelial growth was determined by measuring the colony diameter with vernier callipers every 2 days for 16 days.

Effect of culture media on growth Three kinds of media were used: peptone-yeast extract-glucose-seawater agar (PYGS agar; formula as described above); corn meal-seawater agar (CMS agar; 17.0 g of corn meal agar, 37.6 g of artificial seawater and 1 l of distilled water); and shrimp extract agar (SE agar; 100 g of crushed Penaeid shrimp meat, 37.6 g of artificial seawater, 12.0 g of agar and 1 l of distilled water). Each medium was inoculated as described for the temperature test. All plates were incubated at 15°C for 10 days, and colony diameter was measured every 2 days during the incubation.

Mineral requirement for growth PYG agar was prepared as PYGS agar but with NaCl or KCl added instead of artificial seawater at the concentrations listed in Table 1. PYGS agar was also prepared. Petri dishes containing 20 ml of each medium were inoculated with a piece of agar with the actively growing fungus cut by No. 2 cork borer and kept at 15°C. Mycelial growth was observed 14 days after inoculation.

Effects of five chemicals on zoospores Zoospore formation was conducted by transferring a small block of agar with active mycelium into 30 ml of PYGS broth and incubation at 15°C for 3 days. Hyphae were washed two times with sterilzed artificial seawater, then transferred into 30 ml of sterilized artificial seawater and incubated at 15°C for 2 days. The number of zoospores was calculated to be 3×10^4 spores/ml.

The effects of five chemicals on zoospores were examined: formalin (0.25-125 ppm), malachite green (0.006-31.3 ppm), polyphenon-100 (2-1000 ppm), hydrogen peroxide (6.25-3000 ppm) and povidone iodine (2-1000 ppm). Portions of 200 μl of each concentration of each chemical, 1000 μl of double concentrated PYGS broth and 800 μl of zoospore suspension were serially put into 24-Multiwell Tissue Culture Plate (Becton-Dickinson). All plates were incubated at 15°C for seven days for observation of mycelial growth.

Results

Isolation and identification Morphological characteristics of the fungus isolated from infected abalone, *Haliotis sieboldii*, are as follows:

Atkinsiella awabi Kitancharoen, Nakamura, Wada et Hatai sp. nov. Figs. 1-10

Hyphae endobioticae, holocarpice, crassae, irregulares, ramosae, aseptatae, vacuolatae, guttulis olei numersis praeditae, 16-41 μm diam, aliquando ad 140 μm diam. Sporangium thallum conforme, e latere vel apice tubulos emittanres singulos vel duos formans, 5-8 × 70-175 μm. Zoosporae pyriformes, 4-8 × 7-12 μm, biflagellatae, diplaneticae; cystosporae 8 μm in diam, in fibrarum 62-295 μm longarum germinantes. Reproductio sexualis non visa. Holotypus: NJM 9354, colonia exsiccata e cultura ex corpis *Haliotidis siboldii* Reeve, Yamaguchi Pref. in Japania, 21 Sep. 1993, a N. Kitancharoen isolata et ea collectione cultureae in Universitate Veterinarii et Scientificae Animalis Nipponensis (NJM) conservata.

Colony on PYGS agar growing moderately with an irregular rhizoid margin. Fungal hyphae stout, irregular, 16-41 μm in diam, branched and non-septate. Hyphae contain many vacuoles and shiny granules (Figs. 4, 9A, B). Dense cytoplasmic swollen hyphae generally up to 140 μm. In sterilized seawater, hyphae growing from the PYGS agar block perpetually formed irregular clusters.

Thalli were converted into sporangia of the same size and shape with the formation of septa. Sporangia had 1-2 terminal and/or lateral discharge tubes. The discharge tubes were wavy or coiled, measuring 5-8 × 70-175 μm (Fig. 5). Zoospore formation was initiated by transferring the vegetative hyphae into sterilized seawater. Zoospores were formed and cleaved within the sporangium. After moving for a short time in the sporangium, zoospores were released from discharge tubes and swam freely (Figs. 6, 9C, D). This step took place about 20 h after the transfer into sterilized artificial seawater. Occasionally, some zoospores remained and became encysted within the sporangia (Fig. 9E). Zoospores were pyriform in shape, 4-8 × 7-12 μm, biflagellate, isokont and diplanetic (Fig. 9F). Encystment appeared about 3 h after the transfer into PYGS broth. Encysted spores were globular, about 8 μm in size (Figs. 7, 9G).

Germination of encysted zoospores commenced about 9 h after encystment. A hair-like filament 62-295 μm, average 140 μm, in length developed. The tip of the filament enlarged and germinated a globular hyphal bud with a thin-walled empty spore at the end originally having the encysted spore (Figs. 8, 9H). However, a

Fig. 5. Young zoosporangium with two lateral wavy or coiled discharge tubes. (Scale bar = 40 μm.)
Fig. 6. Zoospores formed within zoosporangium. (Scale bar = 40 μm.)
Fig. 7. Zoospores encysted 3 h after transfer into artificial seawater. (Scale bar = 40 μm.)
Fig. 8. Spore germination by development of a hair-like filament with globular enlarged tip. (Scale bar = 20 μm.)

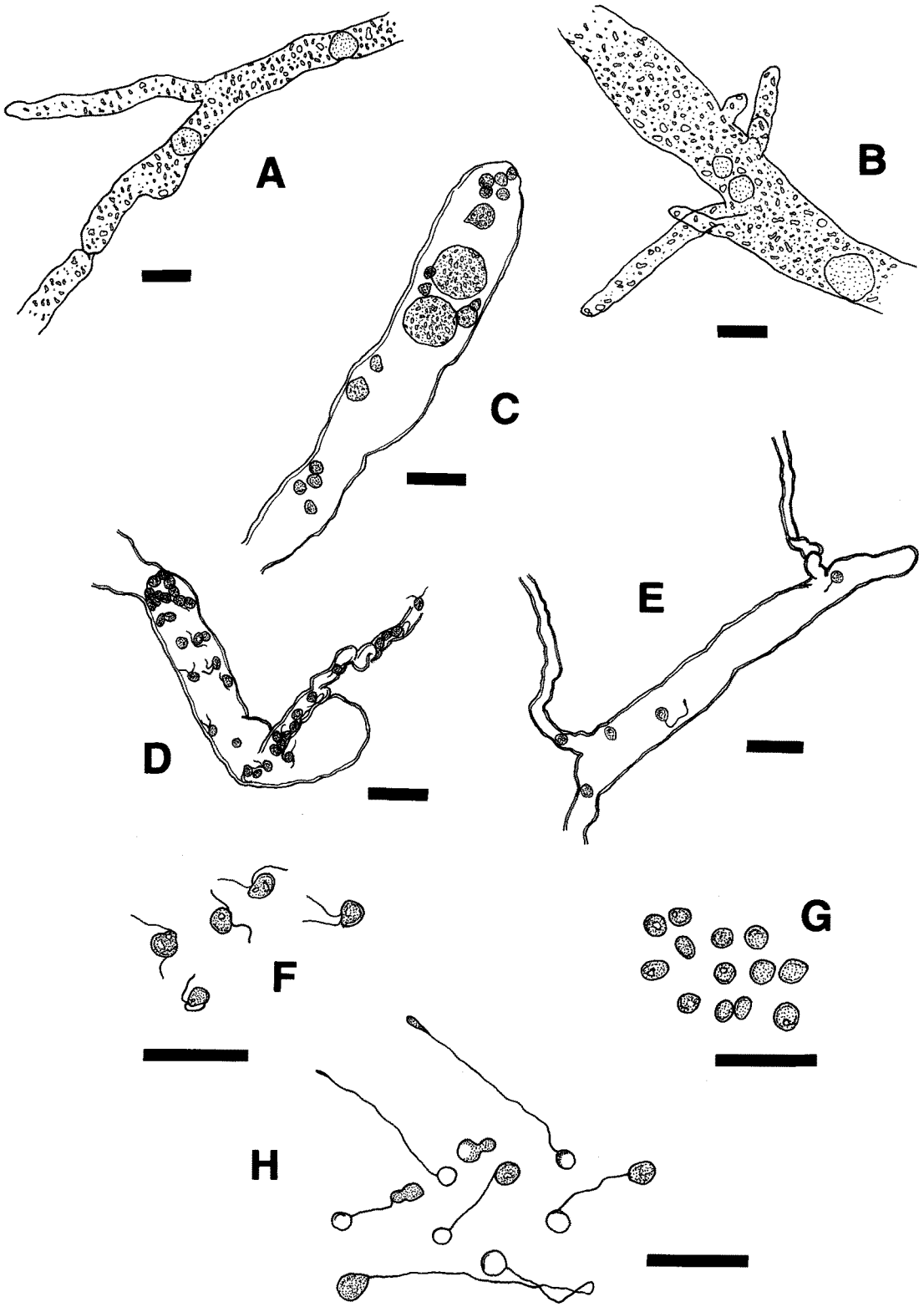

Fig. 9. *Atkinsiella awabi* sp. nov. isolated from abalone. A, B. Vegetative hyphae grown in PYGS broth; C. Zoosporangium during zoospore formation, protoplasm cleaved and formed zoospores; D. Release of zoospores; E. Some zoospores encysted and germinated within zoosporangium; F. Swimming zoospores; G. Encysted zoospores; H. Germination. (Scale bars=50 μm.)

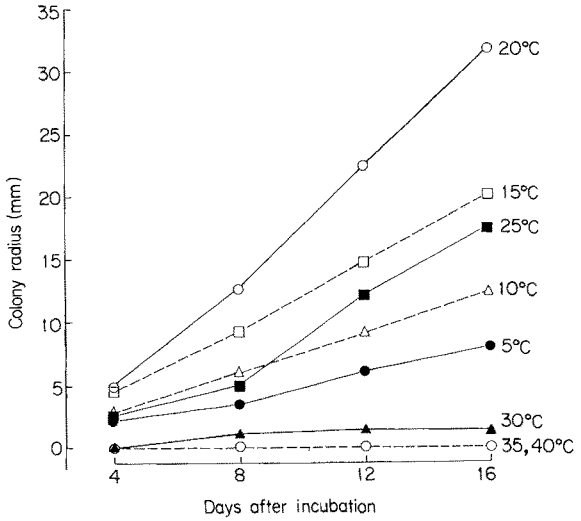

Fig. 10. Effect of temperature on growth of *A. awabi*.

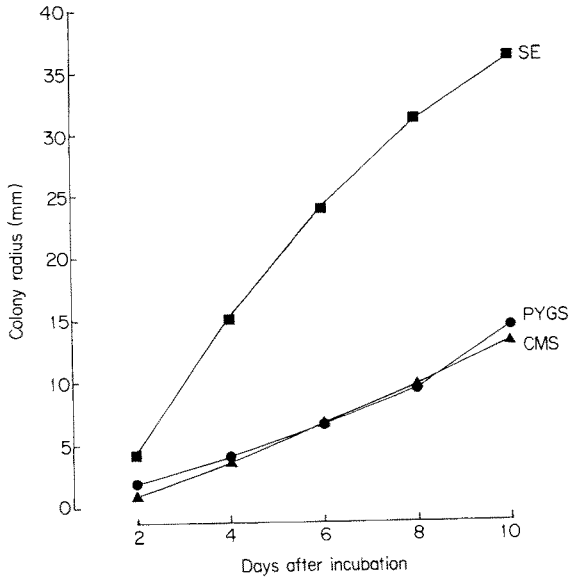

Fig. 11. Mycelial growth of *A. awabi* on different media at 15°C for 10 days.

few encysted spores germinated directly without filament formation.

Histopathology Histologically, large numbers of sparsely branching aseptate, fungal hyphae 8-20 μm in diam, morphologically consistent with the isolated fungus, were present in the tubercle-like swellings on the mantle and the melanized lesions on the peduncle. In the mantle lesions, hyphae penetrated the deeper layer and accumulated in the large cavities containing many haemocytes. Although the hyphae were stained positively by PAS reaction, they were most easily recognized in Grocott-Giemsa preparations. There were no granulomas or cellular infiltration around the hyphae (Fig. 3).

Effect of various temperatures on growth The results are illustrated in Fig. 10. Fungal growth appeared at 5-25°C on PYGS agar with slightly growth at 30°C and no hyphal growth at 35 and 40°C. At 30°C, the growth appeared after 4 days of incubation. The optimum temperature for growth on PYGS agar was 20°C.

Effect of culture media on growth As shown in Fig. 11, hyphal growth occurred at 15°C on all media, PYGS, CMS and SE agar. The fungus grew best on SE agar, followed by PYGS agar.

Table 1. Growth of *A. awabi* sp. nov. on PYG agar containing various amount of KCl and NaCl.

Media	growth*
PYG agar	−
PYGS agar	+
PYG agar with 1.0% KCl	−
PYG agar with 2.5% KCl	−
PYG agar with 5.0% KCl	−
PYG agar with 1.0% NaCl	−
PYG agar with 2.5% NaCl	−
PYG agar with 5.0% NaCl	−

* After incubation of 14 days at 15°C.

Mineral requirement for growth Examination of the fungal growth on PYG media with different mineral compounds revealed clear fungal growth only on PYGS agar (Table 1). Although hyphal growth appeared on PYG agar with 2.5% NaCl, the growth was scant and ceased after 4 days.

Effects of five chemicals on zoospores As shown in Table 2, malachite green was the most effective chemical to inhibit *Atkinsiella awabi* sp. nov. zoospores, having an MIC of 0.05 ppm. Polyphenon-100 was the second most inhibitory with an MIC of 7.8 ppm, while MIC of formalin, hydrogen peroxide and povidone iodine were 31.3 ppm, 50.0 ppm and 250.0 ppm, respectively.

Discussion

The fungus NJM 9354, namely, *Atkinsiella awabi* sp. nov., was isolated from the lesion of diseased abalone and found characteristically develop a non-septate, stout and irregular thallus, and to produce 1-2 wavy or coiled discharge tubes at terminal and/or lateral parts of sporangia during zoosporangiosis. Many vacuoles were found in hyphae. Zoospores were pyriform, biflagellate,

Table 2. Effects of five chemicals against germination of *A. awabi* sp. nov. zoospores.

Chemicals	MIC (ppm)
Formalin	31.3
Malachite green oxalate	0.05
Polyphenon-100	7.8
Hydrogen peroxide	50.0
Povidone iodine*	250.0

* Povidone iodine consists of 7% iodine.

isokont and diplanetic. On the basis of these characteristics, the fungus was ascribed to the genus *Atkinsiella*.

Primary zoospores of this fungus were mostly released from the sporangium through discharge tubes, but some remained and were encysted with in the sporangium. This characteristic is similar to that of *A. hamanaensis*, with a pattern between *A. dubia* and *A. entomophaga*. However, the size of *Atkinsiella awabi* sp. nov. encysted zoospores were about 8 μm, larger than those of *A. hamanaensis* which were about 5 μm. The size of encysted zoospores of *A. dubia* and *A. entomophaga* were respectively about 7-9 μm (Sparrow, 1973) and 10.5 μm (Martin, 1977). The present fungus developed 1-2 unbranched discharge tubes, wavy or coiled, 5-8 $\times$ 70-175 μm in size, thinner and shorter than *A. dubia*, which were 10-30 $\times$ 50-400 μm (Atkins, 1954). *A. entomophaga* has 1-4 discharged tubes, up to 3.7 mm (Martin, 1977). *A. hamanaensis* formed sporangia with several straight or wavy discharge tubes, measuring 40-1150 $\times$ 5-15 μm (Bian and Egusa, 1980). In the germination stage, along the length of *A. awabi* sp. nov. germ tube, no rounded swelling was visible in contrast to *A. entomophaga*, as described by Martin (1977).

Hatai (1982) reported *H. milfordensis* infection in abalone with lesions on flat or tubercle-like swellings on the mantle, epipode and dorsal surface of foot. Lesions found in the present case were similar to those in the *H. milfordensis* infection, as were the non-inflammatory responses to penetrating hyphae. However, the present case was characterized by accumulating hyphae in the large cavities in the deeper layer of the mantle. Regardless of the presence of inflammatory responses, it was considered that the hyphae accumulated in the cavities could cause severe hindrance of circulation of body fluid and affect the host.

Atkinsiella awabi sp. nov. grow at 5-30°C on PYGS agar, and grew best at 20°C. *A. hamanaensis* has the optimum range for growth of 29-32°C (Bian and Egusa, 1980), higher than *A. awabi* sp. nov. *A. hamanaensis* could grow at 1-5% NaCl in PYGS broth (Bian and Egusa, 1980), whereas *A. awabi* sp. nov. grew only on PYGS agar. *A. awabi* sp. nov. is exclusively a marine fungus. *A. awabi* sp. nov. grew best on SE agar, followed by PYGS agar, but it should be noted that SE agar preparation is more costly than PYGS agar.

The in vitro minimum inhibitory concentrations of five chemicals against *A. awabi* sp. nov. zoospores, shown in Table 2, indicated that malachite green is the most effectively inhibits the germation of zoospores. However, we do not recommend using this chemical because of its toxic and mutagenic potential (Bills et al., 1977; Alderman, 1985; Meyer and Jorgenson, 1983; Stoskopf, 1993). From the results, polyphenon-100 was also effective against zoospores, and no side-effects have been reported with this chemical.

Literature cited

Alderman, D. J. 1985. Malachite green: A review. J. Fish Dis. **8**: 289-298.

Atkins, D. 1954. A marine fungus *Plectospira dubia* n. sp. (Saprolegniaceae), infecting crustacean eggs and small crustacea. J. Mar. biol. Ass. U.K. **33**: 721-732.

Bian, B. Z. and Eguza, S. 1980. *Atkinsiella hamanaensis* sp. nov. isolated from cultivated ova of the mangrove crab, *Scylla serrata* (Forsskål). J. Fish Dis. **3**: 373-385.

Bills, T. D., Marking, L. and Chandler, J. H. 1977. Malachite green: Its toxicity to aquatic organisms, persistence and removal with activated carbon. Invest. Fish Cont. **75**: 1-6.

Fuller, M. S., Fowles, B. E. and McLaughein, D. J. 1964. Isolation and pure culture study of marine phycomycetes. Mycologia **56**: 745-756.

Hatai, K. 1982. On the fungus *Haliphthoros milfordensis* isolated from temporarily held abalone (*Haliotis sieboldii*). Fish Pathol. **17**(3): 199-204.

Karling, J. S. 1981. "Predominantly holocarpic and eucarpic simple biflagellate Phycomycetes," Strauss and Cramer, Hirschberg. 252 p.

Martin, W. W. 1977. The development and possible relationships of a new *Atkinsiella* parasitic in insect eggs. Amer. J. Bot. **64**(6): 760-769.

Mayer, F. P. and Jorgenson, T. A. 1983. Teratological and other effects of malachite green on development of rainbow trout and rabbits. Trans. Am. Fish Soc. **112**: 818-824.

Sparrow, F. K. 1960. "Aquatic Phycomycetes, 2nd ed.," Univ. Michigan Press, Ann Arbor. 1187 p.

Sparrow, F. K. 1973. The peculiar marine phycomycete *Atkinsiella dubia* from crab eggs. Arch. Mikrobiol. **93**: 137-144.

Stoskopf, M. K. 1993. Freshwater tropical fish phamacology. In: "Fish Medicine," (ed. by Stoskopf, M. K.). pp. 593-601. Saunders, Philadelphia.