Biology of Marine Fungi

Bearbeitet von
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1. Auflage 2012. Buch. X, 354 S. Hardcover
ISBN 978 3 642 23341 8
Format (B x L): 15,5 x 23,5 cm
Gewicht: 689 g

Chapter 2
Diseases of Fish and Shellfish Caused by Marine Fungi

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Contents

Abstract Fungal diseases are problematic in cultured fish and shellfish, their seeds, and sometimes wild marine animals. In this chapter fungal diseases found in marine animals, especially in Japan, are described. Pathogens in the fungal diseases are divided into two groups. One of them is marine Oomycetes, which cause fungal diseases in marine shellfish and abalones. The diseases caused by the fungi of this group and the fungal characteristics are introduced. The pathogens include members of the genera *Lagenidium, Haliphthoros, Halocrusticida, Halioticida, Atkinsiella*, and *Pythium*. On the other hand, some fungal diseases caused by mitosporic fungi are also known in marine fish and shellfish. The diseases caused

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C. Raghukumar (ed.), *Biology of Marine Fungi*,
Progress in Molecular and Subcellular Biology 53,
DOI 10.1007/978-3-642-23342-5_2,

by these fungi and the fungal characteristics are described. The pathogens include members of the genera *Fusarium, Ochroconis, Exophiala, Scytalidium, Plectosporium*, and *Acremonium.*

2.1 Introduction

Many diseases of fish and shellfish have been observed in studies at monitoring sites in the oceans around the world. The impact of these diseases on population sizes in marine ecosystems in general is poorly understood. Marine fishes and prawns are very popular as seafood especially in Japan, because the Japanese like to eat them raw as "sushi" or "sashimi." Therefore, the culture of fish and shellfish and their seed production are important industries in the sea around Japan. The yield from these industries is gradually increasing along with demand. However, the industry is facing serious problems with infectious diseases. These diseases include fungal infections. No strategies are currently available for effectively controlling fungal diseases with antifungal substances. Therefore, some of these diseases cause high mortality rates, which results in significant economic losses.

In this chapter some of the most economically important diseases caused by species of Oomycetes and mitosporic fungi, found mainly in marine fish and shellfish in Japan, are described. Procedures for identification of these pathogens are also included. Accurate identification of pathogens is necessary in studies designed to improve production rates.

2.2 Fungal Diseases of Shellfish Caused by Oomycetes

Five fungal genera have been reported in Japan as pathogenic Oomycetes of marine shellfish including abalone. For classification of the fungi, an observation on the mode of zoospore production is essential and important. All fungi of the marine Oomycetes were isolated from the lesions using PYGS agar (peptone, 1.25 g; yeast extract, 1.25 g; glucose, 3.00 g; agar, 12–15 g; sea water, 1,000 mL). For inhibition of the most bacterial growth, an addition of ampicillin and streptomycin sulfate in the medium is required. After fungal colonies develop on the agar plates, each one is transferred onto fresh PYGS agar to make a pure culture. The fungi are maintained at 20–25°C and subcultured on PYGS agar approximately at monthly intervals. For morphological observations, the isolates were inoculated into PYGS broth and incubated at 25°C for 3–5 days. Small colonies in PYGS broth are rinsed twice with sterilized artificial seawater and transferred into Petri dishes containing 25 mL of sterilized artificial seawater, and then incubated at 25°C to induce zoospore production. The fungi of the five genera are illustrated in Fig. 2.1.

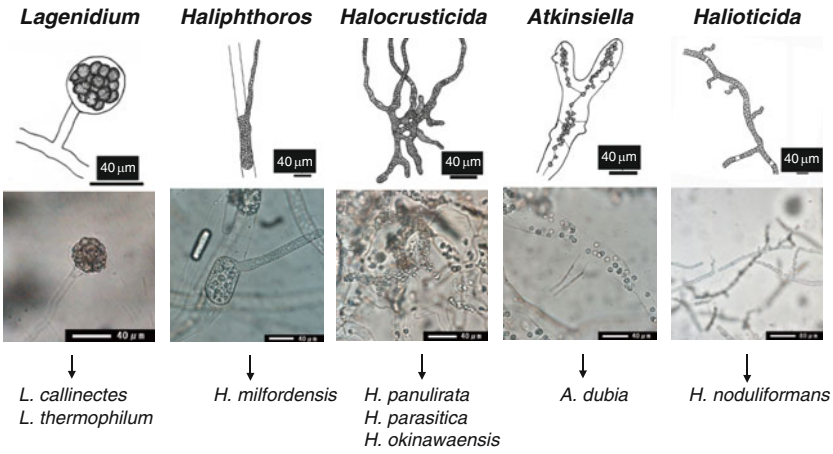

Fig. 2.1 Mode of zoospore production in the fungi of the five genera reported as pathogenic Oomycetes of marine shellfish including abalone in Japan

2.2.1 Lagenidium *Infection*

Species of *Lagenidium* have been found on various hosts from both freshwater and marine habitats (Sparrow 1960, 1973a). As parasites of animals, *L. callinectes* has been described from ova of the blue crab, *Callinectes sapidus* (Couch 1942; Rogers-Talbert 1948; Bland and Amerson 1973) and ova of the barnacle, *Chelonibia patula* (Johnson and Bonner 1960); *L. chthamalophilum* has been isolated from ova of the barnacle, *Chthamalus fragilis* (Johnson 1958), while *L. giganteum* has been reported in mosquito larvae, *Daphne* and copepods (Couch 1935). *Lagenidium callinectes* has been reported from certain marine algae (Fuller et al. 1964). In addition, unidentified species of *Lagenidium* were isolated from cultivated crustaceans, e.g., white shrimp, *Penaeus setiferus* (Lightner and Fontaine 1973), the Dungeness crab, *Cancer magister* (Armstrong et al. 1976) and the American lobster, *Homarus americanus* (Nilson et al. 1976).

A new fungus, *Lagenidium scyllae*, was isolated from ova and larvae of the mangrove crab, *Scylla serrata*, in Philippine (Bian et al. 1979). This fungus was very similar to *L. callinectes*. However, there were some differences between the two. Discharge tubes of *L. scyllae* were longer than those of *L. callinectes*. In the vesicle of *L. callinectes*, a gelatinous envelope is obvious and protoplasmic material never fills more than half of the inside vesicle (Couch 1942), whereas, in that of this fungus, the gelatinous envelope was not seen and the protoplasmic material nearly fills in the whole inside vesicle. According to Couch (1942) and Bland and Amerson (1973), zoospores of *L. callinectes* were discharged by the bursting of vesicle, which was rather persistent for several hours after the spores had emerged. In *L. scyllae*, however, all the spores were simultaneously discharged by rapid deliquescence of the vesicle or the spores were liberated one by one through the

opening of vesicle. Moreover, the collapsed vesicles were never persistent. It seemed to be related to the thin and non-gelatinous characters of the vesicle. The mangrove crab is widely distributed in the tropical Pacific Ocean and the Indian Ocean, while the blue crab is in the Atlantic Coast of North America. In the relation to the distributions of the hosts, there is no overlap between that of *L. callinectes* and that of *L. scyllae*. As a result, it was reported as a new species.

Lagenidium callinectes was isolated from the eggs and zoea of the marine crab, *Portunus pelagicus* for the first time in Japan (Nakamura and Hatai 1995a). Masses of protoplasm flowed into the tip of 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 entry of protoplasmic masses, division into initial zoospores, and active movement of zoospores. The way of zoospore liberation varied: sometimes they were released simultaneously by rupture of the vesicle, sometimes singly through a hole in the vesicle wall. When zoospores were discharged singly, vesicles usually persisted for a few minutes (Fig. 2.2).

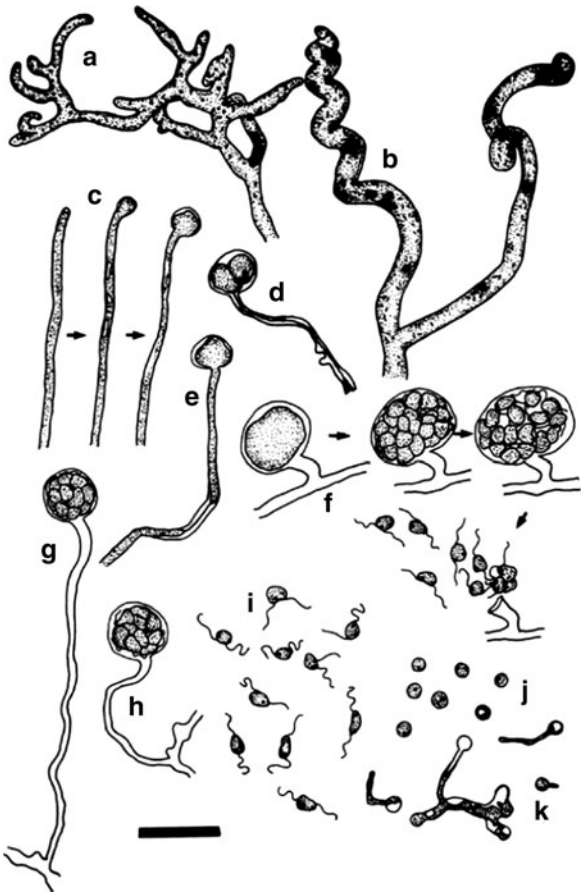

Fig. 2.2 Morphological characteristics of *L. callinectes* isolated from an egg of *P. pelagicus*. Scale = 50 μm. (**a**) Irregularly branched hyphae with numerous shiny rod granules; (**b**) Coiled hyphae in PYGS broth; (**c**) Vesicle formation; (**d**, **e**) Protoplasmic masses flow into the vesicle with a protoplasmic thread; (**f**) Division into initial zoospores and zoospores liberation; (**g**, **h**) Mature vesicles; (**i**) Zoospores; (**j**) Encysted zoospores; (**k**) Germination (Nakamura and Hatai 1995a)

Lagenidium callinectes was also isolated from larvae of mangrove crab, *Scylla serrata*, in Bali, Indonesia (Hatai et al. 2000).

A fungal infection occurred in the eggs and larvae of mangrove crab, *Scylla serrata*, affecting the seed production in Bali, Indonesia. The fungus isolated in August 1993 was a new species in the genus *Lagenidium*, and named *L. thermophilum*, because of its rapid and thermotolerant growth and unique discharge process. Masses of protoplasm occupied nearly all of the vesicles and divided into individual zoospores with two flagellae. The envelopes of the vesicles were not apparent. Zoospore liberation occurred after the vesicles separated from the discharge tubes, namely the vesicles left the discharge tubes before the zoospores were released (Nakamura et al. 1995). 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 opening in the vesicles. Generally, the former was observed among the bigger vesicle and the latter among the smaller ones. Collapsed vesicles were not persistent. The isolate grew at 15–45°C with an optimum at 30–40°C. This species differed from *L. callinectes* in its salt requirements. As *L. callinectes* grew on media containing seawater or 1–2.5% (w/w) NaCl, it seems to be a marine fungus. However, as *L. thermophilum* also grew on media without seawater, it is obvious that it is not exclusively marine.

L. thermophilum was also found in eggs and larvae of black tiger shrimp, *Penaeus monodon*, at a hatchery in August 2000, Thailand (Muraosa et al. 2006). The characteristics feature of asexual reproduction of the fungus was that zoospores swam away in seawater after the vesicle separated from the discharge tube (Fig. 2.3). This was the first report of *L. thermophilum* infection in black tiger shrimp in Thailand.

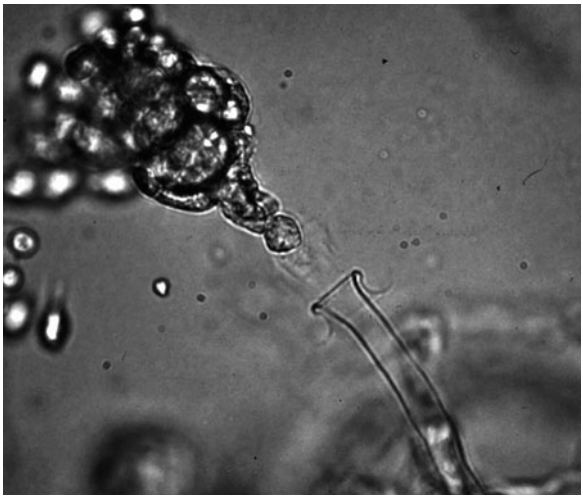

Fig. 2.3 Zoospores swim away in seawater after the vesicle separated from the discharge tube

2.2.2 Haliphthoros *Infection*

The genus *Haliphthoros* was a monotypic genus erected by Vishniac (1958) as the type of the family Haliphthraceae (Saprolegniales). *Haliphthoros milfordensis*, the type species of the genus, has been reported as an endoparasite of eggs of the oyster drill, *Urosalpinx cinerea* (Vishniac 1958). Since then it has been isolated from juveniles of the American lobster, *Homarus americanus* (Fisher et al. 1975), adults of the white shrimp, *Penaeus setiferus* (Tharp and Bland 1977), and a few marine algae (Fuller et al. 1964).

A new species, *H. philippinensis*, was isolated from larvae of the jumbo tiger prawn, *Penaeus monodon* in Philippines (Hatai et al. 1980). The hyphae were stout, branched, irregular, non-septate, developing within the bodies of larvae of the prawn, and it was holocarpic. In pure cultures, the hyphae were homotrichous, at first somewhat uniform, sometimes highly vacuolated, 10–37.5 μm in diameter, becoming fragmentary by means of cytoplasmic constriction with age (Fig. 2.4). Fragments with a dense cytoplasm were variable in size and shape, globose, elongate or tubular, often with protuberances, up to 190 × 100 μm, not disarticulated, connected in bead-like chains, functioning as sporangia and developing discharge tubes which were straight, wavy or coiled, up to 620 × 7.5–12.5 μm. Zoospores were polyplanetic. Encysted spores were spherical, 5–7.5(−12.5) μm in diameter, producing a delicate germ tube. Germ tube was simple, sometimes once branched and up to 250 μm in length. Sexual reproduction was not observed. The fungus showed a close resemblance to *H. milfordensis*, but it differed from the latter in a number of features as described below.

When the fragment with protuberance on the medium was transferred into sea water, the protuberance might again constrict and transform into another sporangium, or might extend and serve as a part of the discharge tube. The sporangia of

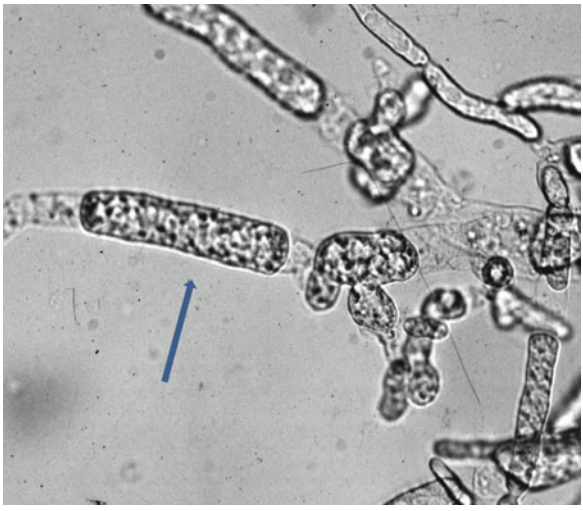

Fig. 2.4 Fragment (*arrow*) formation of genus *Haliphthoros*

this fungus are very variable in shape and often with protuberances: globose, elongate, tubular, or irregular-shaped. These are distinctive for the fungus. In this fungus, discharge of zoospores is also peculiar and diverse from that of *Haliphthoros* reported previously. The zoospores were released not only through the orifice of discharge tube but also through the opening of the sporangium. According to Vishniac (1958) and Fuller et al. (1964), zoospores of *H. milfordensis* were monoplanetic and monomorphic. This fungus, however, has polyplanetic and polymorphic zoospores including primary and secondary types. *H. philippinensis* has a wide range of temperature requirement for its growth. Possibly owing to its tropical habitation, it could be tolerant even up to 36°C. This feature was different from that of *H. milfordensis* which could not grow at 35°C (Vishniac 1958).

Haliphthoros milfoldensis infection was found in abalone, *Haliotis sieboldii*, temporarily held in aquaria with circulating sea water adjusted to 15°C by a cooling system in Japan (Hatai 1982). The typical external symptom of diseased abalones was flat or tubercle-like swelling formed on mantle, epipode and dorsal surface on foot (Fig. 2.5). The mycelium was always observed in the lesions. Zoospores of a fungus, which was isolated from the lesion, formed within the fragment were liberated through the orifice of discharge tube. Encysted spores were spherical, usually 7 μm in diameter. The fungus grew at a temperature range of 4.9–26.5°C, with optimum of 11.9–24.2°C.

In June 1994, fungal diseases occurred in the eggs and zoeae of crab, *Portunus pelagicus*, in Japan. Fragments of the isolated fungus were clearly constructed of concentrated masses of protoplasm in the hyphae, tuberculate, saccate or irregular, and quite variable in size and shape. They changed into zoosporangia-producing discharge tubes. Many vacuoles appeared in the zoosporangia and the extending

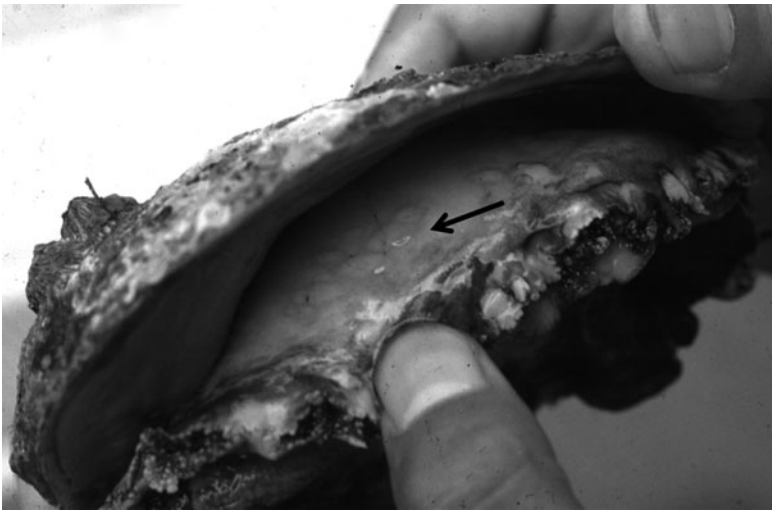

Fig. 2.5 The typical external symptom of diseased abalones was flat or tubercle-like swelling (*arrow*) formed on mantle

discharge tubes, and were also observed in the active mycelia. It was identified as *Haliphthoros milfordens* (Nakamura and Hatai 1995a).

In July 1997, *Haliphthoros milfordens* infection occurred in the eggs and zoeae of the mangrove crab, *Scylla serrata*, in Bali, Indonesia. The mortality rate reached almost 100% in the larvae (Hatai et al. 2000). The colonies on PYGS agar were whitish and reached a diameter of 20–25 mm after 5 days at 25°C. Hyphae in PYGS broth were stout, aseptate, branched with numerous shiny spherical granules, and sometimes concentrated masses of protoplasm were observed in the hyphae. In artificial seawater, fungal fragments were clearly observed to be concentrated masses of protoplasm in the hyphae. They changed into zoosporangia-producing discharge tubes. One discharge tube was usually formed on the lateral side of each zoosporangium. Division of the protoplasm started in the sporangia and continued in the discharge tubes just before zoospore liberation (Fig. 2.6).

In March 2001, *Haliphthoros milfordens* was isolated from larvae of the black tiger prawn, *Penaeus monodon* in Nha Trang, Vietnam (Chukanhom et al. 2003). This was the first report of disease in the black tiger prawn in Vietnam.

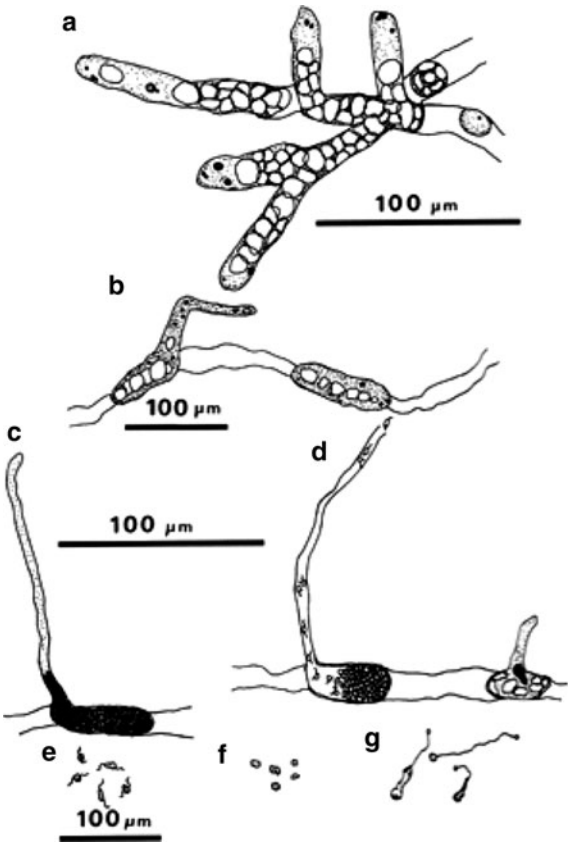

Fig. 2.6 Morphological characteristics of *Haliphthoros milfordensis* isolated from a zoea of *S. serrata*. (**a**) Hyphae in PYGS broth; (**b**) Fragments. Discharge tube formation on the left fragment; (**c**) Zoospore formation; (**d**) Zoospore liberation; (**e**) Zoospores; (**f**) Encysted zoospores; (**g**) Germination (Hatai et al. 2000)

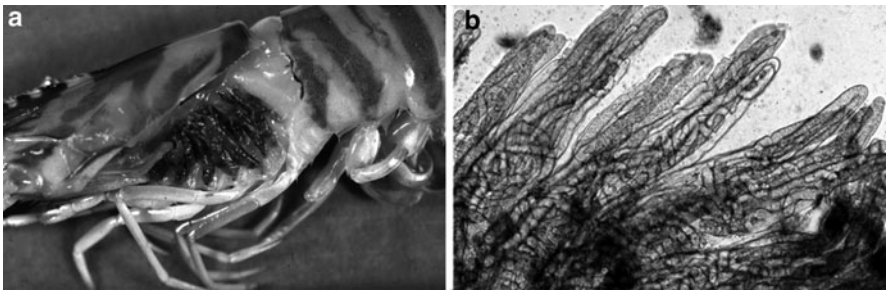

Fig. 2.7 *H. milfordensis* infection in juvenile kuruma prawns, *Penaeus japonicus*. (**a**) Showing *black* gills. (**b**) Hyphae found in gills. Hyphae are only growing within gills

H. milfordensis was also isolated from gill lesions of juvenile kuruma prawns, *Penaeus japonicus*, with black gill disease (Fig. 2.7) at a private farm in August 1989, Japan (Hatai et al. 1992). *H. milfordensis* has been known as a pathogen of various marine organisms. Especially, the fungus has been well known to be an important fungal pathogen of eggs and larvae of crustaceans. However, this was the first case of *H. milfordensis* infection in juvenile kuruma prawn. This condition has previously been known in kuruma prawn as the typical clinical sign of *Fusarium solani* infection.

2.2.3 Halocrusticida *Infection*

A new genus *Halocrusticida* gen. nov. (Lagenidiales, Haliphthoraceae) was proposed for the six species formerly reported as the fungi in the genus *Atkinsiella* except *A. dubia* (Nakamura and Hatai 1995b). These six species of *Atkinsiella* (Table 2.1) were reported from various aquatic animals (Martin 1977; Bian and Egusa 1980; Nakamura and Hatai 1994, 1995a; Kitancharoen et al. 1994; Kitancharoen and Hatai 1995). A key to the species of *Halocrusticida* is described in Table 2.2. Mycelia contained granular clusters without oil droplets and vacuoles on *A. dubia*, but many vacuoles and numerous shiny granules were found on the others. Central protoplasmic masses supported by several protoplasmic threads in the process of zoospore production were observed on *A. dubia*, but not on the others. The most apparent difference between *A. dubia* and the other six species of *Atkinsiella* was the behavior of zoospores in the first motile stage. Zoospores encysted within zoosporangia and discharge tubes following the first motile stage in *A. dubia*, while zoospores in the first motile stage were released from zoosporangia in the other six species.

The definition of the genus *Halocrusticida* is as follows. Thallus is endobiotic, holocarpic, stout, and branched. Zoosporangia are the same in size and shape as thalli. Discharge tubes develop one to several per sporangium. Zoospores in the first motile stage emerge from the zoosporangia. Zoospores are monoplanetic or

Table 2.1 Six species reported previously as *Atkinsiella*[a]

Species	References	Host	Locality
A. entomophaga	Martin (1977)	Insect eggs	USA
A. hamanaensis	Bian and Egusa (1980)	Eggs and larvae of *Scylla serrata*	Japan
A. parasitica	Nakamura and Hatai (1994)	Rotifer (*Brachionus plicatilis*)	Japan
A. awabi	Kitancharoen et al. (1994)	Abalone (*Haliotis sieboldii*)	Japan
A. okinawaensis	Nakamura and Hatai (1995a)	Zoea of the crab (*Portunus pelagicus*)	Japan
A. panulirata	Kitancharoen and Hatai (1995)	Philozoma of spiny lobster (*Panulirus japonicus*)	Japan

[a]Nakamura and Hatai (1995b)

Table 2.2 Key to species of *Halocrusticida*

1 Colonies filamentous, less than two tubes produced from each sporangium . . . . . .*H. awabi*
1 Colonies lobed, bulbous. 2
2 Encysted spores more than 9 μm, parasitic on insect eggs . . . . . .*H. entomophaga*
2 Encysted spores less than 9 μm, parasitic on crustaceans 3
3 Branched discharge tubes present 4
3 Branched discharge tubes absent 5
4 Zoospores generally formed two or more deep in the discharge tubes. . . . . *H. okinawaensis*
4 Zoospores generally formed in a single row in the discharge tubes. . . . . *H. parasitica*
5 Pigmentation from gray to light brown, optimum temperature for growth 30–32°C . . . . . .*H. hamanaensis*
5 No pigmentation, optimum temperature for growth 25°C. . . . . *H. panulirata*

diplanetic, isokont, laterally biflagellate. Germinating zoospore has a slender germ tube. Sexual reproduction is absent. It is parasitic on aquatic animals, especially marine crustaceans.

Halocrusticida hamanaensis was originally reported as *Atkinsiella hamanaensis* (Bian and Egusa 1980). The fungus was isolated from ova of mangrove crab, *Scylla serrata* in Japan. The swollen hyphal tips up to 150 μm in diameter contained dense cytoplasm. Each sporangium was formed through the formation of septa and several lateral or terminal discharge tubes. The discharge tubes were straight or wavy, measuring 40–1,150 × 5–15 μm. Zoospores measured 6.3 (5–10) × 4.5 (3.8–5) μm in size, were pyriform or slipper-shaped, with two lateral flagella. The encysted spores were 5 (4.5–7.5) μm in diameter, spherical, subglobose, or angular. The encysted spore in sterile sea water developed a hair-like filament, 10–270 μm in length.

Halocrusticida awabi was originally reported as *Atkinsiella awabi* (Kitancharoen et al. 1994). The fungus was isolated from diseased abalone, *Haliotis sieboldii* in Japan. It showed external signs of infection of tubercle-like swelling on the mantle and melanized lesions on the peduncle. The hyphae were stout, irregular, branched, 16–140 μm diameter. 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 hair-like filament with globular enlarged tip in PYGS broth. Direct germination without filament formation also occurred occasionally. The fungus was exclusively marine and grew best in shrimp extract medium at 25°C.

Halocrusticida parasitica was originally reported as *Atkinsiella parasitica* (Nakamura and Hatai 1994). In May 1992 the rotifer, *Brachionus plicatilis* did not increase in number when it was bred in a concrete tank as food supply for seed production of crustaceans and fishes. Because protozoa were observed microscopically on the surface of rotifers, a bath treatment with 25 ppm formalin was first conducted to solve the problem in the tank. However, no increase in the number of rotifers in the tank was found following the treatment. Further detailed microscopical observation revealed thick, non-septate hyphae measuring about 10 μm diameter in the eggs and bodies of many rotifers examined. Discharge tubes were extended outside the bodies (Fig. 2.8), and zoospores with lateral biflagella were released into the seawater through the tubes. Vesicles were not formed at the tip of discharge tubes (Nakamura and Hatai 1994; Nakamura et al. 1994a). The fungus isolated from the rotifer was characterized by producing monoplanetic, lateral biflagellate zoospores, and infrequently branched discharge tubes. The zoosporogenesis of the species is shown in Fig. 2.9.

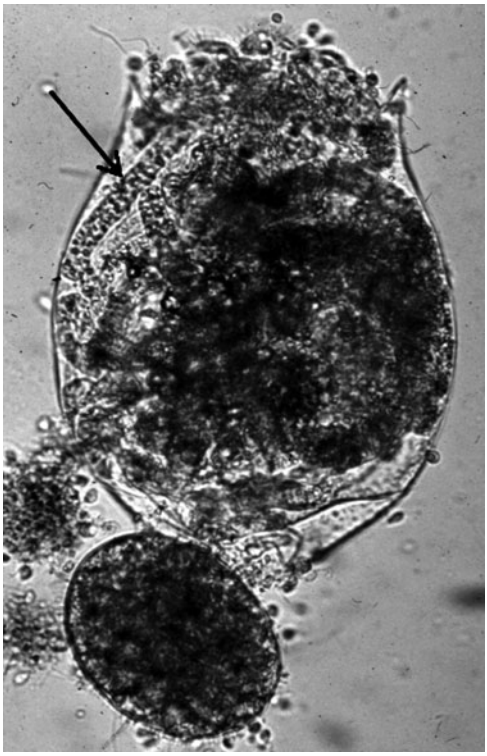

Fig. 2.8 Hyphae in bodies of a rotifer (*arrow*)

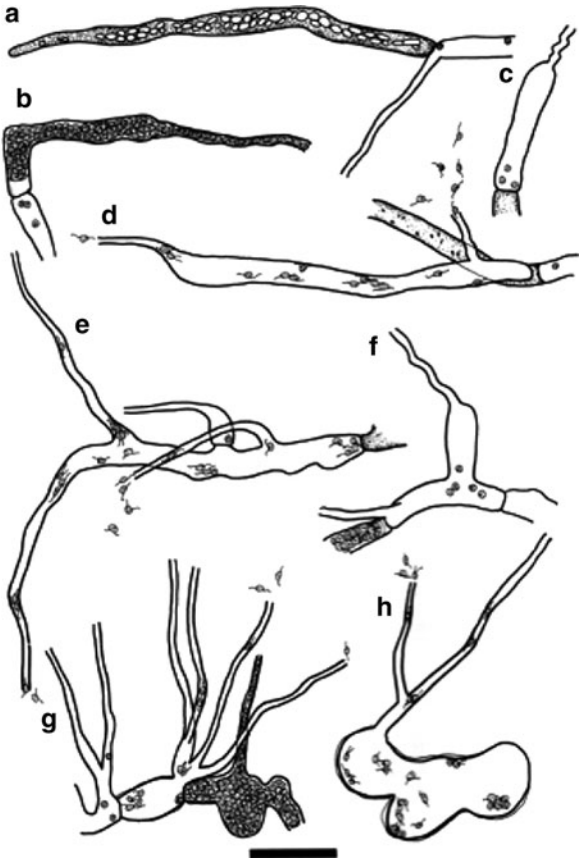

Fig. 2.9 Zoosporogenesis of *Halocrusticida* (*Atkinsiella*) *parasitica*. Scale bar = 50 μm. (**a**) Numerous large vacuoles appeared at an early stage of zoosporogenesis, and later discharge tubes developed. (**b**) Zoospore formation in a zoosporangium and a discharge tube at the final stage of zoosporogenesis. (**c**–**f**) One to three discharge tubes formed from a zoosporangium. (**g**, **h**) Branched discharge tubes

Halocrusticida panulirata was originally reported as *Atkinsiella panulirata* (Kitancharoen and Hatai 1995). This species was isolated from philozoma of the diseased spiny lobster, *Panulirus japonicus* in Japan. 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 diameter, occasionally separated by cross walls into subthalli. Thalli occasionally consisted of swollen features. Sporangia formed from the subthalli had one to three 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 hair-like filament with a globular enlarged tip in sterilized synthetic seawater, and directly as stout initial hyphae in PYGS broth. Gemmae spontaneously occurred in 3-day-old culture in PYGS broth at 25°C (Fig. 2.10). They were characterized by saccate-lobed-chained, thick-walled dense cytoplasmic and non-vacuolate features, width of 179–270 μm and various lengths up to 18 mm. Gemmae not only developed new thalli on PYGS agar or in PYGS broth, but also in sterilized synthetic seawater.

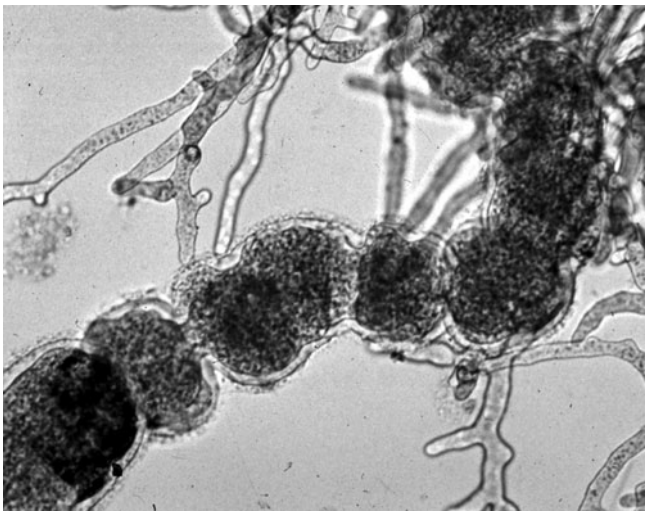

Fig. 2.10 Gemmae (*arrow*) spontaneously occurred in *Halocrusticida panulirata* culture in PYGS broth at 25°C

Halocrusticida okinawaensis was originally reported as *Atkinsiella okinawaensis* (Nakamura and Hatai 1995a). The new fungus was isolated from infected eggs and zoeae of the marine crab, *Portunus pelagicus*. Hyphae were stout, non-septate at first, irregularly branched with numerous shiny rod granules, 10–38 μm width. In seawater, hyphae were divided into subthalli with septa. Gemmae were present with thick walls, 22–190 μm in diameter. Zoosporangia were the same size and shapes as subthalli and gemmae. Discharge tubes were produced laterally or terminally from the sporangia, usually coiled or wavy. Each sporangium extended one to several discharge tubes. In the discharge tubes, zoospores were produced in more than two rows. The discharge tubes were 6–10 μm diameter and 40–510 μm length. Zoospores were laterally biflagellate, diplanetic, 4.7 × 6.3 μm on average. Germination was observed about 3 h after spores had encysted, with a hair-like filament measuring 5–190 μm length (Fig. 2.11).

2.2.4 Halioticida *Infection*

Halioticida infection was reported from abalone, *Haliotis* spp. in Japan (Muraosa et al. 2009). The genus was classified in Peronosporomycetes (formerly Oomycetes) as a new genus. The class Peronosporomycetes contains species that are pathogens of many commercially important plants, fish, and crustaceans (Kamoun 2003). Among the marine invertebrates, infections resulting from some members of the Peronosporomycetes cause problematic diseases, especially in the seed production of marine crustaceans such as shrimp and crabs. On the other hand,

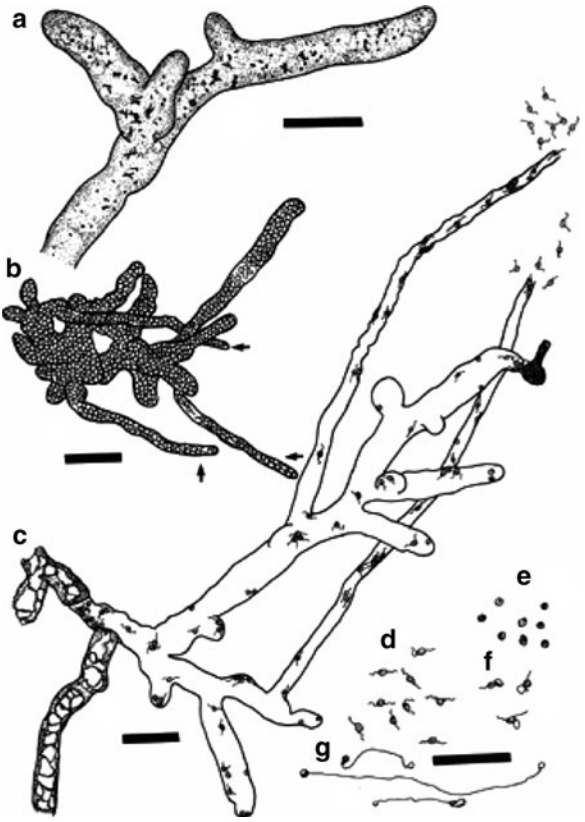

Fig. 2.11 Morphological characteristics of *Halocrusticida okinawaensis* isolated from a zoea of *P. pelagicus*. Scale = 50 μm. (**a**) Hyphae in PYGS broth; (**b**) A zoosporangium with three discharge tubes (*arrows*); (**c**) Zoospores released from the orifices of two discharge tubes. Another zoosporangium with one discharge tube is on the right; (**d**) Zoospores; (**e**) Encysted zoospores; (**f**) Secondary zoospores released from cysts; (**g**) Germination (Nakamura and Hatai 1995a)

Haliphthoros milfordens (Hatai 1982), *Halocrusticida awabi* (Kitancharoen et al. 1994), and *Atkinsiella dubia* (Nakamura and Hatai 1995b) have been reported as causative agents of such diseases in abalone, *Haliotis sieboldii*. Recently, a new fungus belonging to the Peronosporomycetes was isolated from white nodules found on the mantle of three species of abalone, *Haliotis midae* imported from the Republic of South Africa, *Haliotis rufescens* imported from the Republic of Chile and the United Mexican State, and *Haliotis sieboldii* collected in Japan. They were stocked for sale in the same tank and died from the infection. The daily mortality of stocked abalone was about 1%. The clinical sign of a moribund abalone was the presence of white nodules on the mantle (Fig. 2.12). In the lesions of the white nodules, thick and aseptate hyphae were present. The fungus was isolated from moribund abalones using PYGS agar. The manner of zoospore formation in the fungus isolated from the lesion was totally different from that of the genera *Halocrusticida* and *Atkinsiella*, but similar to that of the genus *Haliphthoros*. However, the isolate differed from the genus *Haliphthoros* as follows. In artificial seawater, the fragments were formed by constricting protoplasm in the hyphae such as in the genus *Haliphthoros*, but the protoplasm constriction was weaker, and fragments were longer, with smaller space between them, than those of

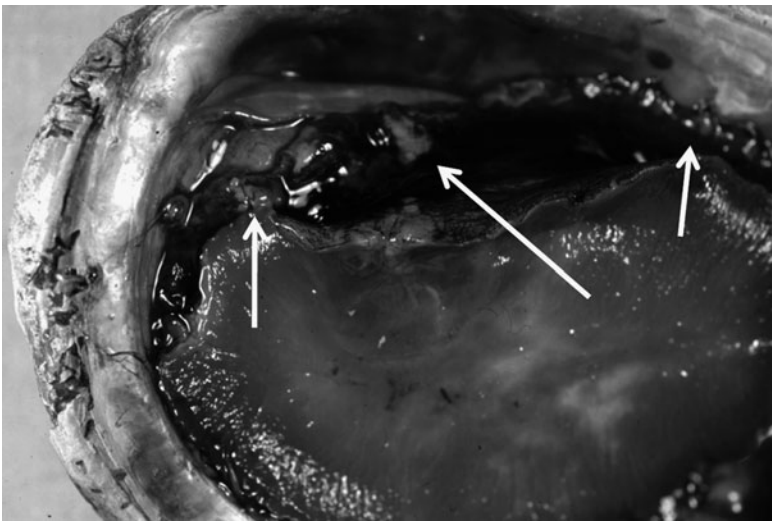

Fig. 2.12 Clinical sign of a moribund abalone. Note the presence of white nodules on the mantle (*arrows*)

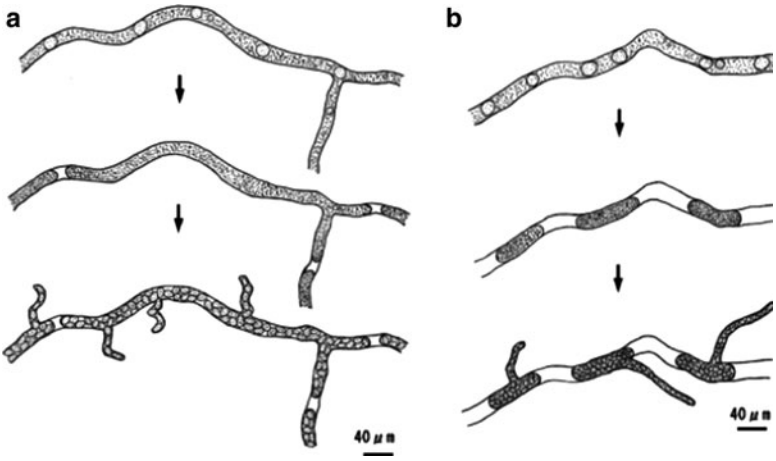

Fig. 2.13 Differences in zoospore formation between *Halioticida noduliformans* (**a**) and *Haliphthoros milfordensis* (**b**). (**a**) Fragments are longer, and spaces between adjacent fragments are smaller than those of *H. milfordensis*. One to several discharge tubes are formed. (**b**) Fragments with only one tube are shorter, and space between fragments are larger than those of *H. noduliformans*

Haliphthoros (Fig. 2.13). The species under the genus *Haliphthoros* form only one discharge tube from a zoosporangium (Vishniac 1958; Hatai et al. 1980, 1992, 2000; Nakamura and Hatai 1995a; Chukanhom et al. 2003), but the fungus from abalone has one or more discharge tubes formed from each zoosporangium.

The size of zoospores was 7.0–8.5 × 9.5–12.5 μm (width × length). From the results mentioned above, the isolate was recognized to have unique morphological characteristics in the family Haliphthoraceae.

Four isolates from white nodules and nine peronosporomycete species isolated from various marine invertebrate animals were used for analysis on the D1/D2 region of LSU rDNA. In the phylogenic tree based on LSU rDNA, the isolate was not classified into the subclass Peronosporomycetidae, Saprolegniomycetidae, or Rhipidiomycetidae, but as a new clade with the genera *Haliphthoros* and *Halocrusticida* (Fig. 2.14). Within this new clade, the four isolates from abalone, *Haliphthoros* spp. and *Halocrusticida* spp. were grouped in the respective independent subclade. *Atkinsiella dubia* and *Lagenidium* spp. were included in Saprolegniomycetidae and Peronosporomycetidae, respectively. The phylogenetic analysis also supported that the four isolates were classified into a new genus and species belonging to the family Haliphthoraceae based on their morphological characteristics. As a result, it named *Halioticida noduliformans* as new genus and species (Muraosa et al. 2009).

Dick (2001) proposed a new taxonomic system for Peronosporomycetes, in which Peronosporomycetes were subdivided into three subclasses: Peronosporomycetidae, Rhipidomycetidae, and Saprolegniomycetidae. Under this taxonomic system, the genera *Haliphthoros*, *Halocrusticida*, and *Atkinsiella* were classified in Haliphthoraceae – Salilagenidiales – Saprolegniomycetidae, and the genus *Salilagenidium*, which was named as a new genus by Dick (2001) for marine species of the genus *Lagenidium*, was classified in Salilagenidiaceae – Salilagenidiales – Saprolegniomycetidae. Molecular phylogenetic analysis by Muraosa et al. (2009) showed that only *Atkinsiella dubia* was included in the subclass Saprolegniomycetidae, but the genera *Haliphthoros*, *Halocrusticida*, and *Halioticida* were not included within the three subclasses proposed by Dick (2001). Furthermore, the genus *Lagenidium* (Salilagenidium) was included in the subclass Peronosporomycetidae in the analysis of Muraosa et al. (2009). Cook et al. (2001) also suggested that the genera *Atkinsiella* and *Lagenidium* (*Salilagenidium*) were classified into the subclass Saprolegniomycetidae and Peronosporomycetidae, respectively, and the genera *Haliphthoros* and *Halocrusticida* were not included in the three subclasses, according to their molecular phylogenetic analysis using the mitochondrially encoded cytochrome *c* oxidase subunit 2 (*cox*2) gene. Thus, the taxonomic position of genera *Haliphthoros*, *Halocrusticida*, *Atkinsiella*, and *Lagenidium* is still confusing.

In December 2006, a *Halioticida* infection was found in wild mantis shrimp, *Oratosquilla oratoria* in Tokyo Bay, Japan (Atami et al. 2009). Fungi were found in the gills of mantis shrimp (Fig. 2.15), isolated from lesions using PYGS agar, and identified by morphological observation and molecular analysis. The fungus formed fragments in the hyphae and several discharge tubes developed from each fragment. Zoospores were formed within the fragments and released into the seawater through the tops of discharge tubes. Based on the characteristics of zoospore production mode, the fungus was classified into the genus *Halioticida*. It was compared by molecular analysis of the D1/D2 region of the large subunit

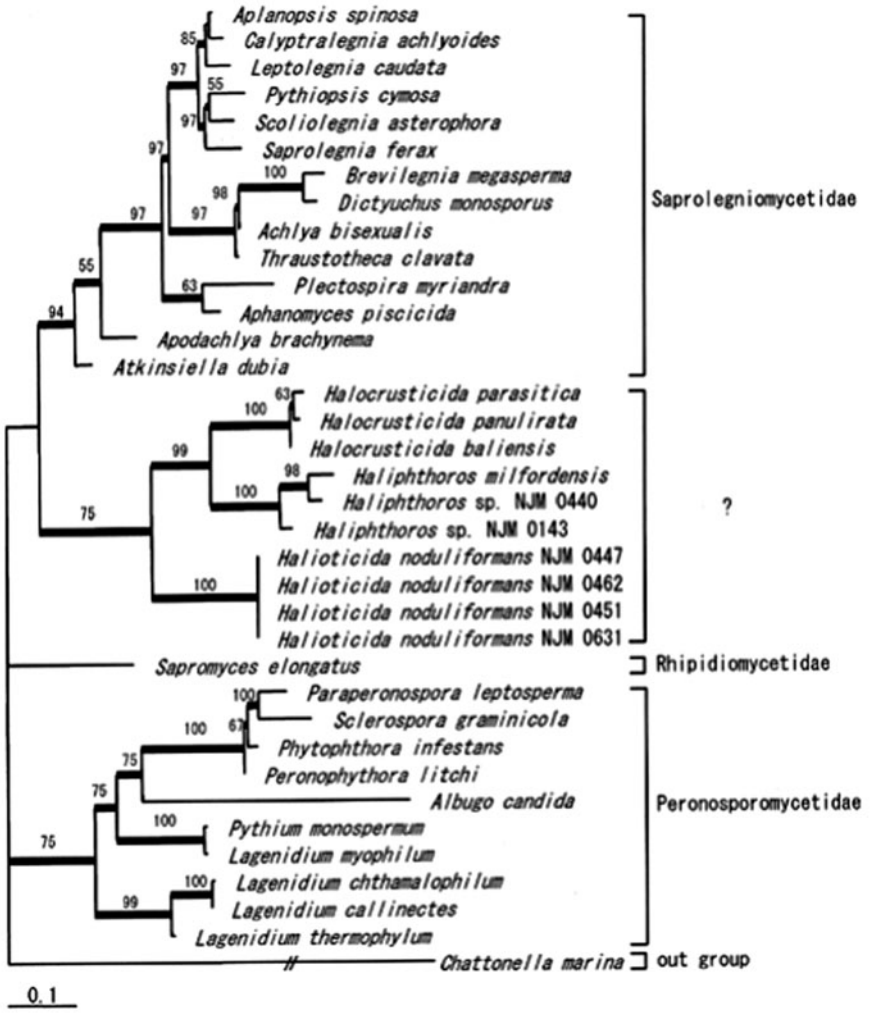

Fig. 2.14 Maximum-likelihood tree based on the D1/D2 region of LSU rDNA. Numbers on branches show bootstrap values (1,000 replicates, above 50% are indicated)

ribosomal RNA gene with *Halioticida noduliformans* isolated from abalone, *Haliotis* spp. (Muraosa et al. 2009). As a result, the sequences of the fungus isolated from mantis shrimp showed 99–100% homology at the D1/D2 domain of the large subunit ribosomal RNA gene sequence with *Halioticida noduliformans*. Histopathological observation indicated that the fungus grew in an aerobic environment, because the hyphae were found mainly in the gills and base of gills. The fungus grew well at 15–25°C, with optimal temperature of 20°C, which corresponds with *H. noduliformans* (Muraosa et al. 2009). The fungus could not

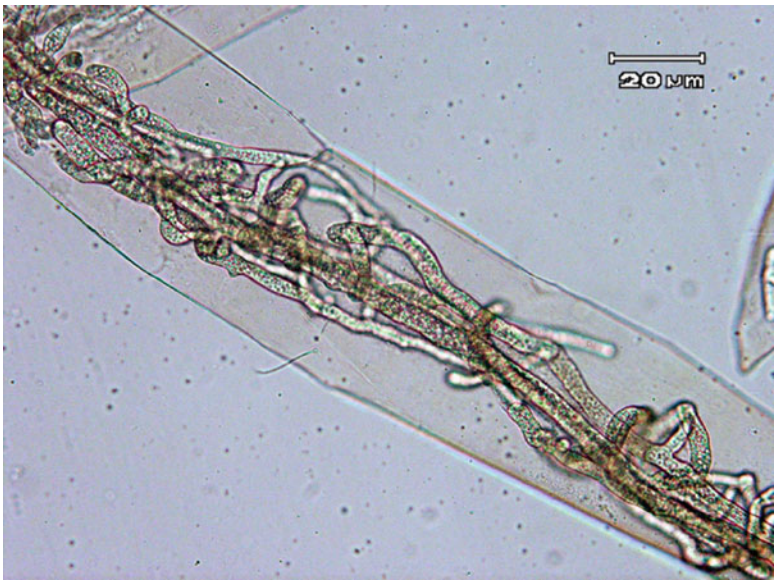

Fig. 2.15 Fungus found in the gills of mantis shrimp

grow on PYG agar or PYG agar with NaCl and KCl, but grew on PYGS agar. This suggested that it was an obligate marine fungus.

2.2.5 Atkinsiella *Infection*

Atkinsiella dubia was originally isolated from eggs of pea crab, *Pinnotheres pisum* in England (Atkins 1954), and assigned to the genus *Plectospira*. Atkins observed the same species on the eggs of *Gonoplax rhomboids* and succeeded in experimentally infecting the eggs of some species of crustaceans. The morphology of the fungal parasite on the eggs of crab was studied at that time. Later, Vishniac (1958) established a new family, Haliphthoraceae (Saprolegniales), for holocarpic biflagellate filamentous fungi, including *Haliphthoros milfordensis* and Atkins' fungus, which was renamed *A. dubia*, although she did not actually observe *A. dubia*. Its morphology and development in pure culture were followed by Fuller et al. (1964) and Sparrow (1973b) from marine algae and the eggs of various crabs, respectively.

Dick (2001) classified *Atkinsiella dubia* and *Haliphthoros* spp. into Saprolegniomycetidae, but at present the genus *Haliphthoros* is classified into different clade, *Haliphthoros*/*Halocrusticida* clade (Sekimoto et al. 2007), or unknown group (Muraosa et al. 2009), because they constructed different clades from phylogenetic analysis.

During the survey of the fungi belonging to Lagenidiales on marine animals without clinical signs, an interesting fungus was isolated from the mantle of abalone, *Haliotis sieboldii*, in Chiba Prefecture, Japan. The same fungus was also obtained from the gills of swimming crab, *Portunus trituberculatus* in Chiba Prefecture. The fungus was characterized by crystalline, tuberculate and moist colonies, dimorphic and diplanetic zoospores, and zoospores which remained in the zoosporangia during the first motile stage (Fig. 2.16), and identified as *Atkinsiella dubia*, new to Japan (Nakamura and Hatai 1995b).

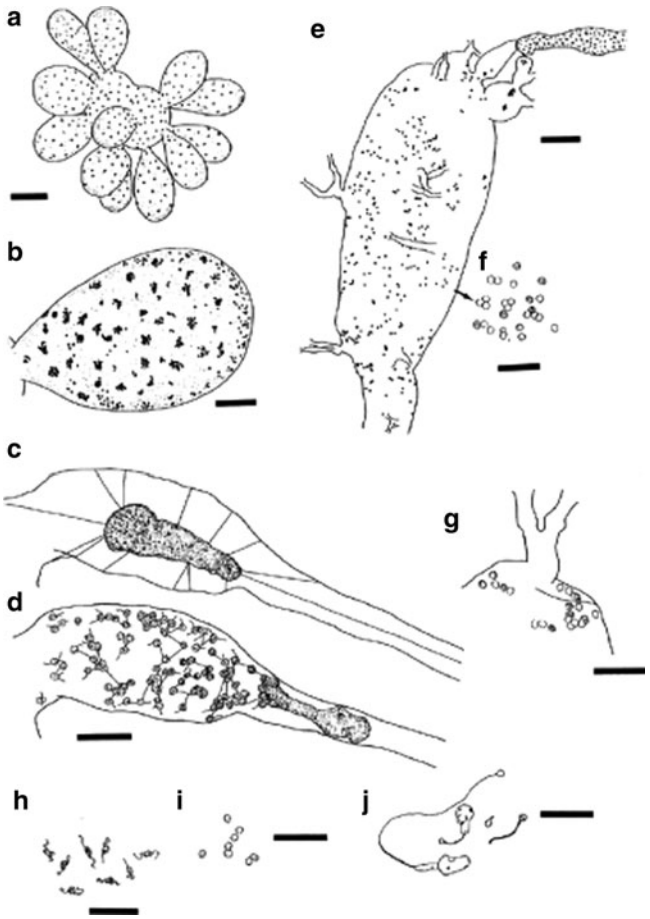

Fig. 2.16 *Atkinsiella dubia*. (**a**, **b**) Mycelium with granular clusters. (**c**) A protoplasmic mass supported by several protoplasmic thread. (**d**) Loose net-works of zoospores. These differentiated into free individual zoospores in the first motile stage. (**e**) A zoosporangium with branched discharge tubes. (**f**) Empty encysted zoospores, and encysted zoospores with protoplasm from which zoospores in the second motile stage will emerge. (**g**) A branched discharge tube with flared openings. (**h**) Zoospores in the second motile stage. (**i**) Encysted zoospores after the second motile stage. (**j**) Germination. *Scales*: (**a**, **e**) 150 μm; (**b**) 70 μm; (**c**, **d**, **g**–**j**) 50 μm; (**f**) = 40 μm

Roza and Hatai (1999) reported that heavy mortalities reaching 100% among larvae of the Japanese mitten crab, *Eriocheir japonicus*, occurred in Yamaguchi Prefecture, Japan. Under the microscope, infected zoeal larvae were filled with numerous aseptate hyphae. The infected fungus was inoculated on PYGS agar and incubated at 25°C for 7–10 days. Colonies on PYGS agar were attaining a diameter of about 25 mm in 15 days, crystalline, tuberculate, and moist; moderately heaped at the center. Mycelia in the broth were aseptate, radially branched, stout, swollen up to 150 μm in diameter, with clusters of shiny spherical granules, without oil droplets and vacuoles. Granular clusters were evenly distributed inside mycelia, generally consisting of several granules. Mycelia in seawater developing narrow branches (discharge tubes) were followed by zoospore production. Gemmae were present. Zoospores in the first motile stage were produced after 30 h at 25°C. Protoplasmic masses due to gathering of granular clusters on zoosporogenesis were supported at the center of zoosporangia by several protoplasmic threads; differentiated into loose networks of zoospores, then into free individual zoospores in the first motile stage. Zoosporangia were the same in size and shape as the mycelia, with several discharge tubes extending from each zoosporangium. Zoospores in the first motile stage were swimming dully and encysting within zoosporangia and discharge tubes, and biflagellate, subglobose to globose, 3–6 μm in size. Zoospores in the second motile stage were released one by one from encysted zoospores within zoosporangia and discharge tubes, swimming freely for a long time; laterally biflagellate, pyriform, slipper-shaped, isokont, 2–7 μm. Zoospores were dimorphic and diplanetic. Encysted spores were globose to subglobose, 3–7 μm in the first motile stage and 3.5–6 μm in the second motile stage. Discharge tubes were unbranched or occasionally branched, straight or tapering with flared openings, rarely with a central swelling, 4–9 μm in width, 5–16 μm in length. Germination was observed at 6–8 h after spores with slender germ tube were transferred to broth. This fungus was identified as *A. dubia*. This was the first report of mass mortality in crustaceans due to *A. dubia* infection. The optimum growth temperature was at 25°C, and grew only on PYG agar containing 2.5% NaCl and PYGS agar.

2.2.6 Pythium *Infection*

This infection was first reported as *Lagenidium myophilum* infection from marine shrimp (Hatai and Lawhavinit 1988). Later, Muraosa et al. (2009) made clear that the fungus was included into the genus *Pythium* by phylogenic tree. *Pythium myophilum* (*Lagenidium myophilum*) infection occurred in the abdominal muscles and swimmerets of adult northern shrimp, *Pandalus borealis*, cultured at the Japan Seafarming Association (JASFA). Pure cultures of *P. myophilum* were consistently isolated from the partly blackened abdominal muscle (Fig. 2.17) and the inside of the swimmerets of the adult northern shrimps. Growth of the fungus on PYGS agar was observed at 2 days after incubation. Microscopical observation of the blackened areas of the lesions showed them to be filled with hyphae and the pathogenic

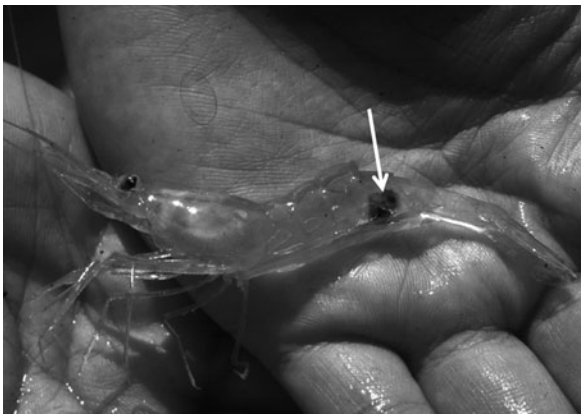

Fig. 2.17 *Pythium myophilum* isolated from the partly *blackened* abdominal muscle (*arrow*)

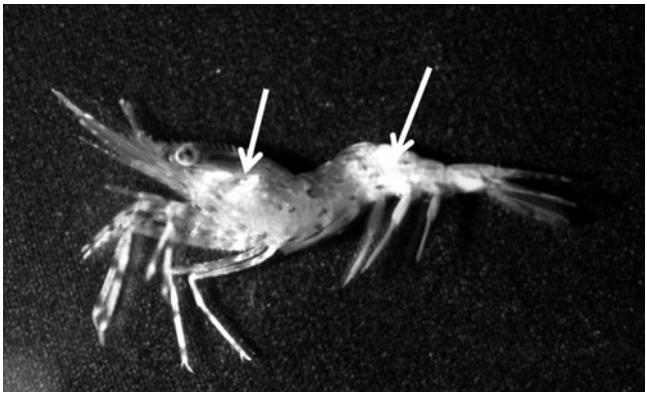

Fig. 2.18 A juvenile coonstripe shrimp infected with *Pythium myophilum*. The lesions look *whitish* (*arrows*)

fungus to grow only in the tissue of shrimp. The optimum temperature for growth of this fungus was 25°C , but it also grew at the low temperature of 5°C. It would thus be able to infect northern shrimps living in cold seawater; the temperature of the Japan Sea was approximately at 5°C. In pure culture, the hyphae were somewhat uniform with a diameter of 7–10 μm and generally vacuolated. Vesicle formed at the end of discharge tube were measuring 86–240 × 7–10 μm in diameter. Zoospores were 12.9 × 9.6 μm, globose, reniform, pyriform or elongate, monoplanetic and laterally biflagellate. Encysted zoospores were spherical, 5.5–12.0 μm in diameter. Sexual reproduction was not observed.

In 1991, a fungal infection occurred in the larvae of coonstripe shrims, *Pandalus hypsinotus*, artificially produced at Hokkaido in Japan. Mortality was 100%. In 1993, the infection also occurred in juvenile coonstripe shrimps (Fig. 2.18), which had been reared in tanks after seed production. Mortality was about 70%

(Nakamura et al. 1994a, b). The pathogenic fungi isolated from the lesions were same as those caused by *Pythium myophilum* reported by Hatai and Lawhavinit (1988). *P. myophilum* is pathogenic toward adult northern shrimp, larval and juvenile coonstripe shrimps and Hokkai shrimp, *Pandalus kessleri* (Hatai, unpublished). *P. myophilum* infections have only been in Japan, and these shrimps of the genus *Pandalus* are known to live only in the deep areas of the sea off the coast of Japan. It was interesting that these hosts seemed to be highly sensitive to *P. myophilum*.

2.3 Diseases of Fish and Shellfish Caused by Mitosporic Fungi

2.3.1 Fusarium *Infection*

Some species in the genus *Fusarium* such as *Fusarium solani*, *F. moniliforme*, and *F. oxysporum* have been isolated from kuruma prawn, *Penaeus japonicus*, with black gill in Japan. Among these species, *F. solani* subsequently was reported as an important pathogen.

F. solani was originally proposed as a genus *Fusarium* (Wollenweber 1913). Later the section included five species, ten varieties, and four forms (Wollenweber and Reinking 1935). Snyder and Hansen (1941) combined three species (*F. solani*, *F. martti*, and *F. coeruleum*) into *F. solani*. This taxonomy, however, was not approved by Gerlach and Nirenberg (1982). Booth (1971) and Gerlach and Nirenberg (1982), included four and six species in the section *Martiella*, respectively, from conidiogenesis and shapes of conidia which are major criterions for the classification. The main species, *F. solani*, in the genus *Fusarium* has been later reported in the literatures as *formae specials* (f. sp.), mating population (MP 1–MP VII), or anamorph of *Nectria haematococca* due to its polytypic appearances (Matuo and Snyder 1973). Because of its pathogenic importance, studies on the biological specification of *F. solani* species has also been developed (O'Donnell 2000). Previous studies on the taxonomy of this complex fungal species have contributed valuable information on the limits of the specification and evolutionary relationships within species, *F. solani* (Matuo and Snyder 1973; Hawthorne et al. 1992; Suga et al. 2000; O'Donnell and Gray 1995; O'Donnell 2000). However, *F. solani* isolated from aquatic animals including marine crustaceans and fishes have never been studied in detail in previous reports.

Black gill disease of pond-cultured kuruma prawn, *Penaeus japonicus*, was first reported in Japan (Egusa and Ueda 1972). They demonstrated that a fungus belonged to the genus *Fusarium* was the causative agent and gave the fungus a temporary designation, BG-Fusarium. Since their report, the disease has often broken out among pond cultured kuruma prawn in various districts. Hatai et al. (1978) investigated a taxonomical position of the BG-Fusarium isolated from gill lesions of kuruma prawn with black gill disease (Fig. 2.19). The fungus produced micro- and macro-conidia on conidiophores and chlamydospores. As a result, the

Fig. 2.19 Kuruma prawn infected with *Fusarium solani*. Note gills showing *black color*

BG-Fusarium was identified as *Fusarium solani* according to Booth (1971, 1977) from the characteristics of the fungus on Potato Sucrose Agar. They demonstrated that the pathogenic fungus could be isolated from wet sand in ponds with fungal infection, but not from it without fungal infection, and was capable of surviving for long time in wet sand. Khoa et al. (2005) also reported *Fusarium solani* infection of kuruma prawn (Fig. 2.20). They demonstrated that the fungus showed pathogenicity to kuruma prawn by intramuscular injection. Phylogenetic analyses based on the sequences of its internal transcribed spacer region, including 5.8 S ribosomal DNA and a partial 28 S ribosomal DNA region, showed that all strains tested were monophyletic. And the strains isolated from the diseased kuruma prawn and the phytopathogenic *Fusarium solani* were clearly distinguished by the morphological and phylogenetical characteristics (Khoa et al. 2005).

Fusarium moniliforme was also isolated from gill lesions of kuruma prawn with black gill disease at a private farm in Japan (Rhoobunjongde et al. 1991). The colonies of the fungus cultured on upper surface of potato dextrose agar (PDA) were floccose, creamy white, undersurface a lavender to violet, but did not grow on mycobiotic agar containing cycloheximide. Fungal hyphae were hyaline and 2.5–6.0 μm in diameter. Conidiogenous cells with long monophialides were abundantly formed laterally on aerial mycelium or on sympodially branched conidiophores, and were hyaline, subulate 2.0–4.0 μm in diameter. Macroconidia were present, but only rarely and their appearance varied from slightly sickle- to cigar-shaped, three to four septa, rarely five septa and 26.0–50.0 μm in length. Microconidia were abundant and variable on shape and size from ovoid to elliptical, zero to one septa, rarely two septa, 6.0–20.0 μm in length, and were produced in chains mostly from a simple conidiophores (Fig. 2.21) and false heads on PDA and KCl medium but especially with longer chains on the KCl medium. Chlamydospore was absent. This was the first case of *F. moliniforme* infection in crustacean.

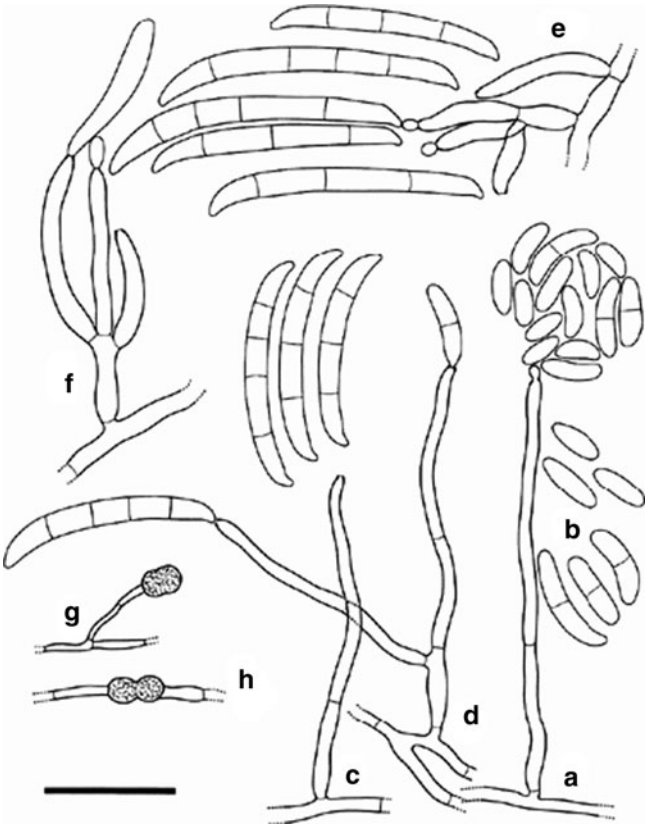

Fig. 2.20 Microscopic morphology of *Fusarium solani* isolated from an infected *Penaeus japonicus*. Scale bar = 25 μm. (**a**) Aerial conidiophores is long and unbranched, slightly narrow toward the apex, monophialidic, producing abundant zero to one-septate conidia that cohere in a false head. (**b**) Oval or ellipsoid one-cell conidia and subcylindric or slightly curved two-cells conodia. (**c**) Unbranched aerial conidiophores bear three to four-septate conidia and are slightly curved with a short and blunt apical cell and slightly notched basal cell. (**d**) A lateral branched aerial conidiophores producing one to four-septate conidia. (**e**) Irregularly and verticillately branched sporodochial conidiophores, bearing monophialides and producing three and four-septate conidia. Conidia extend from the basal part and curve to the apex. The dorsal side is more curved than ventral side, and there is a blunt apical cell and slightly notched basal cell. (**f**) A sporodochial conidiophores verticillately forming monophialides in the early stage of sporulation. (**g**) Terminal chlamydospore from conidiophores is smooth-walled, globose. (**h**) Intercalary chlamydospores in the hyphae are smooth-walled, globose, and in a pair

Khoa and Hatai (2005) reported *Fusarium oxysporum* infection in cultured kuruma prawn *Penaeus japonicus* in Japan. The infection was the first case in kuruma prawn. The infected prawn showed black gills, but the other apparently looked healthy. Fungal hyphae with septa and canoe-shaped conidia were clearly observed in wet-mount preparations of the prawns with black gills. Colony of the *F. oxysporum* grew well on PDA at 25°C. Mycelia were delicate, felt-like, and

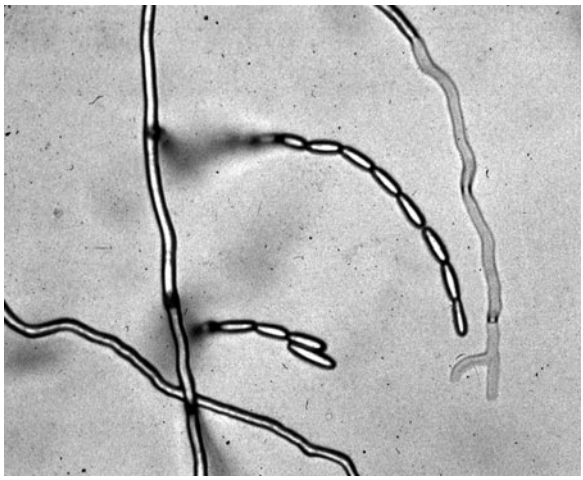

Fig. 2.21 Microconidia are produced in chains mostly from simple conidiophores

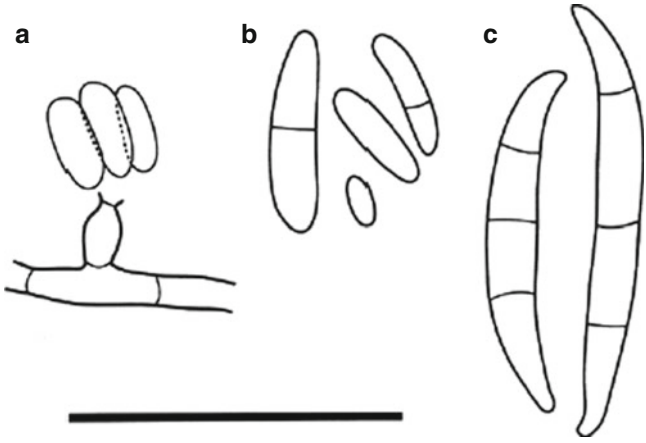

Fig. 2.22 Morphology of *Fusarium oxysporum* . Scale bar = 30 μm. (**a**) Short aerial conidiophore, unbranched, monophialidic conidiophore, producing one-cell conidia in false head. (**b**) Aerial conidia with one to two cells. (**c**) Three-septate sporodochial conidia, tapering toward both ends with a pointed apical cell and a slightly hooked basal cell

funiculous flat appressed. Pigment on PDA was white at first, gradually changed to pale beige in the center of the agar plate, and purple or dark violet in the aged cultures. Chlamydospores were not observed. Arial conidiophores were usually single, unbranched and mostly short, 5–15 μm in length, and produced one cell or two cells conidia in false head at 4-day culture (Fig. 2.22). Aerial conidia were usually oval, cylindrical or ellipsoid, straight or slightly curved. One-cell conidia were $5 \pm 2.5 \times 2.5 \pm 0.5$ μm, and two cells conidia were $8.5 \pm 3.5 \times 0.7$ μm. Sporodochial conidiophores were occasionally observed on SNA cultures (Nirenberg 1990), and monophialidic, irregularly or verticillately branched, and

produced three to five septate conidia, predominantly three septate conidia. Sporodochial conidia were usually curved, equally tapering toward both ends with a pointed apical cell and a slightly hooked basal cell. Three septate conidia were 27 ± 3.7 × 2.7 ± 0.3 μm. The prawns artificially injected with *F. oxysporum* showed typical black gills, and the clinical sign was similar to that of prawn naturally infected with fungus.

F. oxysporum infection was also found in cultured red sea bream, *Pagrus major*, in Japan (Hatai et al. 1986). In almost all cases, no external signs were observed, but kidneys of the fish were remarkably swollen and discolored. The other organs, however, appeared to be normal. The fungus was isolated by inoculating a piece of kidney on Sabouraud dextrose agar (SA agar) at 25°C, and a pure culture was obtained. The fungus was identified as *Fusarium oxysporum* as described by Booth (1971).

In Vietnam a new *Fusarium* infection occurred in black tiger shrimp, *Penaeus monodon* (Khoa et al. 2004). Infected shrimps showed typical signs of black gill disease and mortalities about a month prior to harvest. The isolated fungus was identified as *F. incarnatum* from the detailed morphological and molecular phylogenic analyses. The fungus showed the pathogenicity to kuruma prawn by experimental infection. Optimal temperature for the fungus ranged from 20 to 30°C. The fungus grew drastically at 35°C, but did not at 5 and 40°C.

2.3.2 *Ochroconis Infection*

The fungal infection in fishes caused by *Ochroconis humicola* was first reported from the kidney of coho salmon, *Oncorhynchus kisutch* (Ross and Yasutake 1973). Later, the infection was reported from rainbow trout, *Salmo gairdneri* (Ajello et al. 1977), Atlantic salmon, *Salmo salar* (Schaumann and Priebe 1994).

In Japan, *Ochroconis humicola* infection has been found in marine cultured fish. First description was from devil stinger, *Inimicus japonicas* (Wada et al. 1995). The diseased fish were about 1.4 g in body weight, and had some ulcers on the body surface. The fish examined showed little appetite, but no mortality was recorded. The center of the lesion was necrotic and sloughed, leaving trunk muscles exposed in a crater-shaped cavity surrounded by an erosion periphery. Direct microscopical examination of the exposed trunk muscles revealed numerous septate fungal hyphae. Fungal colonies were slow growing, slightly domed, velvety to floccose, and pale brown in color. Hyphae were septate, pale brown in color, and 1–2 μm in width. Conidia were usually sparse, 1.8–2.2 × 7.0–10.0 μm, two-celled, pale brown in color and cylindrical with rounded ends. The reproductive mode of the conidia was sympodial. The fungus was identified as *Ochroconis humicola* according to de Hoog and von Arx (1973) and Howard (1983). Later, *O. humicola* infection was found in marine-cultured fish, red sea bream, *Pagrus major*, and marbled rockfish, *Sebasticus marmoratus* (Wada et al. 2005). The average body weight of the fish examined was 1.2 g for red sea bream and 1.0 g for marble

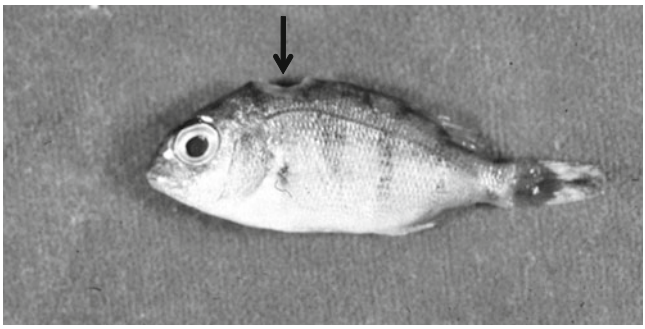

Fig. 2.23 Ochroconis infection in red sea bream. Severe ulceration (*arrow*) formed around the base of the dorsal fins

rockfish. Both cases showed apparent lesions on the body surfaces. In the red sea bream, severe ulceration was found around the base of the dorsal fin (Fig. 2.23), while erosive and/or ulcerative lesions mainly appeared at the mouth regions in the marbled rockfish.

In April 2004, a fungal infection occurred in cultured young striped jack, *Pseudocaranx dentex* at a fish farm in Japan (Munchan et al. 2006). The water temperature in this month was 17–18.5°C. The examined 0-year-old fish were 6–10 cm in body length and 5–10 g in body weight. Moribund fish with fungal infection showed disease sign such as distended abdomen kidney. Numerous brownish hyphae were found in squash preparation of the kidney under microscopy. The cumulative mortality of the disease reached 25% (62,000 out of 250,000 fish) for 1 month after the disease was recognized. Histopathology showed that fungal hyphae were found in the musculature, spleen (Fig. 2.24) and kidney. The granulomas consisted of massive fungal elements and outer layers surrounded by epitheloid cells. No bacteria or parasites were found in the examined tissues. Munchan et al. (2009a) compared the histopathology of young striped jack experimentally infected with dematiaceous fungus *O. humicola* with that of spontaneously infected fish. Moribund and freshly dead fish from both groups showed similar histopathology, and appeared to have been killed due to hyphae penetrating the visceral organs. Fish that survived the infection appeared to be able to suppress the fungal growth by well-established inflammatory reaction involving mycotic granulomas and granulation tissues. The results suggested that two types of *O. humicola* infection occur in young striped jack: an acute type infection, which is characterized by penetrating hyphae that cause direct tissue destruction and a chronic type infection, which is characterized by severe inflammatory reaction that causes function disorders of the affected organs. All fungi isolated from diseased fish were identified as the same fungus. Colony of the isolate showed dark brown to black color when observed from the reverse side of the plate and no visible exudates diffused into the medium, and it was flat, very slow-growing on PDA plate. Colony radii on PDA incubated at 25°C reached 30.1 mm after 4 weeks. Hyphae were septate, 2–3 μm in diameter, pale brown in color, and aerial hyphae

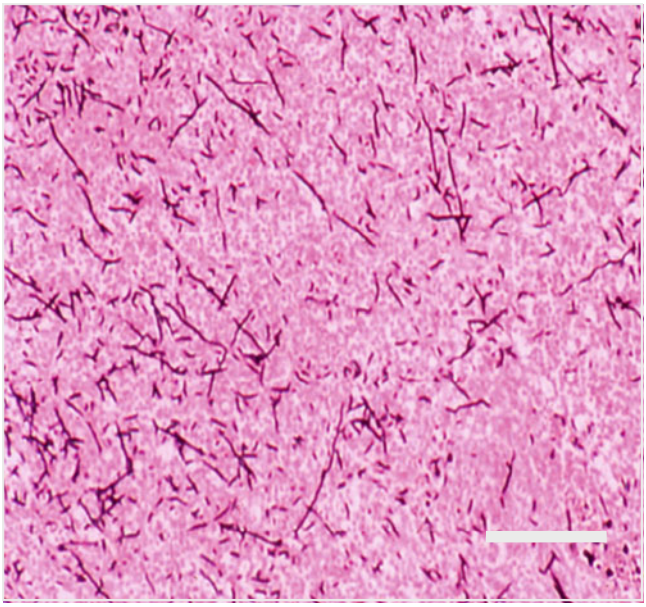

Fig. 2.24 Histopathological finding of spleen in diseased fish. Note many fungal hyphae in the spleen. Grocott-HE stain, Bar = 100 μm

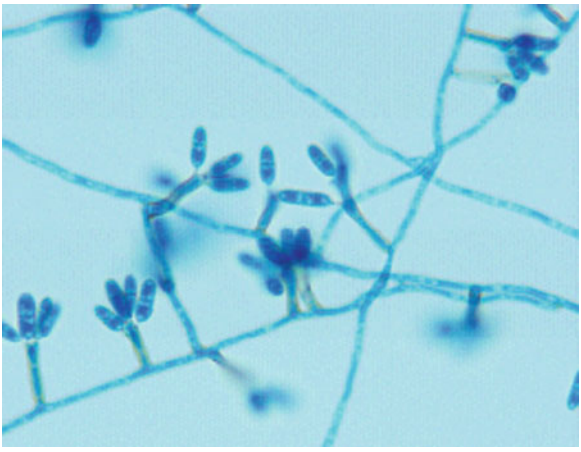

Fig. 2.25 Conidia of *Ochroconis humicola*: two-celled, cylindrical to oblong, constricted at the septum

were sparse. Conidiophores were predominantly cylindrical, average 2.5 × 7.5 μm had denticles at each tip. Conidia were holoblastic, two-celled, cylindrical to oblong with rounded ends, average 2.5–4.5 × 5.5–12.5 μm, smooth-walled, and pale brown in color (Fig. 2.25). The isolate was as *Ochroconis humicola* from these characteristics. The isolate grew at 10–30°C, but not at 35°C. The isolate could grow up to 9% NaCl indicating that *O. humicola* could grow in an environment with

a wide range of salinity. Itraconazole (for oral administration), with an MIC (MFC) range of 0.06–0.13 (0.0625–0.125) μg/mL was chosen for in vivo treatment. In vivo treatment with itraconazole of striped jack experimentally infected with *O. humicola* was conducted for 50 days. No fish died, but gray to white nodules were found in the visceral membrane, kidney, liver, and spleen in the fish. Granulomatous inflammatory reactions were histopathologically found in all fish injected with conidia of *O. humicola*. Clinical signs and histopathological findings indicated that itraconazole showed no efficacy for curing the fish infected with *O. humicola* (Munchan et al. 2009b).

2.3.3 Exophiala *Infection*

Fungal infection caused by the genus *Exophiala*, known as black yeast, has been reported in several species of fish. The first report was by Carmichael (1966) who described a systemic infection of cutthroat trout, *Salmo clarki*, and lake salmon, *Salvelinus namaycush*. The causative agent was initially named a *Phialophora*-like fungus but later classified as *Exophiala salmonis*. Fijan (1969) reported a systemic mycosis in channel catfish, *Ictalurus punctatus*, later the fungus was identified as *E. pisciphilus* (McGinnis and Ajello 1974). Later, *E. salmonis* infection was reported from Atlantic salmon, *Salmo salar* (Richard et al. 1978; Otis and Wolke 1985). On the other hand, *E. pisciphila* infection was reported from smooth dogfish, *Mustelus canis* (Gaskins and Cheung 1986), Atlantic salmon (Langdon and McDonald 1987). *E. psychrophila* infection was also reported from Atlantic salmon (Pedersen and Langvad 1989).

In Japan, *Exophiala* infection occurred in cultured striped jack, *Pseudocaranx dentex*, in 2005 (Munchan et al. 2009c). One hundred out of 35,000 fish died per day and mortalities continued for 1 month. Diseased fish showed swelling of the abdomen and kidney distension. Microscopic examination of the kidney of diseased fish revealed numerous septate hyphae, pale brown in color, in squash preparations. Histology revealed abundant fungal hyphae and conidia in gill, heart, and kidney. Fungal hyphae were accompanied by cell necrosis and in influx of inflammatory, mainly mononuclear cells. The fungus isolated from the diseased fish had septate hyphae, pale brown in color and 1.8–3.0 μm in diameter. The colony morphology of the fungus after 1 week of incubation on PDA at 25°C was initially a black yeast form. It then became woolly and velvety and olive brown in color but black on the reverse side after 4-week incubation. Conidiogenous cells were conspicuous annellides (Fig. 2.26), short or cylindrical or fusiform in shape. Conidia were one-celled, ellipsoidal with smooth walls, accumulated in balls at the apices of annellides that tended to slide down, 1.5–2.0 μm in width and 3.0–5.0 μm in length. The fungus was classified into the genus *Exophila* based on its morphology and as *Exophiala xenobiotica* based on sequences of the ITS1-5.8S-ITS2 regions of rDNA. This is the first record of this fungus in a marine fish.

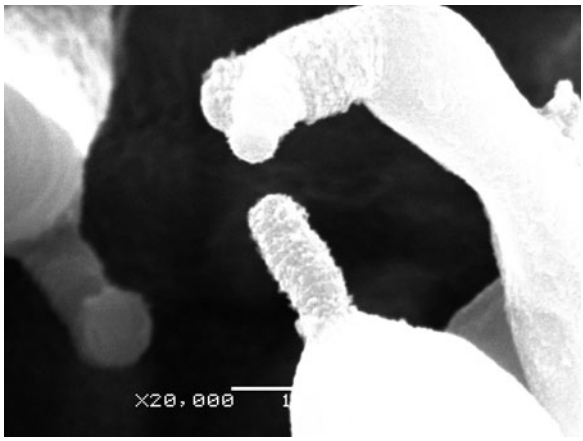

Fig. 2.26 Conidiogenous cells: conspicuous annellated zones, short or cylindrical

Fig. 2.27 The lesion was only limited in the skin, which is involving ulcerative skin lesion (*arrow*) in the fish

On the other hand, different *Exophiala* infection occurred in Japanese flounder, *Paralichthys olivaceus* in Japan (Kurata et al. 2008). The lesion was only limited in the skin, which is involving ulcerative skin lesions in the fish (Fig. 2.27). The water temperature during the period was approximately 17–21°C. A dematiaceous fungus was only isolated from the fish skin with ulcerative and erosion. The fungal colonies were dark brown to olive black in color. The fungus produced conidia (2.0–3.0 × 2.7–5.0 μm) of an elliptic or obovoid shape and with no or one septum. Conidia were formed as a cluster on the tip of conidiogenous cells. Annellations on the tip of conidiogenous cells were observed under scanning electron microscopy, but were inconspicuous under light microscopy. The fungus grew well at 25°C, but no growth was observed at 37°C. The fungus was identified as an *Exophiala* species, with different morphological, biological and molecular characteristics from three previously described pathogenic *Exophiala* species. The fungus had

a high similarity of 99.6% with *Capronia coronate* from the phylogenetic tree of *Exophiala* spp. based on the sequence of the D1D2 domain of large subunit ribosomal DNA (LSU rDNA). Histology showed that fungal hyphae extended laterally in the dermis, and were absent from the epidermis and musculature of the skin lesions and kidneys of the diseased fish. An inflammatory response with granuloma occurred in the dermis involving accumulations of epitheloid cells around the hyphae. The granulomas were surrounded by lymphocyte-like cells. Epidermal degeneration was observed above the inflamed dermis, suggesting that the inflammatory response caused epidermal damage. Experimental infection reproduced hyphal extension and infiltration of inflammatory cells in the dermis of the flounder, confirming the pathogenicity of the fungus.

2.3.4 Scytalidium *Infection*

Iwatsu et al. (1990) reported first *Scytalidium* infection in striped jack, *Pseudocaranx dentex* with systemic mycosis in Japan. The external clinical signs were blackish patches and ulcers formed on the surface, especially at the basement of dorsal fin, at the tip of snout, and the anal area (Fig. 2.28). No apparent clinical signs were found in the internal organs. Numerous pale brown, septate hyphae, and arthroconidia were found in the lesions of the surface and various internal organs by direct microscopical examination. The fish was reared in sea water with a temperature of about 18°C. The mortality was about 6% of the original population. A fungus was isolated from the lesions of the surface and the internal organs. Experimental infection using striped jack showed that the fungus was a causal agent of the mycosis. The fungus was isolated on PYGS agar. The colonies were dark green and conidia showing dark green were abundantly produced. Mycelium immersed or superficial, composed of straight or sinuous, sometimes curled, smooth, cylindrical, hyaline to mid-brown, branched, rather thick-walled, septate. Stromata were absent. Conidiophores were micronematous, mononematous, straight or flexuous, hyaline to pale brown and branched or unbranched, smooth-walled. Conidiogenous cells were undifferentiated, fragmenting and forming

Fig. 2.28 *Scytalidium* infection in striped jack, *Pseudocaranx dentex* with systemic mycosis. The external clinical signs were *blackish patches* and ulcers formed on the surface, especially at the basement of dorsal fin, at the tip of snout, and the anal area

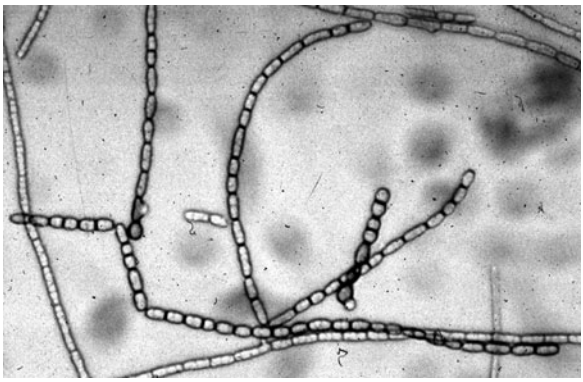

Fig. 2.29 Arthroconidia of *Scytalidium infestans* formed in extended chains

arthroconidia. Arthroconidia of one-type, formed in extended chains (Fig. 2.29), hyaline to mid-brown, dry, simple, rather thick-walled, smooth or verrucose, oblong, dolioform or broadly ellipsoidal, truncate at both ends, 0-1(-3) septate, not easily detached. Chlamydospores were absent. It did not grow at 37°C. As a result, the fungus was a new species of the genus, and named *S. infestans*.

The genus *Scytalidium* was originally erected by Pesante (1957), based on *S. lignicola*. The fungus was characterized by possession of arthroconidia of two types: hyaline, thin-walled, cylindrical ones formed by fragmentation of undifferentiated hyphae, and brown, thick-walled, broadly ellipsoid ones borne in an intercalary fashion. The generic concept was expanded when Sigler and Carmichael (1974) described *S. acidophilum*, a species possessing only dematiaceous arthroconidia. They considered that the genus was characterized by dematiaceous intercalary or terminal arthroconidia formed by fragmentation of undifferentiated hyphae and that the presence of hyaline arthroconidia was not essential for the genus delimitation.

Futhermore, *Scytalidium infestans* infection was first found in red sea bream, *Pagrus major* (Hanjavanit et al. 2004) in Japan. Ulcerative lesions were observed from head to dorsal part of the body surface of red sea bream. Histopathologically, numerous, frequently septate fungal hyphae were observed in the lesions. A fungus was isolated in pure culture from each lesion using PYGS agar. Colonies were dark green in color, and arthroconidia formed in extended chains. It was identified as *S. infestans* according to Iwatsu et al. (1990).

2.4 Infection in Mantis Shrimp by Mitosporic Fungi

The mantis shrimp, *Oratosquilla oratoria*, is an economically important and delicious culinary crustacean species. One of the famous Japanese, sushi dishes, is made from the meat of mantis shrimp. This shrimp is living in mud in the coastal areas of Japan and is the most dominant species. Fungal infection of mantis shrimp

has never been reported in Japan, but it has been known that many mantis shrimp died and the production decreased from 1991. Moribund mantis shrimp were sampled and examined. As a result, it was made clear that the mortality was caused by fungal infection (Duc et al. 2009). They had fungal infection in the gills. Gills of almost mantis shrimp with naturally fungal infection showed brown discoloration (Fig. 2.30). Some gills disappeared due to the fungal infection. Numerous conidia and hyphae inside the gill lamella were observed under microscope (Fig. 2.31). The results of histological examination showed that fungal elements were present in the gills. Fungal hyphae were encapsulated in base of gills.

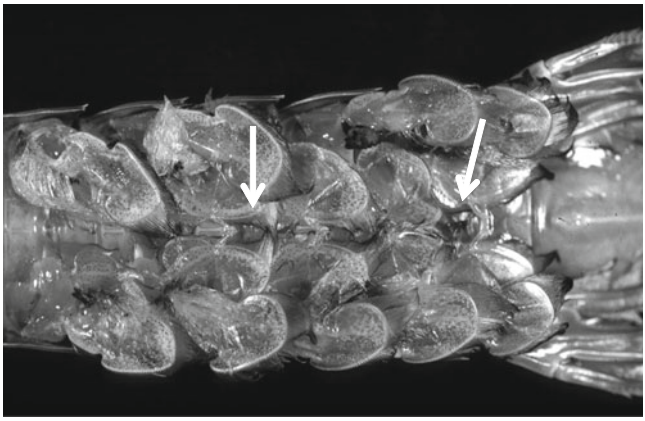

Fig. 2.30 Gills of most mantis shrimp with naturally fungal infection showed *brown discoloration* (*arrows*)

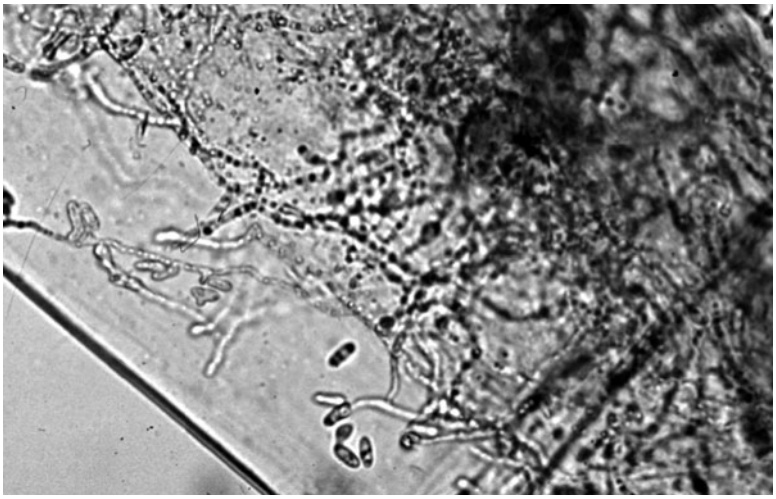

Fig. 2.31 Numerous conidia and hyphae inside the gill lamella observed under microscope

Infected gills were washed three times in sterile physiological saline (0.85% NaCl) and inoculated on PYGS agar. Ampicillin and streptomycin sulfate were added to the medium to inhibit bacterial growth. Plates were incubated at 25°C for 2–4 days. The single spore culture method was applied to obtain pure culture (Ho and Ko 1997). As a result, two kinds of fungi were isolated from the lesions. They were easy to recognize in culture by their growth. The morphological and physiological characteristics, and DNA analysis and sequencing were examined. One of them showed slow growth, and was identified as a new species, *Plectosporium oratosquillae*. The other one exhibited fast growth, and was identified to *Acremonium* sp. (a member of the *Emericellopsis* marine clade) from phylogenetic analysis of ITS and β-tubulin sequences. And it was also a new species, but teleomorph development in culture failed. It was the first report on fungal infection in mantis shrimp (Duc et al. 2009).

Duc and Hatai (2009) carried out experiments to determine pathogenicity of anamorphic fungi *Plectosporium oratosquillae* and *Acremonium* sp., which were isolated from gills of marine shrimp *Oratosquilla oratoria* caught in Japan. Cumulative mortality of the mantis shrimp injected with a high dose (5.0×10^6 conidia/mL) and a low dose (5.0×10^4 conidia/mL) of *P. oratosquillae* reached 100% and 60% at day 25, respectively. Cumulative mortality of the shrimp injected with the high dose and the low dose of the *Acremonium* sp. reached 100% and 80% at day 25, respectively. The gill lesions in the shrimp experimentally infected with the fungi showed many brown spots in the gill filaments, which were similar to the clinical sign of mantis shrimp naturally infected with the fungi. Histopathologically, the hyphae and conidia were found in the gill filaments and heart, and the hyphae were encapsulated by hemocytes in the gill filaments and the base of gills. The result confirmed that these two anamorphic fungi were pathogenic to mantis shrimp.

Duc et al. (2010a) demonstrated the pathogenicity of both the fungi isolated from mantis shrimp to kuruma prawn *Penaeus japonicus* by intramuscular injection of conidial suspensions. These fungi caused mortality in the injected kuruma prawn. Especially cumulative mortality in kuruma prawn injected with 0.1 mL of a conidial suspension with 5×10^6 conidial/mL of *Acremonium* sp. reached 100%. The results indicated that the both fungi were also pathogenic to kuruma prawn. The prawn is important cultured crustacean in Japan, and lives in the same environmental conditions.

Acremonium sp. isolated from diseased mantis shrimp was susceptible *in vitro* to three kinds of antifungal agents: voriconazole, amphotericin B, and terinafine hydrochloride (Duc et al. 2010b). They selected voriconazole to treat kuruma prawn, which had been intramuscularly injected with 0.1 mL of 5.0×10^4 conidia/mL of *Acremonium* sp. Voriconazole was administered orally at doses of six and 2 mg/kg body weight per 7 consecutive days, or intramuscularly injected at doses of 4 and 2 mg/kg body weight per day for 3 consecutive days. Both treatments were started 6 h after injection of the conidial suspension. They demonstrated that voriconazole was an efficient antifungal agent against *Acremonium* sp. from the gross features, mortality, and histopathological observations.

2.5 Future Research

The culture of fish and shellfish and their seed production are important industries in Japan. Diseases caused by fungi result in significant economic losses. Thorough descriptions of many important diseases of fish and shellfish and procedures for identification have been presented in this chapter. It is hoped that these data will be helpful for future research in Japan and in other parts of the world desiring to increase production of commercial fisheries. Some of these obligate host–parasite associations offer excellent tools for research on disease development at cellular and molecular levels. The defense reactions of animals that escape infection will be worth investigating.

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