

FULL PAPER

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A new peronosporomycete, *Halioticida noduliformans* gen. et sp. nov., isolated from white nodules in the abalone *Haliotis* spp. from Japan

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Abstract Four strains belonging to the Peronosporomycetes (formerly Oomycetes) were isolated from white nodules found on the mantle of three species of abalone. In artificial seawater, the four isolates formed fragments such as in the genus *Haliphthoros*, but the protoplasm constriction was weaker, and fragments were longer, with smaller spaces between them, than those of *Haliphthoros*. The four strains form one or more discharge tubes from each zoosporangium. The four strains were similar, but not identical, to the genus *Haliphthoros* based on morphological characteristics. As a result, the four isolates were classified in a new genus and species, *Halioticida noduliformans* gen. et sp. nov. Phylogenetic analysis of the D1/D2 region of the large subunit ribosomal RNA gene (LSU rDNA) was performed, and the four isolates showed 100%–99.8% concordance. In the phylogenetic tree, the four isolates were not classified in the subclass Peronosporomycetidae, Saprolegniomycetidae, or Rhipidiomycetidae. However, the four isolates formed a new clade with genera *Haliphthoros* and *Halocrusticida* in Peronosporomycetes. Within this new clade, the four isolates, *Haliphthoros* spp. and *Halocrusticida* spp., were grouped in their respective independent subclades. These results showed that these were the new genus and species from the morphological characteristics.

Key words Abalone · D1/D2 region of LSU rDNA · *Halioticida noduliformans* · *Haliotis* spp. · Peronosporomycetes

Introduction

The class Peronosporomycetes (formerly Oomycetes) 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. *Haliphthoros milfordensis* Vishniac, *Halocrusticida awabi* (Kitancharoen et al.) Nakamura & Hatai, and *Atkinsiella dubia* (Atkins) Vishniac have been reported as causative agents of such diseases in *Haliotis sieboldii* Reeve (Hatai 1982; Kitancharoen et al. 1994; Nakamura and Hatai 1995b). The taxonomic position of genera *Haliphthoros* Vishniac, *Halocrusticida* Nakamura & Hatai, and *Atkinsiella* (Atkins) Vishniac in the class Peronosporomycetes has been in confusion. Dick (2001), however, classified them into Haliphthoraceae – Salilagenidiales – Saprolegniomycetidae in his new taxonomy system of Peronosporomycetes.

The taxonomy of the Peronosporomycetes has been based on morphological characteristics of asexual and sexual reproductive structures. Unfortunately, sexual reproduction has not been found in the Peronosporomycetes isolated from marine invertebrates. Furthermore, asexual reproduction often declines with repeated subculturing. Recently, DNA sequence-based molecular phylogenetic studies of the Peronosporomycetes have been carried out to assist morphologically based taxonomy. Nuclear ribosomal RNA gene regions such as the small subunit ribosomal RNA gene (18S rDNA), the D1/D2 region of the large subunit of ribosomal DNA (LSU rDNA), and internal transcribed spacer (ITS)1-5.8S-ITS2 regions have been used to analyze phylogenetic relationships in the Peronosporomycetes (Dick et al. 1999; Riethmüller et al. 1999, 2002; Cooke et al. 2000; Petersen and Rosendahl 2000; Sekimoto et al. 2007). In these gene regions, the D1/D2 region of LSU rDNA was used for the identification and classification of genus in fungi such as Geoghefferales (Bauer et al. 2005) and was also used for the phylogenetic relationships of

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genera in Peronosporomycetes (Voglmayr et al. 2004). Mitochondrially encoded cytochrome *c* oxidase subunit 2 (*cox2*) gene sequences have also been used to analyze phylogenetic relationships in representative marine Peronosporomycetes (Hudspeth et al. 2000; Cook et al. 2001; Sekimoto et al. 2007).

From January 2004 to January 2006, three species of abalone, *Haliotis midae* Linnaeus imported from the Republic of South Africa, *Haliotis rufescens* Swainson imported from the Republic of Chile and the United Mexican States, and *Haliotis sieboldii* collected at Nagasaki, Japan, died from infection. They were stocked for sale in the same tank in Chiba Prefecture, Japan. Several moribund abalones about 64.0 g in body weight were examined. White nodules with thick and aseptate hyphae were present on the mantle.

In this study, we attempted to isolate the causative Peronosporomycetes from lesions of infected abalone (*Haliotis* spp.) to study the morphological characteristics and to perform molecular phylogenetic analyses of the D1/D2 region of LSU rDNA.

Materials and methods

Isolation

Tissues from white nodules were stained with Fungiflora Y (Biomate, Tokyo, Japan), and observed under a fluorescence microscope. Portions of the white nodule were inoculated on PYGS agar plates [0.125% peptone, 0.125% yeast extract, 0.3% glucose, 1.2% agar, and 37.6 g artificial seawater (Aqua-Ocean; Japan Pet Drugs, Tokyo, Japan)]. Powdered streptomycin sulfate and ampicillin were directly added on the PYGS agar plate. After 3 days incubation at 15°C, agar blocks at the edge of growing colonies were transferred onto a fresh PYGS agar plate. Before the commencement of experiments, one spore culture was performed to make a pure culture.

Morphological characteristics

For morphological observation, isolates were inoculated into PYGS broth and incubated at 15°C for 4 days. To observe zoospore formation, mycelia were rinsed three times in sterile artificial seawater before being transferred into sterile artificial seawater and then incubated at 15°C for 24 h. Isolates were identified according to Sparrow (1960), Karling (1981), and Nakamura and Hatai (1995b).

Effect of temperature on growth

Each isolate was inoculated onto a PYGS agar plate and incubated at 15°C for 10 days to make a giant colony. Agar blocks were taken from the edge of a growing colony with a cork borer (5.5 mm diameter), and fresh PYGS agar plates were inoculated at the center. Each plate was incubated at

one of seven different temperatures (5°, 10°, 15°, 20°, 25°, 30°, 35°, 40°, 45°C), and the colony radius was measured 10 days after inoculation.

Molecular phylogeny

Four isolates from white nodules and nine peronosporomycete species isolated from various marine invertebrate animals were used for analysis of the D1/D2 region of LSU rDNA (Table 1). Strains were incubated in PYGS broth at 15° or 25°C for 5 days. Young growing hyphae were washed three times with phosphate-buffered saline (PBS) and frozen at -85°C. Total genomic DNA was extracted using DNAZOL Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

The D1/D2 region of LSU rDNA was amplified using the polymerase chain reaction (PCR) with NL1 and NL4 primers (O'Donnell 1993). Each 50 µl of PCR reaction mixture contained 2.5 ng genomic DNA, 10 µl 10 × Ex Taq Buffer (Takara Bio, Shiga, Japan), 8 µl 2.5 mM dNTP Mixture (Takara Bio), 1 µM each primer, and 0.8 units Takara Ex Taq (Takara Bio). PCR was performed using the Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) under the following conditions: 94°C for 1 min, followed by 25 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 2 min, with a final extension at 72°C for 7 min. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and were then used directly for DNA sequencing. Direct sequencing of the PCR products was performed using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) according to the sequencer manufacturer's instructions. Forward primers (NL1, NL3) and reverse primers (NL2, NL4) were used for cycle sequencing (O'Donnell 1993). Sequences were assembled using ATGC version 3.0 (GENETYX, Tokyo, Japan) and GENETYX-WIN version 5.2 (GENETYX). Sequence profile alignments were performed with ClustalX (Thompson et al. 1997). The initial aligned data set was obtained from the European ribosomal RNA Database at the University of Gent (<http://www.psb.ugent.be/rRNA/index.html>). Fourteen new sequences from this study were aligned with sequences from 22 peronosporomycete species and an outgroup species obtained from GenBank (Table 2). Phylogenetic analyses were performed with PAUP version 4.0β8 (Sinauer Associates, Sunderland, MA, USA) for the maximum-parsimony (MP) and the maximum-likelihood (ML) methods. MODELTEST 3.8 via ModelTest Server 1.0 (http://darwin.uvigo.es/software/modeltest_server.html) by David Posada was used to select the appropriate model of substitution for MP analysis of nucleotide sequences. All analyses were performed using heuristic search with a tree bisection and reconnection (TBR) branch-swapping algorithm and random addition of taxa (10 replicates). The reliability of internal branches was assessed using the bootstrap method (Felsenstein 1988), with 1000 replicates. Phylogenetic trees were edited using TreeView (Page 1996).

Table 1. Sources of peronosporomycetes used in this study for D1/D2 region of large subunit (LSU) rDNA sequencing

Species	Strains	Host	Locality	Month, year	GenBank accession no.
<i>Halioticida</i>					
<i>H. noduliformans</i>	NJM^a 0451^b	Abalone, <i>Haliotis midae</i>	Chiba, Japan ^c	Jan. 2004	AB285227
<i>H. noduliformans</i>	NJM 0462	Abalone, <i>Haliotis rufescens</i>	Chiba, Japan ^d	Apr. 2004	AB285228
<i>H. noduliformans</i>	NJM 0447	Abalone, <i>Haliotis rufescens</i>	Chiba, Japan ^e	Jun. 2004	AB285229
<i>H. noduliformans</i>	NJM 0631	Abalone, <i>Haliotis sieboldii</i>	Chiba, Japan ^f	Jan. 2006	AB285230
<i>Lagenidium</i>					
<i>L. callinectes</i>	ATCC ^g 24973	Blue crab, <i>Callinectes sapidus</i>	USA	1973	AB285217
<i>L. thermophilum</i>	NJM 9338^h	Mangrove crab, <i>Scylla serrata</i>	Bali, Indonesia	Aug. 1993	AB285219
<i>L. myophilum</i>	NJM 8601ⁱ	Northern shrimp, <i>Pandalus borealis</i>	Ishikawa, Japan	Feb. 1986	AB285220
<i>Haliphthoros</i>					
<i>H. milfordensis</i>	NJM 0131 ^j	Black tiger shrimp, <i>Penaeus monodon</i>	Nha Trang, Vietnam	Mar. 2001	AB285218
<i>Haliphthoros</i> sp.	NJM 0443	Kuruma prawn, <i>Penaeus japonicus</i>	Mie, Japan	Jun. 2001	AB285226
<i>Haliphthoros</i> sp.	NJM 0440	Swimming crab, <i>Portunus trituberculatus</i>	Fukuoka, Japan	Jun. 2004	AB285225
<i>Halocrusticida</i>					
<i>H. baliensis</i>	GSM^k 9703	Mangrove crab, <i>Scylla serrata</i>	Bali, Indonesia	Jun. 1997	AB285222
<i>H. panulirata</i>	NJM 9832	Mangrove crab, <i>Scylla serrata</i>	Bali, Indonesia	Aug. 1998	AB285224
<i>H. parasitica</i>	NJM 0468	Swimming crab, <i>Portunus trituberculatus</i>	Fukuoka, Japan	Jun. 2004	AB285223
<i>Atkinsiella</i>					
<i>A. dubia</i>	NJM 0132	Swimming crab, <i>Portunus trituberculatus</i>	Okayama, Japan	Jun. 2001	AB285221

Strains shown in bold are the ex-type strains

^a Culture collection in the Division of Fish Diseases, Nippon Veterinary and Animal Science University, Musashino, Tokyo, Japan

^b NBRC 104969.

^c Imported from the Republic of South Africa

^d Imported from the Republic of Chile

^e Imported from United Mexican States

^f Captured at Nagasaki, Japan

^g American Type Culture Collection, Manassas, VA, USA

^h ATCC 200318

ⁱ ATCC 66280

^j ATCC MYA-3264

^k Culture collection in the Gondol Research Station for Coastal Fisheries, Singaraja, Bali, Indonesia

Results

Isolation

The daily mortality of stocked abalone (*Haliotis* spp.) was about 1%. The clinical sign of a moribund abalone was the presence of white nodules on the mantle (Figs. 1, 2). Thick and aseptate hyphae were observed in tissues from white nodules stained with Fungiflora Y (Biomate) under the fluorescence microscope (Fig. 3a–c). After 3 days incubation on PYGS agar, single colonies were observed. Four isolates – NJM 0451, NJM 0462, NJM 0447, and NJM 0631 – were isolated from three species of abalone: *H. midae* imported from the Republic of South Africa, *H. rufescens* imported from the Republic of Chile and the United Mexican States, and *H. sieboldii* collected at Nagasaki, Japan (see Table 1). The isolate NJM 0451 was deposited in the Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan, as accession number NBRC 104969.

Morphological characteristics

The four isolates, NJM 0451, NJM 0462, NJM 0447, and NJM 0631, show the same morphological characteristics. The manner of zoospore formation in the four isolates is

obviously different from that of the genera *Halocrusticida* and *Atkinsiella* but similar to that of the genus *Haliphthoros*. However, the isolates differ 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 *Haliphthoros* (Figs. 7, 8, 11B). One or more discharge tubes were formed from each zoosporangium (Figs. 9, 11C). The size of zoospores was 7.0–8.5 × 9.5–12.5 μm (width × length) (Figs. 10, 11F,G). From the results mentioned above, the present isolates are recognized to have unique morphological characteristics in the family Haliphthoraceae.

Effect of temperature on growth

The results are shown in Table 3. Four isolates from white nodules grew between 10° and 25°C with an optimum of 20°C. No growth was observed at 5°, 30°, 35°, or 40°C.

Molecular phylogeny

The sequences data presented in this study were deposited in GenBank as accession numbers AB285217–AB285230 (see Table 1). The alignment data were deposited in

Table 2. Lists of the peronosporomycete species obtained from GenBank

Taxon	GenBank accession no.
Subclass Peronosporomycetidae	
Peronosporales	
Peronosporaceae	
<i>Bremia lactucae</i> ^a	AY035510
<i>Paraperonospora leptosperma</i> ^a	AY250149
Albuginaceae	
<i>Albugo candida</i> ^a	AY035538
Pythiales	
Pythiaceae	
<i>Pythium monospermum</i> ^a	AY035535
<i>Lagenidium chthamalphilum</i> ^a	AF235946
<i>Phytophthora infestans</i> ^a	AF119602
<i>Peronophythora litchi</i> ^a	AY035531
Subclass Rhipidiomycetidae	
Ripidiales	
Ripidiaceae	
<i>Sapromyces elongatus</i> ^a	AF235950
Subclass Saprolegniomycetidae	
Saprolegniales	
Saplelegniaceae	
<i>Aplanopsis spinosa</i>	AF119589
<i>Brevilegnia megasperma</i>	AF119592
<i>Calyptralegnia achlyoides</i> ^a	AF119593
<i>Dictyuchus monosporus</i> ^a	AF119595
<i>Scoliolegnia asterophora</i> ^a	AF119619
<i>Thraustotheca clavata</i> ^a	AF119620
<i>Pythiopsis cymosa</i> ^a	AF218172
<i>Achlya bisexualis</i>	AF218203
<i>Saprolegnia ferax</i> ^a	AF235953
Leptolegniaceae	
<i>Leptolegnia caudata</i> ^a	AF218176
<i>Aphanomyces piscicida</i>	AF235941
<i>Plectospira myriandra</i> ^a	AF119606
Sclerosporales	
Sclerosporaceae	
<i>Sclerospora graminicola</i> ^a	AY035514
Leptomitales	
Leptomitaceae	
<i>Apodachlya brachynema</i>	AF119590
Outgroup	
<i>Chattonella marina</i>	AY704162

^aThe type species of the respective genera, orders, and higher taxa are according to Dick (2001)

Table 3. Effect of temperature on growth

Isolates	Temperature (°C)								
	5	10	15	20	25	30	35	40	45
NJM 0451	–	3.5	5.0	8.0	7.5	–	–	–	–
NJM 0462	–	4.0	8.0	10.5	6.0	–	–	–	–
NJM 0447	–	5.0	11.0	15.5	5.5	–	–	–	–
NJM 0631	–	3.5	4.5	6.5	6.0	–	–	–	–

–, no growth

Measurements are colony radius (mm) after incubation on PYGS agar plate for 10 days

Bold type indicates optimum temperatures

TreeBASE (<http://treebase.org/treebase/>) as matrix accession number M4339. As a result, sequences showed 100%–99.8% concordance among the four isolates NJM 0451, NJM 0462, NJM 0447, and NJM 0631. In the phylogenetic tree based on LSU rDNA, the four isolates were not classified

into the subclass Peronosporomycetidae, Saprolegniomycetidae, or Rhipidiomycetidae but as a new clade with the genera *Haliphthoros* and *Halocrusticida* (Fig. 12). Within this new clade, the four isolates, *Haliphthoros* spp. and *Halocrusticida* spp., were grouped in the respective independent subclade. *Atkinsiella dubia* and *Lagenidium* spp. were included in Saprolegniomycetidae and Peronosporomycetidae, respectively.

Taxonomy

Halioticida Muraosa & Hatai, gen. nov.

Coloniae in agar PYGS flavae, applanatae, margine irregulares, filamentosae, exhyphis vegetativis compositae. Coloniae in liquido PYGS pubescentes, Glutinosae. Fragmentum hyphae in aqua marina artificiali formatum, ex guttis constrictis includens. Zoosporangia, cum vel aliquot tubules emittentibus.

Etymology: *Haliotis* = generic name of abalone, and *cida* = destroyer, murderer (Latin).

Species typica: *Halioticida noduliformans* Muraosa & Hatai.

Colony on PYGS agar yellowish, flat, filamentous, irregular. Colonies in PYGS broth downy, sticky. Fragments formed by constricted protoplasm in hyphae in artificial seawater. Constriction of protoplasm weaker than in genus *Haliphthoros*. Fragments longer, up to 1600 µm, with smaller space between them, than those of genus *Haliphthoros*. One to several discharge tubes were formed from each zoosporangium. Zoospore size obviously larger than that of *Haliphthoros* spp.

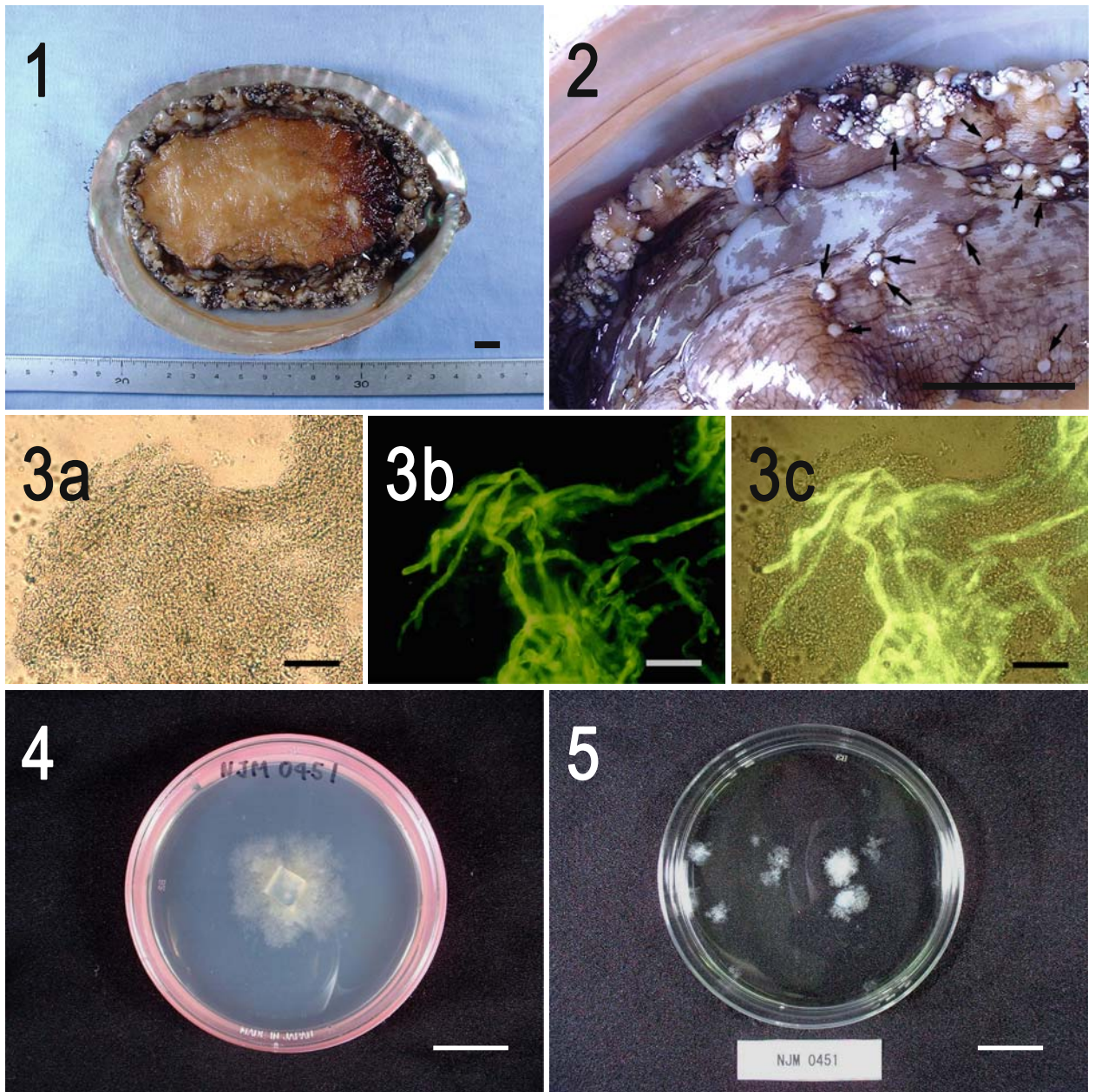
Halioticida noduliformans Muraosa & Hatai, sp. nov.

Figs. 4–10

Colonies in agar PYGS flavae, applanatae, filamentosae, margine irregulares, post 2 hebdomates ad 15°C 11 µm in diametro attingentes. Coloniae in liquido PYGS pubescentes, glutinosae, ex hyphis crassis aseptatis ramosis cum guttis numerosis praeditis 8–35 µm latis compositae. Zoosporangia longe cylindracea, 86–1600 µm longa, 8–35 µm lata, cum 1 vel aliquot tubules emittentibus circinatis 7–15 µm latis 38–300 µm longis formantia. Zoosporae per apicem tubuli in aqua marina liberatae, deinqua in ca 5 dies formata, lateraliter biflagellatae, pyriformes vel subglobosae, 7.0–8.5 × 9.5–12.5 µm, monoplaneticae, post natantem incystatae. Zoosporae incystatae globosae, sine flagellis. 8–10 µm in diametro, post 12 h cum filamento pileis simili germinantes. Status sexualis non visus.

Etymology: nodulus = nodule, formans = forming. Referring to its nodule-forming habit on the host.

Type: Figure 11 showing the strain NJM 0451 is designated as the holotype according to Article 37.5 in the International Code of Botanical Nomenclature (Vienna Code) 2006, because there are technical difficulties in preserving the type specimen: i.e., in slide preparation of hyphae with zoosporangia, their characteristic structures of this new taxon are easily destroyed. NJM 0451 was isolated from



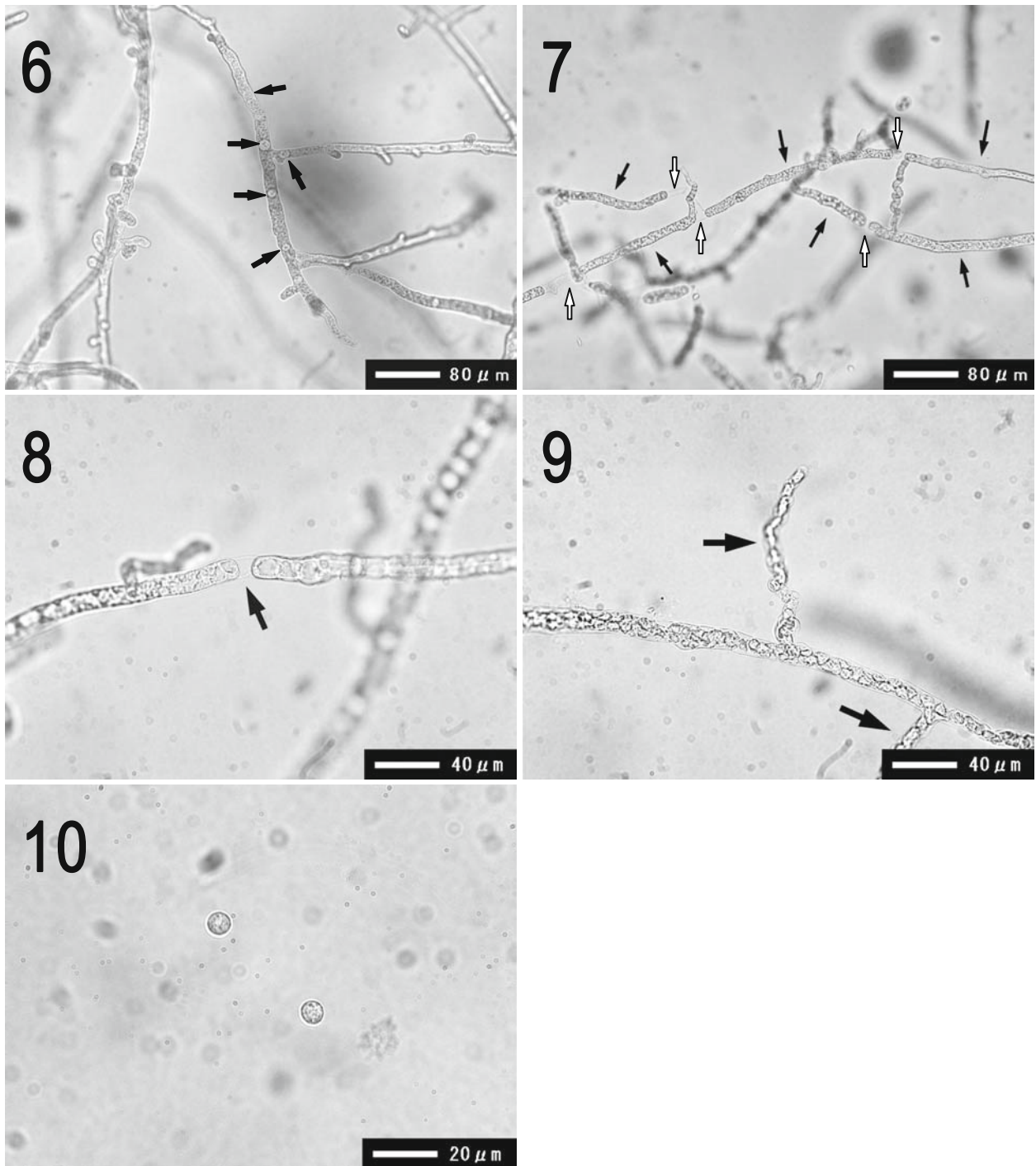
Figs. 1–5. *Halioticida noduliformans* on host and in culture. **1** A moribund abalone, *Haliotis sieboldii*, with white nodules. **2** Note white nodules (arrows). **3** Thick and aseptate hyphae in the tissues from white nodules, stained with Fungiflora Y under a fluorescence microscope: light micrograph (a); fluorescence micrograph (b); light micro-

graph + fluorescence micrograph (c). **4** Yellowish, flat, and filamentous colony with irregular edge (NJM 0451), growing at 20°C for 14 days on PYGS agar. **5** Downy and stinky colonies (NJM 0451), growing at 20°C for 7 days in PYGS broth. Bars **1, 2** 1 cm; **3** 100 µm; **4, 5** 2 cm

diseased abalone, *Haliotis midae*, Chiba, Japan, 14 January 2004, coll. Y. Muraosa, which is preserved at the Laboratory of Fish Diseases, Nippon Veterinary and Life Science University, Tokyo, Japan, and also deposited as NBRC 104969 in the Department of Biotechnology, National Institute of Technology and Evaluation, Chiba, Japan.

Colony on PYGS agar yellowish, flat, filamentous, about 11 mm in diameter after 2 weeks at 15°C, with irregular edge (Fig. 4). Colonies in PYGS broth downy, sticky (Fig. 5). Vegetative hyphae in PYGS broth stout, aseptate,

branched with numerous protoplasmic oil droplets, 8–35 µm in width (Figs. 6, 11A). Zoospore formation is induced under the starved condition. Fragments in artificial seawater, long, constructed by weakly constricted protoplasm (Figs. 7, 11B). Spaces between fragments small, 8–35 µm in width and 3–88 µm in length (Figs. 7, 8, 11B, 13A). Zoosporangia with one to several discharge tubes, 8–35 µm in width and 86–1600 µm in length (Figs. 9, 11C). Discharge tubes coiled, 7–15 µm in width and 38–300 µm in length (Figs. 9, 11D). Protoplasm in the zoosporangium



Figs. 6–10. Light micrographs of *Halioticida noduliformans* NJM 0451. **6** Stout, aseptate, and branched vegetative hyphae with numerous protoplasmic oil droplets (*arrows*), growing in PYGS broth. **7** Fragments in artificial seawater. Fragments (*black arrows*) are longer and constructed of weakly constricted protoplasm, and spaces between frag-

ments (*white arrows*) are smaller than those of genus *Haliphthoros*. **8** Note a narrow space between adjacent fragments (*arrow*). **9** Zoosporangium with two discharge tubes (*arrows*). Zoospores are formed in the zoosporangium and also in discharge tubes (*arrows*). **10** Two globose encysted zoospores

and discharge tubes cleaved into zoospores. Zoospores liberated into seawater through the top of the discharge tube (Fig. 11C,E), laterally biflagellate, pyriform to subglobose, monoplanetic, $7.0\text{--}8.5 \times 9.5\text{--}12.5 \mu\text{m}$ (Fig. 11F), encysting after swimming for several hours. Encysted zoo-

spore globose without flagella, $8\text{--}10 \mu\text{m}$ in diameter (Figs. 10, 11G). Germination observed about 12 h after being encysted, with a hair-like filament (Fig. 11H). Zoospore formation continued for about 5 days. Sexual reproduction not observed.

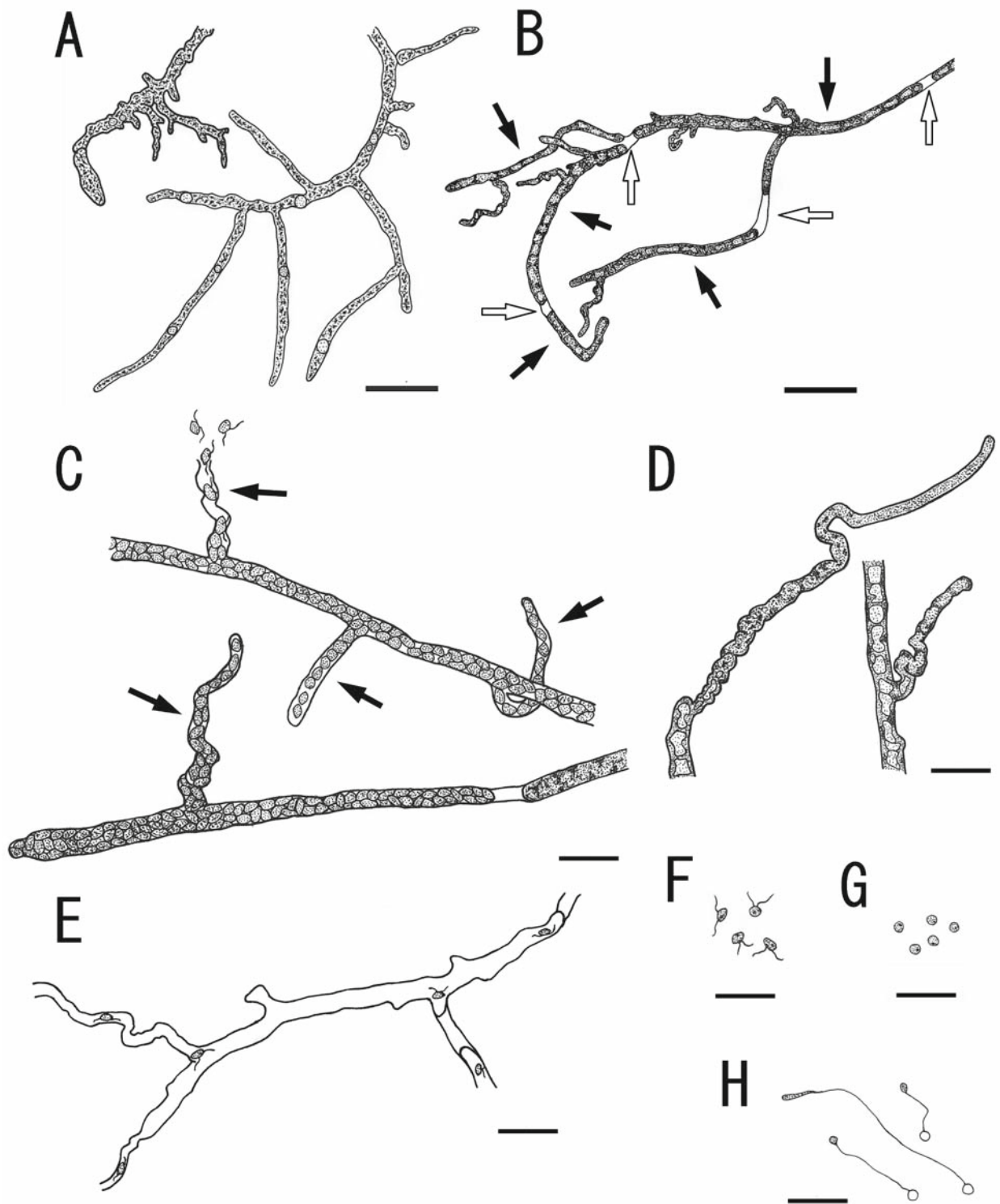


Fig. 11. Morphological characteristics of *Haliotis noduliformans* NJM 0451 isolated from the abalone *Haliotis midae*. **A** Vegetative hyphae growing in PYGS broth. The hyphae are stout, aseptate, and branched with numerous protoplasmic oil droplets. **B** Fragments in artificial seawater. Fragments (*black arrows*) are longer and constructed of weakly constricted protoplasm, and spaces between adjacent fragments (*white arrows*) are smaller than those of genus *Haliphthoros*. **C** Zoospores produced in the zoosporangia and also in discharge tubes.

Zoospores are liberated into seawater through the top of the discharge tube (*arrows*). **D** Discharge tubes developed in seawater. **E** Empty zoosporangium with discharge tubes. Some zoospores remained in the zoosporangium. **F** Swimming zoospores: laterally biflagellate, pyriform to subglobose, and monoplanetic. **G** Encysted zoospores: globose without flagella. **H** Germination of encysted zoospores, with a hair-like filament. *Bars A, B* 100 μm ; *C-H* 40 μm

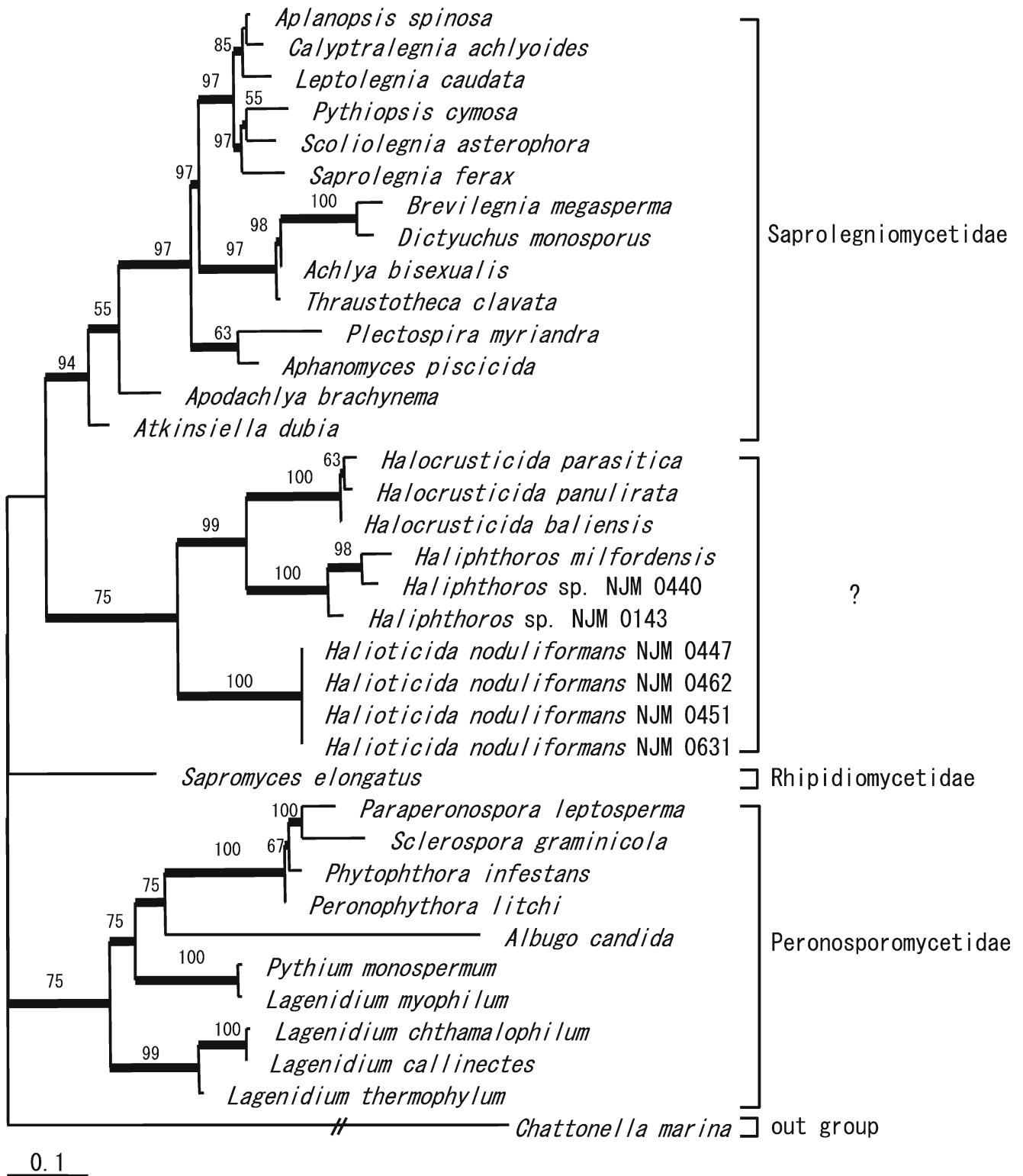


Fig. 12. Maximum-likelihood tree based on the D1/D2 region of LSU rDNA. Numbers on branches show bootstrap values (1000 replicates above 50% are indicated). Classification into subclass is according to Dick (2001)

Discussion

The manner of zoospore formation in the four isolates from abalones with white nodules is similar to the genus *Haliph-*

thoros. However, some morphological characteristics are different from the genus *Haliphthoros*. In artificial seawater, the four isolates form the fragments 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 that of *Haliphthoros*. The fungi of 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 four isolates do have one or more discharge tubes from each zoosporangium. As a result, we name them *Halioticida noduliformans* gen. et sp. nov. as a new genus and species in the family Haliphthoraceae. Differences in zoospore formation between the four isolates and *Haliphthoros milfordensis* NJM 0131 are shown in Fig. 13. In addition, the size of zoospores is obviously larger than that of *Haliphthoros* spp. (Table 4).

The optimum growth temperature estimation test indicated that *Halioticida noduliformans* is adapted to the temperate zone climate. The optimum growth temperature of *Halioticida noduliformans* and *Halocrusticida awabi* was at 20°C (Kitancharoen et al. 1994), but it was lower than that of *Haliphthoros milfordensis* (Chukanhom et al. 2003) and *Atkinsiella dubia* (Nakamura and Hatai 1995b).

Four strains isolated from abalone showed 100%–99.8% concordance in sequence of the D1/D2 region of LSU rDNA, which supported the evidence from morphological characteristics that they were the same species. In the phylogenetic tree based on the D1/D2 region of LSU rDNA, the four isolates were not nested into the subclass Peronosporomycetidae, Saprolegniomycetidae, or Rhipidiomycetidae but formed a new clade with the genera *Haliphthoros* and *Halocrusticida*. Within this new clade, the four isolates, *Haliphthoros* spp. and *Halocrusticida* spp., were grouped in their respective independent subclades. This result indicates that the D1/D2 region of LSU rDNA is useful to identify and classify the genus in the Haliphthoraceae. The phylogenetic analysis supports that the four isolates are classified into a new genus and species belonging to the family Haliphthoraceae based on their morphological characteristics.

Recently, Dick (2001) proposed a new taxonomic system for Peronosporomycetes, in which Peronosporomycetes were subdivided into three subclasses: Peronosporomyceti-

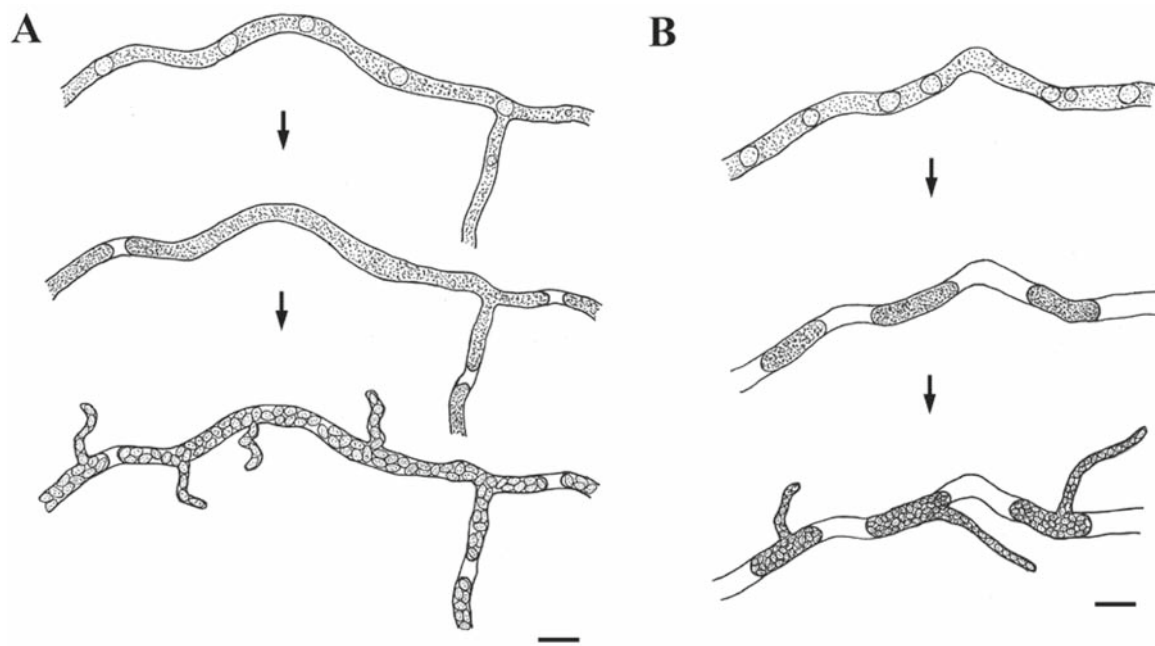


Fig. 13. Differences in zoospore formation between *Halioticida noduliformans* NJM 0451 and *Haliphthoros milfordensis* NJM 0131. **A** Manner of zoospore formation in *Halioticida noduliformans*. Fragments are longer and constructed of weakly constricted protoplasm, and spaces between adjacent fragments are smaller than those of *Haliphthoros milfordensis*. One to several discharge tubes are formed

from each zoosporangium. **B** Manner of zoospore formation in *Haliphthoros milfordensis*. Fragments are shorter and constructed of strongly constricted protoplasm, and spaces between fragments are larger than those of *Halioticida noduliformans*. Only one discharge tube is formed from each zoosporangium. Bars 40 µm

Table 4. Comparison of zoospore size of *Halioticida noduliformans* with *Haliphthoros* species

Species and strain	Swimming zoospore (width × length, µm)	Encysted zoospore (diameter, µm)	Reference
<i>Halioticida noduliformans</i> NJM 0451	7.0–8.5 × 9.5–12.5	8.0–10.0	Present study
<i>Haliphthoros milfordensis</i> GSM 9701	6.0–7.5 × 7.0–12.0	3.0–7.0	Hatai et al. (2000)
<i>Haliphthoros philippinensis</i> IMI ^a 241639	5.0–7.5 × 7.5–10.0	5.0–7.5	Hatai et al. (1980)
<i>Haliphthoros</i> sp. NJM 0443	3.0 × 7.0	3.5–6.5	Present study
<i>Haliphthoros</i> sp. NJM 0440	4.0 × 7.0	4.5–7.0	Present study

^a CABI Genetic Resource Collection, CABI Bioscience UK Centre (Egham), Surrey, UK

dae, Rhipidiomycetidae, 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. Our molecular phylogenetic analysis 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 our analysis. 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 (*cox2*) gene.

Thus, the taxonomic position of genera *Haliphthoros*, *Halocrusticida*, *Atkinsiella*, and *Lagenidium* has been still confused. Their higher taxonomic positions should be classified by further studies based on their morphological characteristics and molecular phylogenetic analysis.

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