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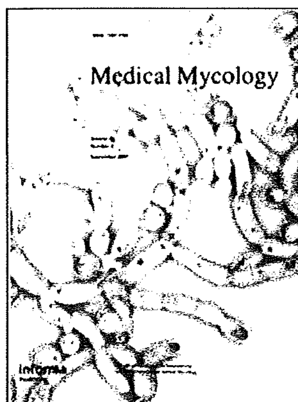

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An atypical *Paracoccidioides brasiliensis* clinical isolate based on multiple gene analysis

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An atypical isolate of *Paracoccidioides brasiliensis* (IFM54648), recovered from the sputum of a Brazilian man, was not detected in immunodiffusion tests for paracoccidioidomycosis and in species-specific PCR for the major antigen 43-kDa glycoprotein coding gene (*gp43*). The mycological characteristics of the isolate were similar to those of a typical *P. brasiliensis.* A total of 8 genes were sequenced from IFM54648, and the sequences were compared between the new isolate and other reference isolates and database sequences. We analyzed fragments of the gene sequences that code for *gp43*, the internal transcribed spacer regions of ribosomal RNA, the D1/D2 domains of the large subunit ribosomal RNA, glucan synthase, chitin synthase, glyoxalase I mRNA, 70-kDa heat-shock protein mRNA and urease. The gene sequences were 98.9–100% identical between IFM54648 and Pb01 (another atypical isolate). When compared to the other typical isolates, the identities were generally lower than 98%. A phylogenetic tree constructed using *gp43* sequences showed that IFM54648 clustered with Pb01 at a considerable distance from other isolates. Therefore, this isolate is likely related to Pb01, which has recently been shown to be genetically distinct from other isolates of this species.

Keywords atypical isolate, *gp43*, *Paracoccidioides brasiliensis*, paracoccidioidomycosis

Introduction

Paracoccidioidomycosis is endemic in Latin American countries and the causative agent, *Paracoccidioides brasiliensis*, is a temperature-dependent, dimorphic fungus. At ambient temperature, it assumes a mycelial phase, while in host tissue or at temperatures above 35°C on certain culture media, it has a yeastlike form. The fungus has been associated with infections of the lung, lymph nodes, skin, mucosa, liver, spleen and various other organs of humans and dogs. It has a chronic adult form accompanied by granulomatous lesions and an acute juvenile form with severe systemic dissemination [1–3].

P. brasiliensis is considered to belong to the family Onygenaceae (Order Onygenales, Ascomycota), in the same group as *Blastomyces dermatitidis*, *Coccidioides immitis*, *Histoplasma capsulatum*, and *Lacazia loboi* [4]. Recently, many *P. brasiliensis* gene sequences have been deposited in GenBank which can be used for molecular epidemiology, identification, diagnosis and sequence diversity analysis [5–11]. According to Matute *et al.* [8–10], there are three different *P. brasiliensis* phylogenetic species, i.e., S1 (species 1 from Brazil, Argentina, Paraguay, Peru and Venezuela), PS2 (phylogenetic species 2 from Brazil and Venezuela) and PS3 (phylogenetic species 3 from Colombia).

Carrero *et al.* [11] described an atypical isolate of *P. brasiliensis* (Pb01) that, based on phylogenetic analysis, was clearly separate from all other isolates

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of the fungus. We recently recovered an isolate, IFM54648, from a 64-year-old Brazilian man with chronic multi-focal (adult form) paracoccidioidomycosis. Through molecular analyses we accidently determined that it was an atypical form of *P. Brasiliensis.* Based on Carrero *et al.*, isolate IFM54648 might have a close phylogenetic relationship to isolate Pb01 [11]. Furthermore, Theodoro and colleagues [12] confirmed that Pb01 does not belong to any of the groups previously described because of the high level of divergence compared to the three different genetic groups [8–10]. These authors proposed that this isolate may be a new species of *Paracoccidioides.*

Although some gene sequences of IFM54648 are available in PubMed, there is no description of the isolate. The present study provides mycological and molecular characteristics of this atypical *P. brasiliensis* isolate.

Materials and methods

Background of the isolate IFM54648

P. brasiliensis isolate IFM54648 (LDR 2) was accidentally found to be an atypical isolate through species-specific PCR testing which indicated that it was negative for the major antigen 43-kDa glycoprotein coding gene (*gp43*) [13]. In addition, IFM54648 yielded an atypical band pattern in loop-mediated isothermal amplification (LAMP) for detection of *gp43* [13] (Fig. 1) and it showed lower identity of a partial sequence of *gp43* to that of the species (S1, PS2, and PS3) described by Matute *et al.* [8–10].

IFM54648 was 99.1% similar to the partial sequence of *gp43* and *P. brasiliensis* isolate Pb01 from the database of BROAD Institute (http://www.broad.mit.edu/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html).

Isolate IFM54648 was initially recovered from a 64-year-old Brazilian man with chronic multi-focal (adult form) paracoccidioidomycosis. Five sera samples collected from the patient over a period of 7 years did not react with an immunodiffusion test for paracoccidioidomycosis which used an antigen derived from the *P. brasiliensis* isolate B339 [14]. The diagnosis of paracoccidioidomycosis was based on cytological observation of the sputum, which revealed typical multiple-budding yeast cells and the isolation of *P. brasiliensis* from a mass in the patient's lower jaw. In addition, the patient had lived in Botucatu, São Paulo, Brazil, and now lives in Londrina, Parana, Brazil, which are endemic areas of paracoccidiodomycosis.

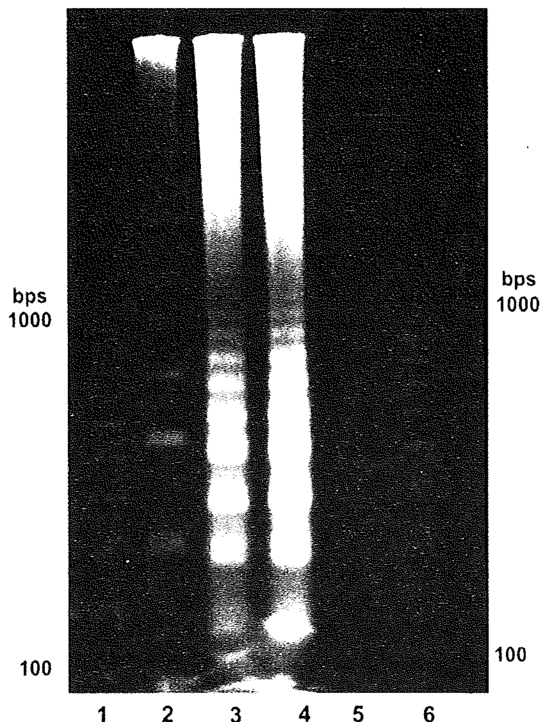

Fig. 1 Detection of *gp43* by LAMP. 1 and 6, marker; 2, B 339; 3 and 4, LDR 2; 5, negative control.

Mycological studies

Colonies were grown at 25°C for 2 months on Sabouraud dextrose agar (SDA) that contained 2% dextrose (Wako Pure Chemical Industries, Osaka, Japan), 1% neopeptone (BD, Becton Drive, Franklin Lakes, NJ, USA) and 1.5% agar (Bacto agar; BD), and on potato dextrose agar (PDA; BD). Morphologic features were microscopically examined on these media. Temperature-dependent dimorphism was observed when mycelia that had been cultured on PDA slants at 25°C for 4 weeks were inoculated onto brain heart infusion agar (BHIA) supplemented with 1% dextrose slants and cultured at 35°C for 1 week. Maximal cell growth was measured at 37, 38, 39 and 40°C. The yeast-form cells were maintained on BHIA slants by weekly subculture for up to 10 weeks.

Molecular studies

DNA was extracted from yeast-form cells cultured on BHIA at 35°C for 1 week by use of a commercial kit (DEXPAT®, TaKaRa, Otsu, Japan). The partial sequences of *gp43* was amplified [15], as were the internal

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transcribed spacer regions (*ITS*), the D1/D2 domains of large subunit ribosomal RNA (*D1/D2*) [16], glucan synthase (*FKS*) [4], chitin synthase (*CHS2*) [6,10], glyoxalase I mRNA (*glyoxalase*; referred from AY252117), 70-kDa heat-shock protein mRNA (*HSP70*; referred from AF386787), and a partial urease gene sequence (*URE*) [17]. The PCR primers and conditions used are shown in Table 1. Note that the primer set used to amplify *gp43* [15] was distinct from those previously employed in the diagnostic assay [13]. Primers MAE and ATO (Table 1), which amplified the fragment from nucleotide 629 to 1217 (GenBank sequence accession number U26160), successfully amplified a DNA fragment from IFM54648.

The PCR products were visualized by electrophoresis purified by use of a purification kit (QIAquick®, Qiagen, Hilden, Germany), and labelled with Big-Dye® Terminator Ver. 1.1 (Applied Biosystems, Foster City, CA, USA). The by-fragments were sequenced by use of an ABI PRISM® 3100 sequencer (Applied Biosystems) and were aligned using GENETYX-MAC genetic information processing software (Software Development Co., Tokyo, Japan).

The gene sequences from IFM54648 were compared to the whole genome sequences of isolates Pb01, Pb03 and Pb18 at the genome project of the BROAD Institute (http://www.broad.mit.edu/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html). Thirty-two clinical isolates and two *P. brasiliensis* isolates from armadillos (*Dasypus novemsinctus*) stored at Medical Mycology Research Center, Chiba University, Japan were used as references. In addition, Matute *et al.* [8–10] phylogenetic species based on the reference isolates were estimated based on the *gp43* sequences through the BALAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi, ID: E7GFFX01016 and EPSB307R01R) using distance trees drown by neighbour joining method (Table 2).

To determine the phylogenetic status of IFM54648, a tree of *gp43* sequences was constructed by aligning sequences from 34 reference isolates and isolate Pb01 from the website (BROAD Institute Pb01 supercontig 15 from 823856 to 824439) with CLUSTALX (Version 2.0.8) [18]. Phylogenetic analyses were performed with PAUP v4.0b10 [19] using a heuristic search for maximum parsimony. Bootstrap values were calculated based on over 1,000 replicates to assess branch topology. A phylogenetic tree was selected from 1,000 unrooted trees and was drawn using the Tree View PPC program (Roderic D. M. Page, Glasgow, Scotland, UK, 1998; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Bootstrap values greater than 50% were indicated. In addition, phylogenetic analyses on the other seven genes and a combination of eight genes

Table 1 PCR primers and conditions

Gene	Primer set	Annealing temperature (°C)	Reference no. or Accession no.
Partial 43 kDa glycoprotein coding gene (*gp43*)	MAE: 5′-TGC TGC GGC GGG GTT AAA CCA TGT C-3′ ATO: 5′-GTT GTG GTA TGT GTC GAT GTA GAC G-3′	48–52	[12]
ITS regions of rRNA] (*ITS*)	ITS-5: 5′-GGA AGT AAA AGT CGT AAC AAG G-3′ ITS-4: 5′- TCC TCC GCT TAT TGA TAT GC-3′	48–53	[13]
D1/D2 regions of rRNA (*D1/D2*)	NL-1: 5′- GCA TAT CAA TAA GCG GAG GAA AAG-3′ NL-4: 5′- GGT CCG TGT TTC AAG ACG G-3′	50–58	[13]
Glucan synthase (*FKS*)	F: 5′- CCT TGT ATT GTT AAG AAG GGA GTT C-3′ R: 5′- GGA CTT TCC GCT TAT AAC CCG TGA-3′	53–55	[4]
Chitin synthase (*CHS2*)	F: 5′- CTT AAC GGT GCC TTC TTT GCG GCT G-3′ R: 5′- GTG AAA GTA TTG TTG CCC AGC GAC A-3′	53–55	[5,9]
Glyoxalase I mRNA (*glyoxalase*)	PbGLYF: 5′- ATG GCC ACA GAT CCA TCA AAA TAC-3′ PbGLYR: 5′- ATG AAG GAT ACG CCA GGA AGT ACA G-3′	53–58	AY252117
70 kDa heat-shock protein mRNA (*HSP70*)	PbHSP70F: 5′- AAG AAG GCC GAG GGT GAA CGC AA-3′ PbHSP70R: 5′- ACC GAC AGA TAG AGG AGC GAC GTC AA-3′	55–58	AF386787
Partial urease coding gene (*Urease*)	PbUreF: 5′- CGG GTA TTT ACA AGG CTG ATA TTG G-3′ PbUreR: 5′- GAC ACC CTG AAC GAA TCT GGC TTC-3′	50–57	[14]

The primer designs for *glyoxalase* were based on the sequence AY252117. The forward primer corresponded to the 1st to 24th bases, and the reverse primer was a complementary sequence corresponding to the 613th to 636th bases. The primer set for *HSP70* was based on the sequence AF386787. The forward primer corresponded to the 984th to 1004th bases and the reverse primer was a complementary sequence corresponding to the 1609 th to 1634th bases. The primers for detecting *Urease* were designed on the basis of sequences from *Coccidioides* spp. [14] because of the homologous sequence in the *P. brasiliensis* database (http://143.107.203.68/est/default.html).

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were performed using sequences from 34 reference isolates (data not shown).

To determine the phylogenetic diversity of *gp43*, a nucleotide–nucleotide BLAST search was performed using registered sequences. The sequences AB047690 from isolate IFM41620 (ID: E7GFFX01016) and AB304693 from IFM54648 (ID: EPSB307R01R) were used as query sequences to identify distant trees.

Nucleotide–nucleotide BLAST searching was also performed to determine the phylogenetic diversity of *FKS* and *CHS2*. The sequences AB304670 (ID: EPU8PPTB01R) for *FKS* and AB304565 (ID: EPYMK5FP016) for *CHS2* from isolate IFM54648 were used as query sequences.

Susceptibility to antifungal drugs

IFM54648 yeast cells that had been grown on BHIA slants at 35°C for 1 week were used for *in vitro* drug susceptibility testing. Tests were performed according to a modified broth microdilution method of the CLSI M27-A2 protocol (Clinical and Laboratory Standards Institute/NCCLS, 2002) [20]. RPMI 1640 medium (Sigma, Poole, UK) buffered with

Table 2 Isolates

IFM Number	Strain	Country (City)	Source (Remarks)	Phylogenetic species[#]
IFM 41620	Pb-9	Brazil	Human patient	S1
IFM 41621	Pb-18	Brazil	Human patient	S1
IFM 41622	Bt-2	Brazil (Botucatu, São Paulo)	Human patient	S1
IFM 41623	Bt-3	Brazil (Botucatu, São Paulo)	Human patient	S1
IFM 41624	Bt-4	Brazil (Botucatu, São Paulo)	Human patient	S1
IFM 41625	Bt-7	Brazil (Botucatu, São Paulo)	Human patient	S1
IFM 41626	Bt-9	Brazil (Botucatu, São Paulo)	Human patient	S1
IFM 41627	Bt-19	Brazil (Botucatu, São Paulo)	Human patient	S1
IFM 41629	PbLev	Brazil	Human patient	S1
IFM 41630	B339	Brazil	Human patient (=CBS 372.73, =ATCC 32069)	S1
IFM 41631	Recife	Brazil (Recife)	Human patient	S1
IFM 41632	Pb-HM-AOK	Japan (Tokyo)*	Human patient	S1
IFM 41633	Hachisuga	Japan (Fukuoka)*	Human patient	S1
IFM 46215	WAG	Japan (Osaka)**	Human patient	S1
IFM 46240	Tateishi	Japan (Ibaragi)*	Human patient	S1
IFM 46463	Tatu	Brazil (Tocantins, Para)	Armadillo	S1
IFM 46464	Bt-1	Brazil (Botucatu, São Paulo)	Human patient	S1
IFM 46465	Pb-267	Brazil	Mutant of Pb-9	S1
IFM 46466	Pb-265	Brazil	Mutant of Pb-9	S1
IFM 46467	Recife-Pb-HC	Brazil (Recife)	Human patient	S1
IFM 46468	P-25	Costa Rica (San Jose)	Human patient	ND
IFM 46469	P-29	Costa Rica (San Jose)	Human patient	ND
IFM 46470	P-30	Costa Rica (San Jose)	Human patient	ND
IFM 46930	UMK	Japan (Chiba)*	Human patient	S1
IFM 47183	PRT1	Brazil (Botucatu, São Paulo)	Armadillo	S1
IFM 47633	F23A	Brazil (Campinas, São Paulo)	Human patient	S1
IFM 50887	HR	Japan (Okayama)*	Human patient	S1
IFM 52933	Iwamizawa	Japan (Hokkaido)*	Human patient	S1
IFM 54647	LDR 1	Brazil (Londrina, Parana)	Human patient	PS2
IFM 54648	LDR 2	Brazil (Londrina, Parana)***	Human patient (Present isolate)	ND
IFM 54649	LDR 3	Brazil (Londrina, Parana)	Human patient	PS2
IFM 54650	LDR 4	Brazil (Londrina, Parana)[§]	Human patient	S1
IFM 54651	LDR 5	Brazil (Londrina, Parana)	Human patient	PS2
IFM 54652	LDR 6	Brazil (Londrina, Parana)	Human patient	PS2
IFM 54653	LDR 7	Brazil (Londrina, Parana)	Human patient	PS2

IFM: Institute of Food Microbiology, Chiba University, the former name of the Medical Mycology Research Center, and deposited as the official abbreviation of the world culture collection of pathogenic fungi and actinomycetes. *The patient was infected in Brazil. **The patient was infected in Paraguay. ***The patient lived in Botucatu, São Paulo, Brazil. [§]The patient lived in São Paulo, Brazil. [#]Phylogenetic species was estimated from gp43 sequence based on BLAST search ID (E7GFFX01016 and EPSB307R01R). ND: Not determined.

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MOPS (Sigma) to pH 7.0 and serial concentrations of amphotericin B (AMPH-B), flucytosine (5-FC), fluconazole (FLCZ), itraconazole (ITZ), miconazole (MCZ) and micafungin (MCFG) were used. The latter three antifungal drugs were included even though the method was originally described for use with AMPH-B, 5-FC and ITZ alone. Testing was performed in 96-well round-bottom plastic plates with 100 ml RPMI 1640 medium with fungal cells and antifungal substances (Dry plate, Koubo Yo, Eiken Co. Ltd., Tokyo, Japan).

Results

The mycelia and yeast morphology of IFM54648 was typical of *P. brasiliensis* and it displayed temperature-dependent dimorphism. Colonies grown on SDA and PDA at ambient temperature were white, with fissures at the centre and formed aleurioconidia and chlamydospores. After incubation at 35°C on BHIA slants, the isolate converted to cerebriform colonies consisting of multiple-budding yeast cells (Fig. 2a–f). The maximum temperature for growth was 38°C. The yeast-form colonies survived on BHIA slants at 35°C with weekly subculture.

The nucleotide sequences were deposited in the DNA Data Bank of Japan (DDBJ) under the accession numbers AB047690–AB04770 and AB304676–AB304698 (*gp43*); AB304414–AB304448 (*ITS* through *D1/D2*); AB304641–AB304675 (*FKS*); AB304536–AB304570 (*CHS2*); AB304606–AB304640 (*glyoxalase*); AB304571–AB304605 (*HSP70*), and AB070575 and AB304699–AB304732 (*urease*).

Table 3 shows a comparison of the gene sequences of IFM54648 and isolates Pb01, Pb03 and Pb18. The largest degree of diversity was in the sequence of *gp43*. For this gene, IFM54648 showed more than 98.9% identity to Pb01, 90.2% to Pb03 and 89.0% to Pb18.

A representative *gp43* phylogenetic tree was constructed using IFM54648, 34 reference isolates (Table 2; 26 isolates of S1, 5 of PS2, and 3 of unidentified sequences located between S1 and PS2 based on *gp43* sequence) and isolate Pb01 (BROAD Institute Pb01 supercontig 15 from 823856–824439). In this tree, IFM54648 and Pb01 were located together at an extremely distant branch from the rest of reference isolates (Fig. 3).

A distance tree of *gp43* diversity was created based on nucleotide–nucleotide BLAST searching with accession number AB47690 from isolate IFM 41620 as a query (ID:E7GFFX01016, http://www.ncbi.nlm.nih.gov/blast/treeview/blast_tree_view.cgi?request = page&rid = E7GFFX01016&queryID = dbj|AB047690 &distmode = on &screenWidth = 1024). This tree indicated that isolate IFM54648 is an outgroup.

A distance tree of *gp43* diversity was also created based on nucleotide–nucleotide BLAST searching with accession number AB304693 from IFM54648 as the query (ID: EPSB307R01R, http://www.ncbi.nlm.nih.gov/blast/treeview/blast_tree_view.cgi?request = page& rid = EPSB307R01R&dbname = nr&queryID = dbj|AB304693). This analysis suggested that the *gp43* sequence from isolate IFM54648 is closely related to 14 other sequences of *gp43* (EU870196–EU870209, directly deposited by Teixeira *et al.*).

The gene identities between isolate IFM54648 and the furthermost sequence from reference isolates was 89.3% for *gp43*, 95.6% for *ITS*, 99.4% for *D1/D2*, 98.1% for *FKS*, 96.4% for *CHS2*, 94.9% for *glyoxalase*, 96.9% for *HSP70*, and 98.6% for *URE*. The identity between IFM54648 and all eight of these genes combined was 96.3%. In addition, the identities among the reference isolates for all gene sequences tested were more than 98%. Sequences from IFM54648 in phylogenetic trees using 34 references isolates based on other genes, i.e., *ITS*, *D1/D2*, *FKS*, *CHS2*, *glyoxalase*, *HSP70*, *URE*, and with the combination of 8 genes were located at extremely distinct branches (data not shown).

Distance tree analyses of the phylogenetic relationship between IFM54648 and the other *P. brasiliensis* isolates based on *FKS* (ID: EPU8PPTB01R, (http://www.ncbi.nlm.nih.gov/blast/treeview/blast_tree_view.cgi?request = page&rid = EPU8PPTB01R&dbname = nr&queryID = dbj|AB304670) and *CHS2* (ID: EPYMK5FP016; http://www.ncbi.nlm.nih.gov/blast/treeview/blast_tree_view.cgi?request = page&rid = EPYMK5FP016&queryID = dbj|AB304565&distmode = on&screenWidth = 1024)showed that 17 sequences of *FKS* (EU870263–EU870279, directly deposited by Teixeira *et al.* and 15 sequences of *CHS2* (EU870229, EU870230, EU870232–EU870236 and EU870238–EU870245, directly deposited by Teixeira *et al.* were closely related to the sequences derived from Pb01 and the atypical isolate IFM54648.

IFM54648 was susceptible to AMPH-B, 5-FC, FLCZ, ITZ and MCZ at 0.25, 0.125, 0.5, 0.05, and 0.06 µg/ml, respectively, while resistant to MCFG having an MIC of greater than 16 µg/ml.

Discussion

Isolate IFM54648 was definitively identified as *P. brasiliensis* based on mycological studies. However,

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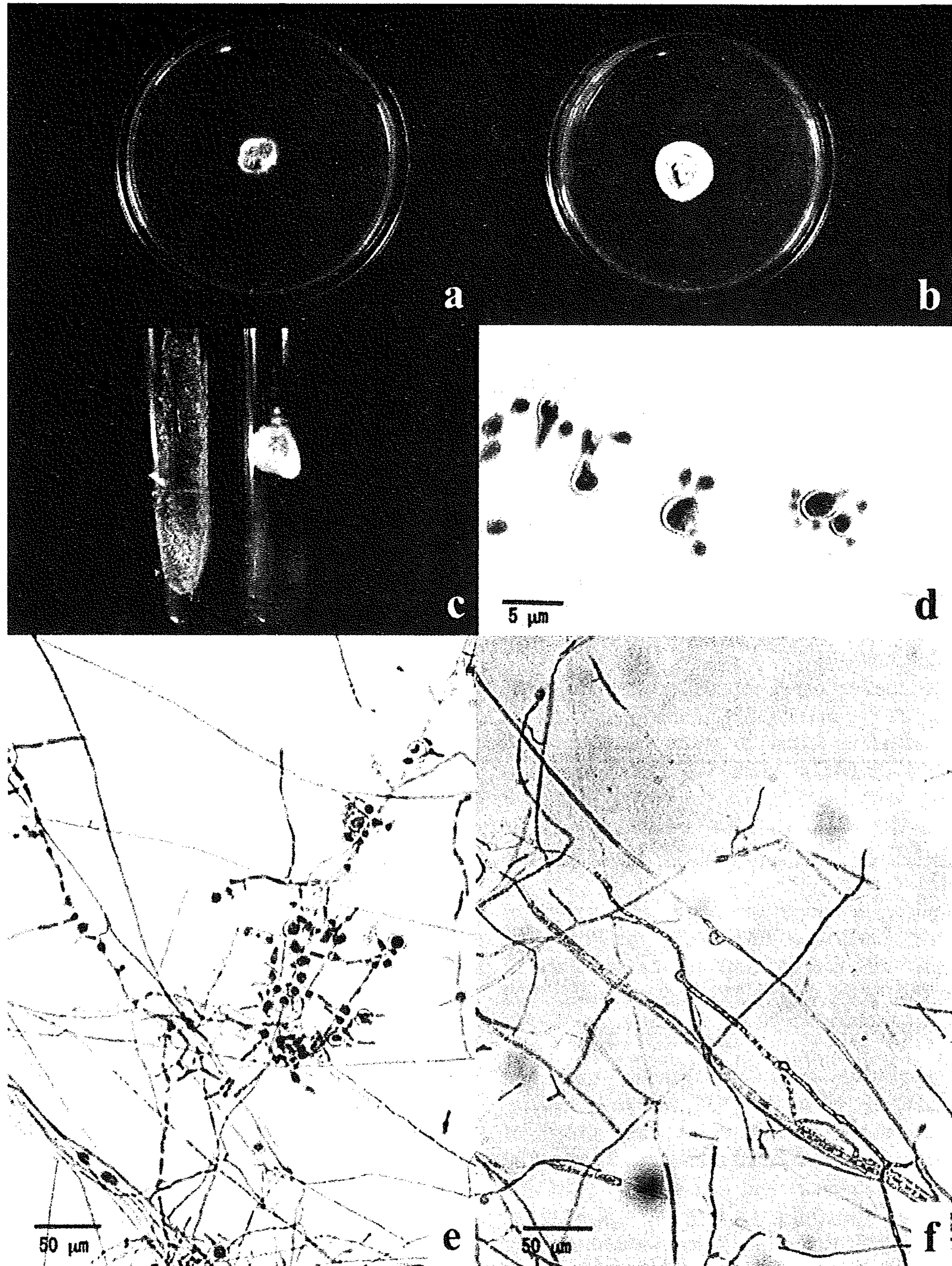

Fig. 2 Colonies on SDA (a) and PDA (b) at 25°C for 60 days, yeast-form colony on BHIA slant at 35°C for 7 days (left) and mycelial form on PDA slant at 25°C for 60 days (right) (c), cells on BHIA at 35°C for 7 days (d), mycelial form on SDA at 25°C for 60 days (e) and those on PDA at 25°C for 60 days (f).

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Table 3 Homology to Pb01, Pb03 and Pb18

Gene	IFM 54648 (LDR2) Accession number (Bps)	Homology to Pb01 (%) (Bps-Supercontig: position)	Homology to Pb03 (%) (Bps-Supercontig: position)	Homology to Pb18 (%) (Bps-Supercontig: position)
Gp43	AB304693	99.1	90.2	89
	587	587-15: 823856–824442	589-11: 757178–757766	589-12: 126603–127191
ITS	AB304443	99.8	97.7	97.4
	615	616-7: 491093–491659	565-14: 380–944	565-32: 37247–37811
D1D2	AB304443	99.8	99.7	99.7
	655	655-72: 57097–57751	655-14: 9222–9876	655-32: 97609–98263
FKS	AB304670	100	97.8	97.8
	643	643-13: 330559–331201	643-6: 1419271–1419913	643-5: 1463819–1464461
CHS2	AB304565	99.7	96.6	96.4
	614	614-33: 77891–78504	614-25: 32391–33004	614-25: 80046–79433
Glyoxalase	AB304635	100	95.2	95.1
	806	806-52: 56139–56944	811-30: 26667–27477	811-29: 27761–28571
HSP 70	AB304600	100	97.1	97.5
	651	651-28: 303849–304499	651-8: 1128750–1128100	651-14: 293447–294097
URE	AB304727	98.9	97.9	97.7
	536	522-2: 98034 –979824	522-2: 997466–997987	522-4: 1137061–1137582

its molecular characteristics were quite distinct from other previously identified *P. brasiliensis* isolates.

The present study demonstrates the unreliability of identifications of atypical *Paracoccidioides* isolates through the use of immunological tests and molecular techniques that employ *gp43* sequences, a gene which codes for a major glycoprotein antigen of *P. brasiliensis.* This was shown by repeated negative results in immunodiffusion tests in our patient and in a species-specific PCR using primers F3 5′-TCA CGT CGC ATC TCA CAT TG-3′encoding from 391st to 410th and B3 5′- AAG CGC CTT GTC CAA ATA GTC GA -3′ designed from the complementary sequence from 718th to 740th correspondent to gp43 sequence at GenBank data base, as well as an atypical band pattern in LAMP for detection of *gp43* [13].

The negative reactions in the immunodiffusion tests might have been caused by the different profile of *gp43*. In fact, the identity of *gp43* derived from B339 (AB304681) was 89.8%. Therefore, cytological observations and mycological studies are important for diagnosing paracoccidioidomycosis caused by atypical *P. brasiliensis* isolates.

Matute *et al.* proposed three different phylogenetic species of *P. brasiliensis* (S1, PS2, PS3) [8–10], but IFM54648 was located at an extreme distance from the reference isolates for all 8 examined genes in the phylogenetic trees or BLAST searches. Although, we could not estimate the phylogenetic species of isolates IFM 46468, 46469, and 46470, they were located between S1 and PS2 phylogenetic species on the bases of *gp43* sequence. Therefore, it was confirmed that the isolate IFM54648 was completely distinct from the other *P. brasiliensis* isolates identified to date.

Interestingly, this isolate might not be an orphan or an atypical isolate. The present study could confirm that our isolate IFM 54648 has a closer phylogenetic relationship to isolate Pb01 at the BROAD genome project and other reports [5,6,11,21]. Furthermore, there are likely more atypical isolates of *P. brasiliensis* than just Pb01 and IFM54648. In total, 14 sequences of *gp43* (EU870196–EU870209), 17 sequences of *FKS* (EU870263–EU870279), and 15 sequences of *CHS2* (EU870229, EU870230, EU870232–EU870236 and EU870238–EU870245) from the central–western part of Brazil have been shown to have a closer relationship to IFM54648 and Pb01 than to the other typical *P. brasiliensis* isolates. These results suggest that atypical isolates related to isolate Pb01 are increasingly being found throughout Brazil. Information about IFM54648 and other isolates with a closer relationship to Pb01 may result in the proposal of a new species of *Paracoccidioides.*

The present isolate showed no resistance to antifungal compounds except for MCFG. It suggested that therapeutic benefits by candin antifungal agents might not be expected for paracoccidioidomycosis caused by atypical *P. brasiliensis.* However, at present it is impossible to confirm genotype-specific sensitivity to antifungal agents.

In conclusion, the genotype of the present isolate is extremely different from that of other *P. brasiliensis* isolates classified by Matute *et al.* [8–10], and is more closely related to the atypical *P. brasiliensis* isolate Pb01 reported by Carrero *et al.* [11]. Sufficient numbers of

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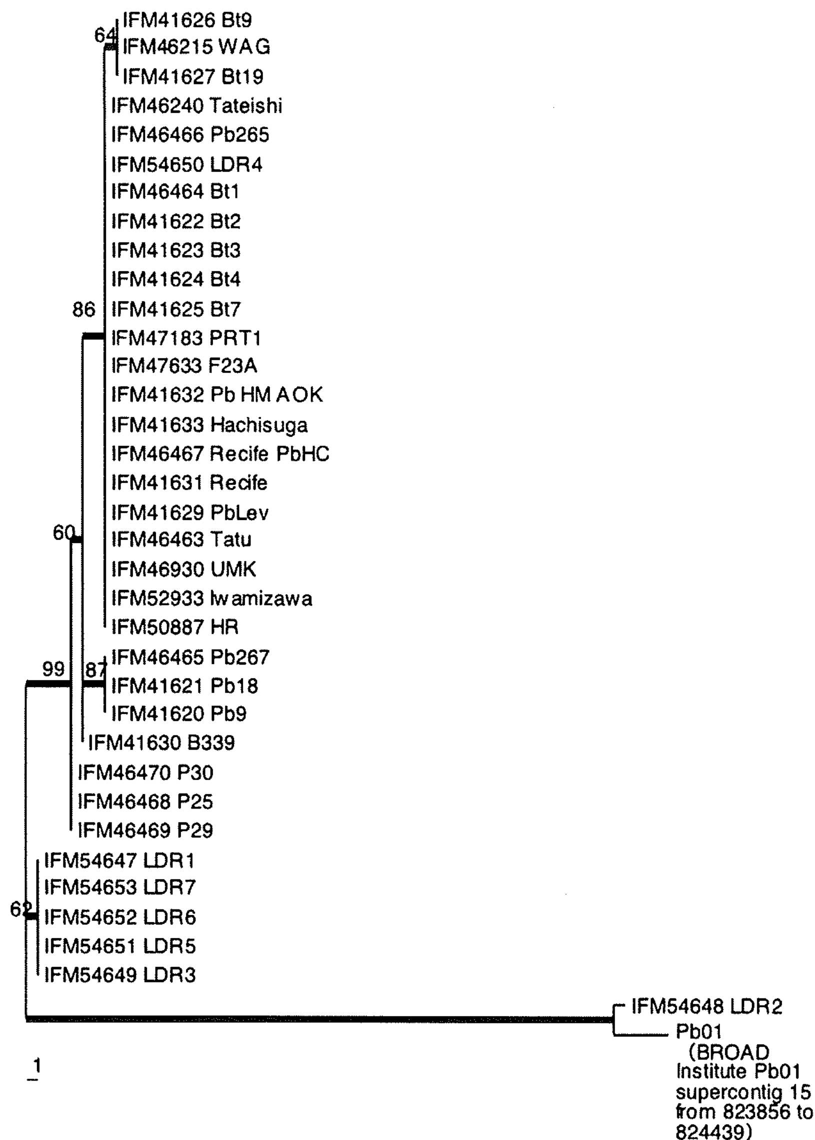

Fig. 3 Phylogenetic tree based on partial sequences of *gp43*. Only one tree with a strict consensus obtained from heuristic searches was produced from *gp43* sequences consisting of 589 base pairs. The bar indicates 1 base pair. Bootstrap support values of more than 50% are indicated at the nodes.

atypical *P. brasiliensis* isolates may permit a new species of *Paracoccidioides* in the near future.

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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

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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 Georgefischeriales (Bauer et al. 2005) and was also used for the phylogenetic relationships of

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.
Halioticida					
H. noduliformans	**NJM[a] 0451[b]**	Abalone, *Haliotis midae*	Chiba, Japan[c]	Jan. 2004	AB285227
H. noduliformans	NJM 0462	Abalone, *Haliotis rufescens*	Chiba, Japan[d]	Apr. 2004	AB285228
H. noduliformans	NJM 0447	Abalone, *Haliotis rufescens*	Chiba, Japan[e]	Jun. 2004	AB285229
H. noduliformans	NJM 0631	Abalone, *Haliotis sieboldii*	Chiba, Japan[f]	Jan. 2006	AB285230
Lagenidium					
L. callinectes	ATCC[g] 24973	Blue crab, *Callinectes sapidus*	USA	1973	AB285217
L. thermophilum	**NJM 9338[h]**	Mangrove crab, *Scylla serrata*	Bali, Indonesia	Aug. 1993	AB285219
L. myophilum	**NJM 8601[i]**	Northern shrimp, *Pandalus borealis*	Ishikawa, Japan	Feb. 1986	AB285220
Haliphthoros					
H. milfordensis	NJM 0131[j]	Black tiger shrimp, *Penaeus monodon*	Nha Trang, Vietnam	Mar. 2001	AB285218
Haliphthoros sp.	NJM 0443	Kuruma prawn, *Penaeus japonicus*	Mie, Japan	Jun. 2001	AB285226
Haliphthoros sp.	NJM 0440	Swimming crab, *Portunus trituberculatus*	Fukuoka, Japan	Jun. 2004	AB285225
Halocrusticida					
H. baliensis	**GSM[k] 9703**	Mangrove crab, *Scylla serrata*	Bali, Indonesia	Jun. 1997	AB285222
H. panulirata	NJM 9832	Mangrove crab, *Scylla serrata*	Bali, Indonesia	Aug. 1998	AB285224
H. parasitica	NJM 0468	Swimming crab, *Portunus trituberculatus*	Fukuoka, Japan	Jun. 2004	AB285223
Atkinsiella					
A. dubia	NJM 0132	Swimming crab, *Portunus trituberculatus*	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	
Bremia lactucae[a]	AY035510
Paraperonospora leptosperma[a]	AY250149
Albuginaceae	
Albugo candida[a]	AY035538
Pythiales	
Pythiaceae	
Pythium monospermum[a]	AY035535
Lagenidium chthamalophilum[a]	AF235946
Phytophthora infestans[a]	AF119602
Peronophythora litchi[a]	AY035531
Subclass Rhipidiomycetidae	
Ripidiales	
Ripidiaceae	
Sapromyces elongatus[a]	AF235950
Subclass Saprolegniomycetidae	
Saprolegniales	
Saplelegniaeae	
Aplanopsis spinosa	AF119589
Brevilegnia megasperma	AF119592
Calyptralegnia achlyoides[a]	AF119593
Dictyuchus monosporus[a]	AF119595
Scoliolegnia asterophora[a]	AF119619
Thraustotheca clavata[a]	AF119620
Pythiopsis cymosa[a]	AF218172
Achlya bisexualis	AF218203
Saprolegnia ferax[a]	AF235953
Leptolegniaceae	
Leptolegnia caudata[a]	AF218176
Aphanomyces piscicida	AF235941
Plectospira myriandra[a]	AF119606
Sclerosporales	
Sclerosporaceae	
Sclerospora graminicola[a]	AY035514
Leptomitales	
Leptomitaceae	
Apodachlya brachynema	AF119590
Outgroup	
Chattonella marina	AY704162

[a]The 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 phylogenic 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 agaro PYGS flavae, applanatae, margine iregulares, filamentosae, exhyphis vegetativiis 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.

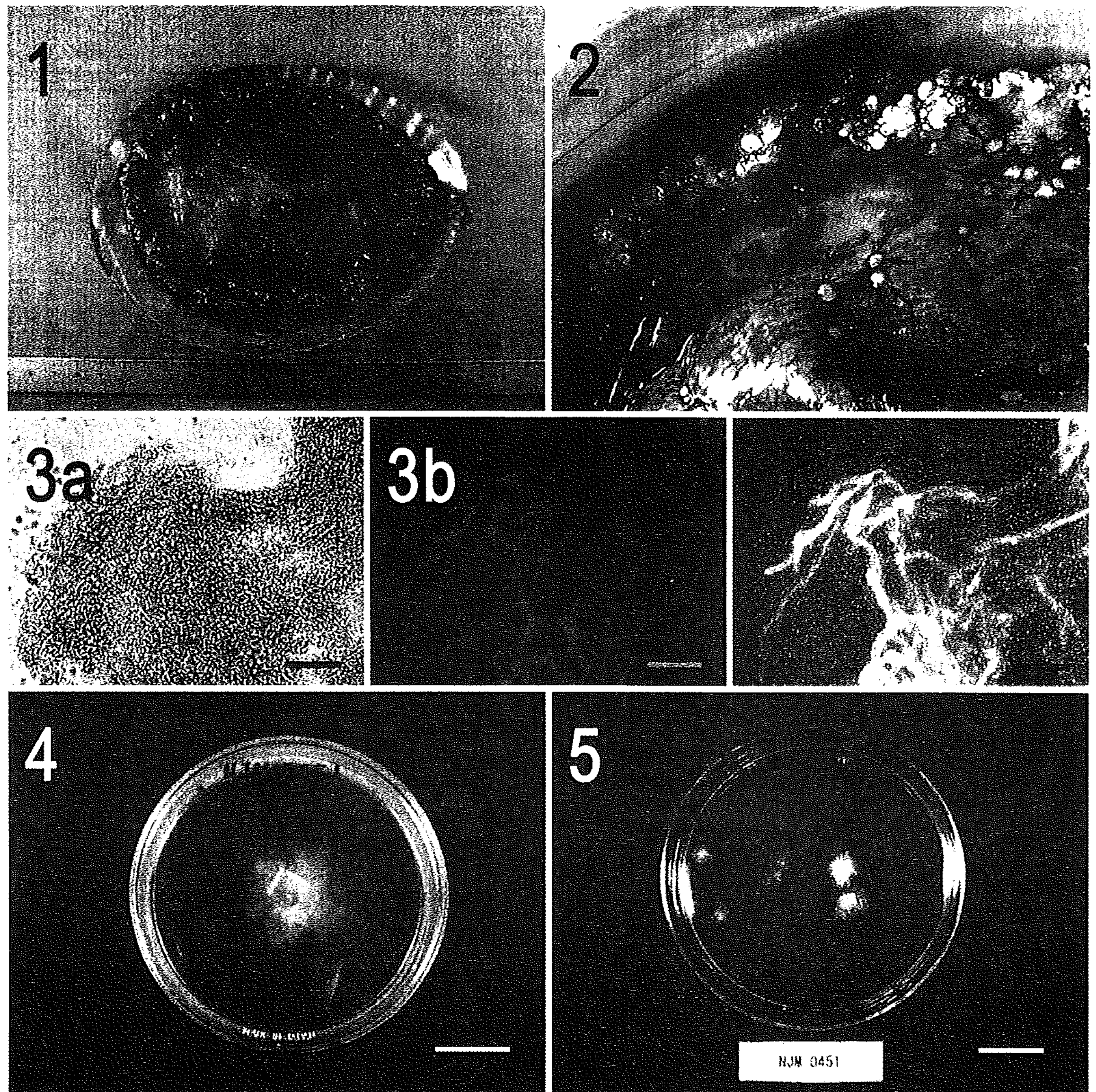
Halioticida noduliformans Muraosa & Hatai, sp. nov.

Figs. 4–10

Colonies in agaro 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 emttantibus 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.

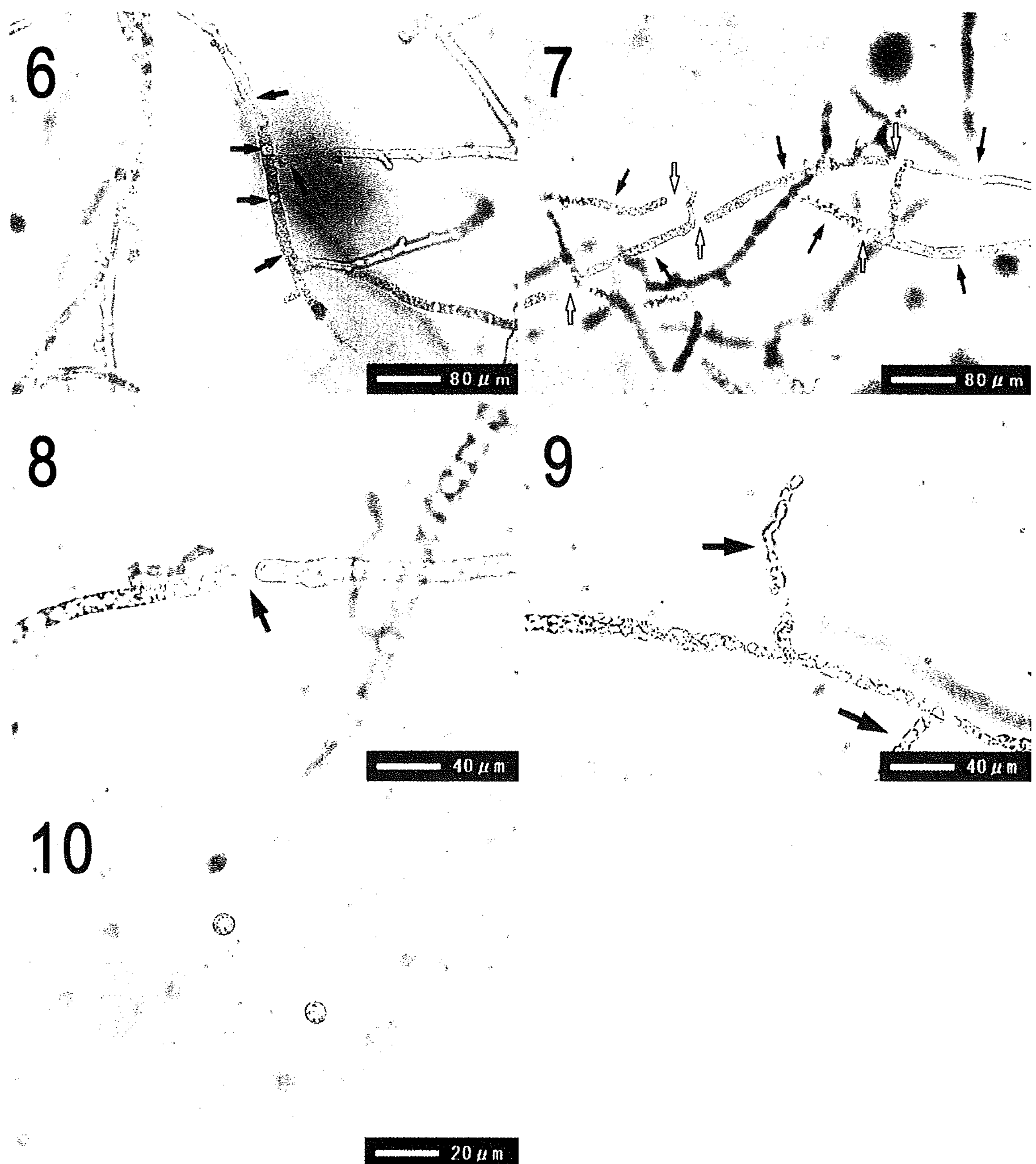
Type: Figure 11 showing the strain NJM 0451 is designated as the holotype according to Article 37.5 in the International Code of Botanical Nomenclature (Vienna Code) 2006, because there are technical difficulties in preserving the type specimen: i.e., in slide preparation of hyphae with zoosporangia, 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 micrograph + 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 fragments (*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, 7.0–8.5 × 9.5–12.5 μm (Fig. 11F), encysting after swimming for several hours. Encysted zoospore globose without flagella, 8–10 μm 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.

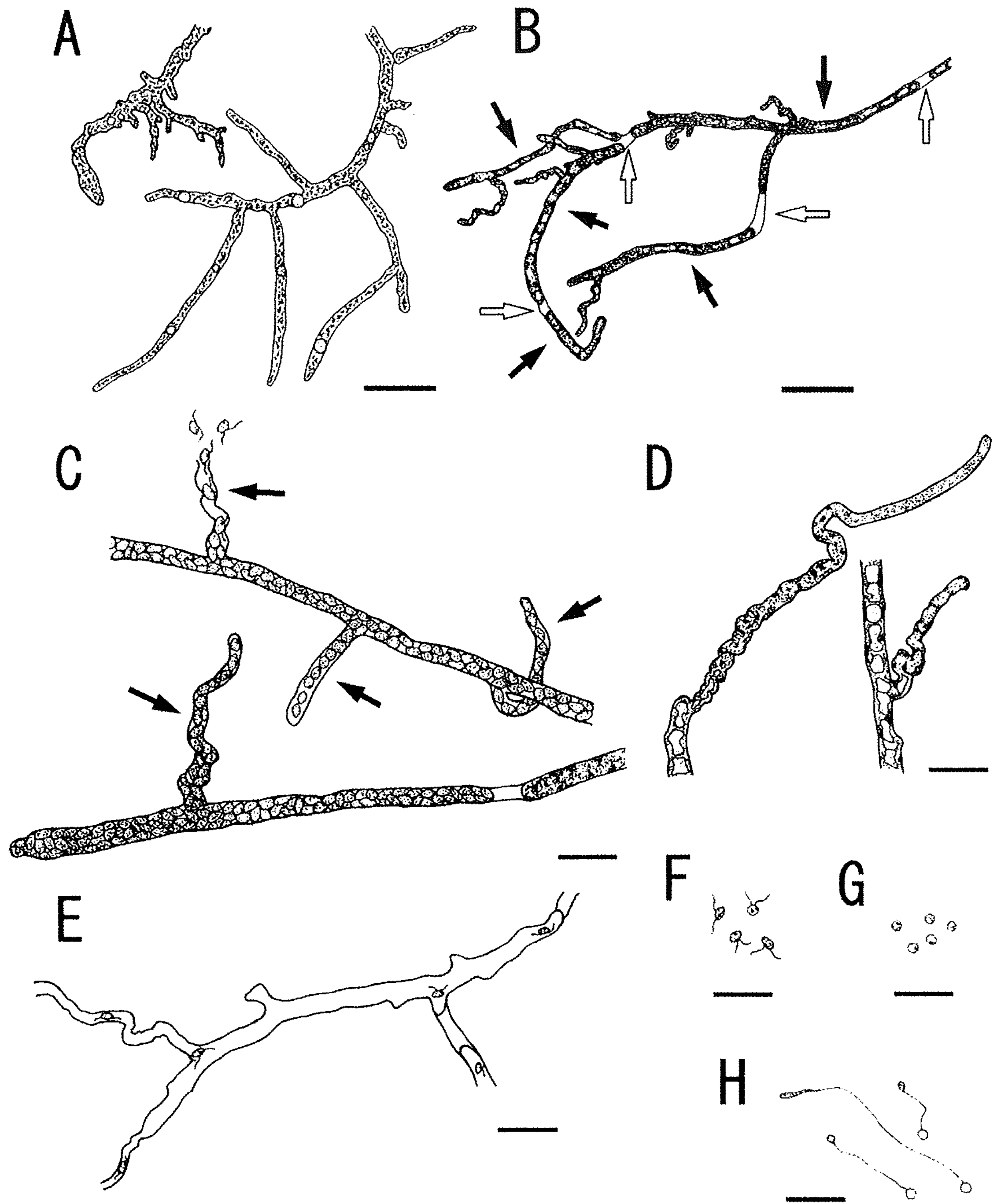


Fig. 11. Morphological characteristics of *Haliotis noduliformans* NJM 0451 isolated from the abalone *Haliotis midae*. **A** Vegetative hyphae growing in PYGS broth. The hyphae are stout, aseptate, and branched with numerous protoplasmic oil droplets. **B** Fragments in artificial seawater. Fragments (*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 *Haliphthoros*. 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-

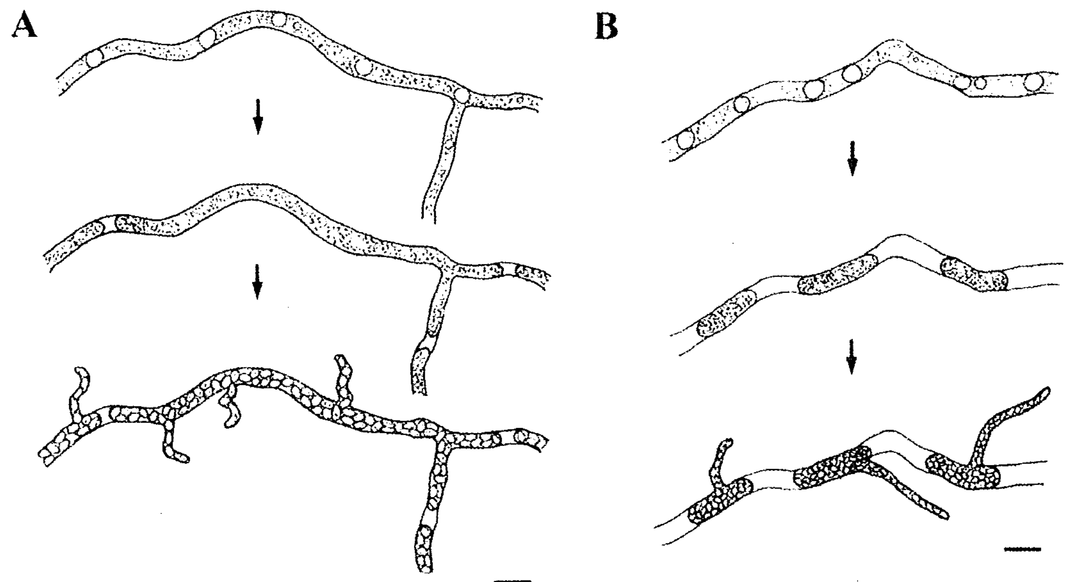


Fig. 13. Differences in zoospore formation between *Halioticida noduliformans* NJM 0451 and *Haliphthoros milfordensis* NJM 0131. **A** Manner of zoospore formation in *Halioticida noduliformans*. Fragments are longer and constructed of weakly constricted protoplasm, and spaces between adjacent fragments 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
Halioticida noduliformans NJM 0451	7.0–8.5 × 9.5–12.5	8.0–10.0	Present study
Haliphthoros milfordensis GSM 9701	6.0–7.5 × 7.0–12.0	3.0–7.0	Hatai et al. (2000)
Haliphthoros philippinensis IMI[a] 241639	5.0–7.5 × 7.5–10.0	5.0–7.5	Hatai et al. (1980)
Haliphthoros sp. NJM 0443	3.0 × 7.0	3.5–6.5	Present study
Haliphthoros 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 (*cox*2) 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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