FULL PAPER

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Molecular phylogeny of an unidentified *Haliphthoros*-like marine oomycete and *Haliphthoros milfordensis* inferred from nuclear-encoded small- and large-subunit rRNA genes and mitochondrial-encoded cox2 gene

Abstract The SSU rRNA, LSU rRNA, and cox2 genes of an unidentified *Haliphthoros*-like marine oomycete (NJM0034) and *Haliphthoros milfordensis* (NJM0131) were sequenced, and their phylogenetic relationships are analyzed and discussed. All phylogenetic trees showed that NJM0034 and NJM0131 were branched before separation of the two main saprolegnian and peronosporalean clades. These data suggest that the clear phylogenetic separation of those marine oomycete endoparasites from the two main oomycete clades. Excepting the LSU rRNA gene tree, NJM0034 and *Haliphthoros* spp. did not form a monophyletic group. On the other hand, *H. milfordensis* NJM0131 clustered with *H. philippinensis* SANK 15178, not with *H. milfordensis* NJM9434 in the cox2 amino acid sequence (COII) tree. This result strongly suggests that a taxonomic reinvestigation of the genus *Haliphthoros* should be considered.

Key words *Haliphthoros* · Marine holocarpic endoparasite · Oomycetes · Stramenopiles

Introduction

The genus *Haliphthoros* Vishniac was first described as a filamentous, holocarpic parasite on eggs of the oyster drill *Urosalpinx cinerea* Say, and the family Haliphthoraceae was established to accommodate *Haliphthoros* (type genus) and *Atkinsiella* Vishniac in the order Saprolegniales (Vishniac 1958). *Haliphthoros* species are known as parasites of a wide range of marine crustaceans and some other marine animals (Vishniac 1958; Lightner 1981; Alderman 1982; Hatai 1989; Hatai et al. 1992; Diggles 2001). To date, the genus *Haliphthoros* contains two species, *H. milfordensis* Vishniac (type species; Vishniac 1958) and *H. phillippinensis* Hatai et al., that were distinguished on the basis of morphological differences associated with zoosporogenesis and zoospore release (Hatai et al. 1980). Because *Haliphthoros* species have been frequently isolated from diseased organisms and are considered to be serious pathogens of economically important marine crustaceans, they were studied so they could be characterized both morphologically and physiologically (Nakamura and Hatai 1995; Hatai et al. 2000; Diggles 2001; Chukanhom et al. 2003). *Haliphthoros* has been isolated from all over the world, and nearly all the isolates have been identified as *H. milfordensis*.

In August 2000, an unidentified *Haliphthoros*-like fungus (NJM0034) was isolated from tissues in white nodules formed in the mantle of an abalone, *Haliotis rubra* Leach, that was imported from South Australia to Japan. The basic morphological characters of this fungus, such as fragmentation of the hyphae by cytoplasmic constriction, show closest affinity with those of the genus *Haliphthoros*, although zoosporogenesis was not observed. Because the manner of asexual reproduction (zoosporogenesis) is one of the key characters for identifying asexual genera such as *Haliphthoros* in the class Oomycetes, the precise identity of this particular isolate consequently remains unknown.

Cook et al. (2001) sequenced the gene for mitochondrial-encoded cytochrome *c* oxidase subunit II (cox2) for two *Haliphthoros* isolates [*H. philippinensis* SANK 15178, which is the type strain of this species (Hatai et al. 1980) and *H. milfordensis* NJM9434 (Nakamura and Hatai 1995)]. The resulting molecular phylogenetic trees showed that both *Haliphthoros* isolates form a monophyletic clade, which rather surprisingly clustered with another marine parasite genus, *Halocrusticida* Nakamura et Hatai, at the base of the oomycete clade, diverging before separation of the main saprolegnialian and peronosporalean clades (Cook et al. 2001). In this study, we sequenced the partial nuclear-

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encoded small-subunit ribosomal RNA (SSU rRNA) gene, the partial large-subunit ribosomal RNA (LSU rRNA) gene, and the cox2 gene of the isolate of NJM0034, and analyzed these to investigate the molecular phylogenetic position of NJM0034, to verify its affinity with the genus *Haliphthoros*, and to further confirm the monophyly of the genus *Haliphthoros*. We also used another isolate of *Haliphthoros* (NJM0131) for this study, which was originally isolated from a prawn in Vietnam in 2001 and described as the typical *Haliphthoros milfordensis* based upon morphological observations (Chukanhom et al. 2003). We performed this multigene approach using new sequence data of the SSU and LSU rRNA genes of *Haliphthoros* isolates to contribute to the overall understanding of the phylogenetic position of this economically important genus.

Materials and methods

Isolation, growth, DNA extraction, and DNA sequencing

The methods for isolation and subcultivation of NJM0034 and NJM0131 were as described by Hatai et al. (1992). DNA extraction from mycelia was carried out as follows. Portions of fungal mycelia (approximately 0.1 g dry weight), grown in PYGS broth (0.125% peptone, 0.125% yeast extract, 0.3% glucose and 4.0% artificial seawater; Hatai et al. 1992) at ambient temperature (~20°C) for 10 days, were transferred into 2.0-ml cryotubes (Sarstedt AG& Co., Nümbrecht, Germany) and then 50 μl glass beads and 600 μl cell lysis solution, from the GenomicPrep Cells and Tissue DNA Isolation Kit (GE Healthcare UK, Buckinghamshire, England), were added. The tubes were shaken at 5000 rpm for 120 s using a cell homogenization machine (Mini Bead-Beater; BioSpec Products, Bartlesville, OK, USA) to homogenize the mycelia. After 3 μl RNase A solution (GE Healthcare UK) was added into the tubes, they were incubated for 1 h at 37°C, then 200 μl protein precipitation solution (GE Healthcare UK) was added. As the final stage in

the process, the total genomic DNA was purified using the standard phenol and chloroform /isoamyl alcohol protocol (Murray and Thompson 1980). Finally, the purified DNA from the mycelia of both isolates was resuspended in 30 μl TE solution.

The SR1 and SR12 (Nakayama et al. 1996), LSU-0021F (5′-ATTACCCGCTGAACTTAAGC-3′) and LSU-1170R (Petersen and Rosendahl 2000), and COX2-For3 (5′-GCHACHCCWGTWATGGARGG-3′ and COX2-Rev3 (5′-TACATTGDCCRTAAAAAAYMCC-3′) primers were used for polymerase chain reaction (PCR) to amplify SSU rRNA, LSU rRNA, and cox2 genes of both isolates, respectively. PCR programs are as follows: an initial denaturation at 95°C for 2 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C (SSU rRNA) / 63°C (LSU rRNA) / 40°C (cox2) for 30 s, extension at 72°C for 60–120 s, and a final extension at 72°C for 5 min. All PCR products were checked by 1.4% TBE agarose gel electrophoresis to confirm the purity and concentration of the total genomic DNA solution. The PCR products were purified with Suprec-PCR kit (Takara Biomedicals, Otsu, Japan) or TA cloned with Qiagen PCR Cloning Kit (Qiagen, Hilden, Germany), and sequenced using a primer-walking approach with a BigDye Terminator v3.0 Cycle Sequencing Kit on a DNA autosequencer model 310 from Applied Biosystems (Foster City, CA, USA).

Molecular phylogeny

All the taxa used for the molecular phylogenetic analyses in this study are shown in Table 1. For the SSU and LSU rRNA gene molecular phylogeny, the initial aligned data set was downloaded from The European Ribosomal RNA Database at the University of Gent [http://www.psb.ugent.be/rRNA/index.html (Wuyts et al. 2004)]. Our determined sequences and the sequences not recorded in this database, but available from GenBank, were individually added to the aligned data set through a profile alignment process by Clustal W, version 1.81 (Thompson et al. 1994), and then

Table 1. Small-subunit (SSU) rRNA, large-subunit (LSU) rRNA, and cytochrome c oxidase subunit II (cox2) gene sequences used in this study

Taxon	Strain no.	Gene	GenBank accession no.	Reference[a]
Unassigned taxon				
Unidentified oomycetes	**NJM0034**	**SSU rRNA**	**AB178865**	**1**
Unidentified oomycetes	**NJM0034**	**LSU rRNA**	**AB178866**	**1**
Unidentified oomycetes	**NJM0034**	**cox2**	**AB178867**	**1**
Haliphthoros/*Halocrusticida* clade				
Saprolegniales				
Haliphthoros milfordensis	**NJM0131, ATCC MYA-3264**	**SSU rRNA**	**AB178868**	**1**
Haliphthoros milfordensis	**NJM0131, ATCC MYA-3264**	**LSU rRNA**	**AB178869**	**1**
Haliphthoros milfordensis	**NJM0131, ATCC MYA-3264**	**cox2**	**AB178870**	**1**
Haliphthoros milfordensis	NJM9434, ATCC 200320	cox2	AF290305	2
Haliphthoros philippinensis	SANK 15178, ATCC 58303	cox2	AF290307	2
Halocrusticida okinawaensis	NJM9435, ATCC 200327	cox2	AF290306	2
Saprolegnian clade				
Saprolegniales				
Achlya ambisexualis	CBS 383.79	LSU rRNA	AF218202	9
Achlya ambisexualis	E87, ATCC 11400	cox2	AF086687	7
Achlya bisexualis	Unknown	SSU rRNA	M32705	5

Table 1. *Continued*

Taxon	Strain no.	Gene	GenBank accession no.	Reference[a]
Aphanomyces euteiches	n-63, ATCC 201684	LSU rRNA	AF235939	11
Aphanomyces euteiches	A466	cox2	AF086692	7
Aphanomyces invadans	IMI836083	SSU rRNA	AF396684	15
Aplanes androgynus	AR 46	LSU rRNA	AF119588	12
Aplanopsis spinosa	CBS 112.61	LSU rRNA	AF119589	12
Aplanopsis terrestris	3102b	SSU rRNA	AF238658	3
Atkinsiella dubia	NJM 9455, ATCC 200323	cox2	AF290312	2
Brevilegnia megasperma	AR 4	LSU rRNA	AF119592	12
Dictyuchus sterilis	CBS 164.38, ATCC 44890	LSU rRNA	AF218193	9
Dictyuchus sterilis	CBS 164.38, ATCC 44890	cox2	AF086691	7
Eurychasma dicksonii	Eury96	SSU rRNA	AY032607	8
Isoachlya toruloides	Unknown	LSU rRNA	AF235947	11
Leptolegnia caudata	3501b	SSU rRNA	AJ238659	3
Leptolegnia caudata	CBS 680.69	LSU rRNA	AF218176	9
Lepotlegnia caudata	ATCC 48818	cox2	AF086693	7
Plectospira myriandra	CBS 523.87	LSU rRNA	AF218196	9
Plectospira myriandra	84-209, ATCC 64139	cox2	AF086694	7
Pythiopsis cymosa	3601j	SSU rRNA	AJ238657	3
Pythiopsis cymosa	CBS 261.38	LSU rRNA	AF218172	9
Pythiopsis cymosa	B-2, ATCC 26880	cox2	AF086689	7
Saprolegnia ferax	2004b	SSU rRNA	AJ238655	3
Saprolegnia ferax	Sf5.6	LSU rRNA	AF235953	11
Saprolegnia ferax	ATCC 36051	cox2	AF086690	7
Thraustotheca clavata	CBS 557.67	LSU rRNA	AF235951	11
Thraustotheca clavata	371a, ATCC 34112	cox2	AF086688	7
Leptomitales				
Apodachlya brachynema	5001a	SSU rRNA	AJ238663	3
Apodachlya brachynema	61-020	LSU rRNA	AF235936	11
Apodachlya pyrifera	Unknown	cox2	AF086695	7
Leptomitus lacteus	AR 80	LSU rRNA	AF119597	12
Leptomitus lacteus	ATCC 38076	cox2	AF086696	7
Peronosporalean clade				
Rhipidiales				
Sapromyces elongatus	CBS 213.82	LSU rRNA	AF235950	11
Sapromyces elongatus	Unknown	cox2	AF086700	7
Pythiales				
Lagenidium callinectes	NJM 9433, ATCC 200337	cox2	AF290308	2
Lagenidium caudatum	CBS 584.85, ATCC 58383	cox2	AF290309	2
Lagenidium giganteum	ATCC 52675	SSU rRNA	M54939	4
Lagenidium giganteum	ATCC 36492	cox2	AF086697	2
Lagenidium humanum	ATCC 76726	cox2	AF290310	2
Lagenidium myophilum	NJM8601, ATCC 66280	cox2	AF290311	2
Lagenidium thermophilum	NJM 9338, ATCC 200318	cox2	AF290304	2
Peronophythora litchii	CBS 100.81	LSU rRNA	AF235949	11
Peronophythora litchii	ATCC 28739	cox2	AF086698	7
Phytophthora megasperma	P3114	SSU rRNA	M54938	4
Phytophthora megasperma	MUCL 11644	LSU rRNA	X75631	13
Phytophthora megasperma	695T	cox2	L04457	7
Pythium aphanidermatum	no. 170	LSU rRNA	AF235956	11
Pythium monospermum	unknown	SSU rRNA	AJ238653	3
Pythium ultimum	67-1, ATCC32939	cox2	AF086699	7
Peronosporales				
Peronospora ficariae	AR 78	LSU rRNA	AF119600	12
Plasmopara pygmaea	AR 86	LSU rRNA	AF119605	12
Outgroup taxa				
Developayella elegans	ATCC 50518	SSU rRNA	U37107	10
Hyphochytrium catenoides	BR217	SSU rRNA	AF163294	6
Rhizidiomyces apophysatus	BR296	SSU rRNA	AF163295	6
Hyphochytrium catenoides	IMI 143642, ATCC 18719	LSU rRNA	X80345	14
Cyanidium caldarium	RK-1	cox2	Z48930	16
Hyphochytrium catenoides	IMI 143642, ATCC 18719	cox2	AF086701	7
Prototheca wickerhamii	263-11	cox2	U02970	17

Taxonomy is fundamentally based on Dick et al. (1984)

The sequences determined in this study are in boldface

[a] References: [1] This study; [2] Cook et al. (2001); [3] Dick et al. (1999); [4] Forster et al. (1990); [5] Gunderson et al. (1987); [6] Hausner et al. (2000); [7] Hudspeth et al. (2000); [8] Küpper et al. (2006); [9] Leclerc et al. (2000); [10] Leipe et al. (1996); [11] Petersen and Rosendahl (2000); [12] Riethmüller et al. (1999); [13] Van der Auwera et al. (1994); [14] Van der Auwera et al. (1995); [15] Vandersea et al. (2006); [16] Viehmann et al. (1996); [17] Wolff and Kuck (1990)

manually refined as a final process. For the cox2 gene (and for translated amino acid, COII) molecular phylogeny, our determined DNA sequences were manually aligned with the alignment of partial COII amino acid sequences (Cook et al. 2001), based on the translated protein sequences. Sequences have been deposited in DNA Data Bank of Japan (DDBJ) (see Table 1) and the alignments in Tree-BASE [matrix accession numbers are M3150 (SSU rRNA), M3152 (LSU rRNA), M3151 (cox2), and M3149 (three genes combined). http://treebase.org/treebase/].

The molecular phylogenetic trees of the SSU rRNA, LSU rRNA, and cox2 genes and the three-genes-combined tree were processed with PAUP*4.0b10 software (Swofford 2003). All gaps were treated as missing for SSU rRNA, LSU rRNA, and cox2 gene trees. For the tree combining DNA sequence data from all three genes, alignments of the three genes were combined in a row and gaps were treated as the ignored sites for pairwise comparisons. A heuristic search procedure using the tree bisection-reconnection (TBR) branch-swapping algorithm was performed to find the optimal maximum-likelihood (ML) tree topology. The transition/transversion (ti/tv) ratio was estimated by maximizing the likelihood value for the neighbor-joining (NJ) topology (Saitou and Nei 1987). The NJ topologies calculated with the HKY85 model (Hasegawa et al. 1985) were used as the initial topology for a heuristic search. The bootstrap values were calculated based on 1000 bootstrap replicates of NJ method with HKY85 model and 100 bootstrap replicates of ML method calculated from a heuristic search procedure using the subtree pruning regrafting (SPR) branch-swapping algorithm.

A maximum-likelihood tree inferred from COII amino acid sequences was constructed using the proml program with the "global rearrangements" option, in a PHYLIP version 3.65 package (Felsenstein 1989). The Jones-Taylor-Thornton (JTT) model (Jones et al. 1992) was used for the

analysis. The bootstrap values were calculated based on 2000 bootstrap replicates of the NJ method performed with protdist and neighbor programs, and 500 bootstrap replicates of ML method performed with the proml program (Felsenstein 1989).

Results

SSU rRNA gene tree phylogeny

In this study, 1782 bp (NJM0034) and 1755 bp (NJM0131) of the partial SSU rRNA gene sequences were determined; 260 of 1916 aligned nucleotides were excluded as gaps, and 1656 nucleotides were used for analysis. The ML tree inferred from the SSU rRNA gene (Fig. 1) shows that both NJM0131 and NJM0034 isolates are located at the basal position of the class Oomycetes together with *Eurychasma dicksonii* (E.P. Wright) Magnus, branching before separation of the main saprolegnian and peronosporalean clades. The separation of three basal oomycetes from the two main clades and the separation between three basal branches are supported by high bootstrap values (95%/98%, and 100%/100%, 99%/97%, and 81%/99% of ML/NJ bootstrap values from the basal to inside positions, respectively). Difference of topology between ML and NJ trees was not observed at the inside of the basal oomycete lineage or between the basal lineage and the two main clades (data not shown).

LSU rRNA gene tree phylogeny

In this study, 1063 bp (NJM0034) and 850 bp (NJM0131) of the partial LSU rRNA gene sequences were determined; 629 of 1186 aligned nucleotides were excluded as gaps, and

Fig. 1. Maximum-likelihood tree (1656 sites) based on 16 small-subunit (SSU) rRNA gene sequences of unknown oomycete NJM0034 and *Haliphthoros milfordensis* NJM0131 (in *boldface*), the organisms in the class Oomycetes, *Developayella elegans*, and two hyphochytrids (*Hyphochytrium catenoides* and *Rhizidiomyces apophysatus*). Two hyphochytrids were defined as an outgroup in the tree. Estimated transition/transversion (ti/tv) ratio is 1.531683. Bootstrap values (%) correspond to maximum-likelihood (ML) (100 replicates) and neighbor-joining (NJ) (1000 replicates) trees, respectively
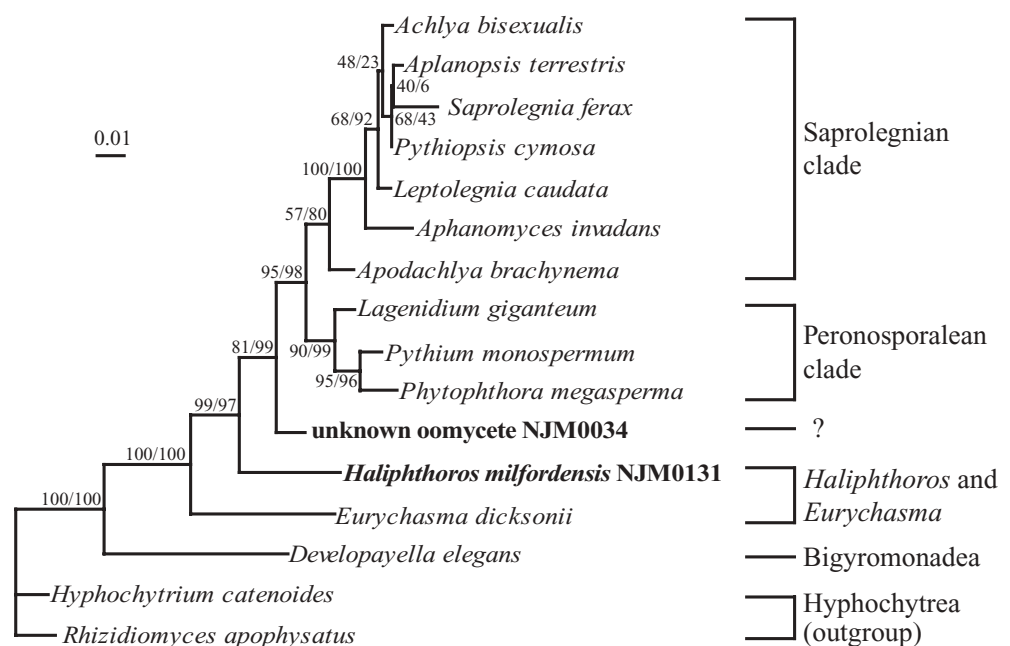
Fig. 2. Maximum-likelihood tree (537 sites) based on 23 large-subunit (LSU) rRNA gene sequences of unknown oomycete NJM0034 and *Haliphthoros milfordensis* NJM0131 (in *boldface*), the organisms in the class Oomycetes, and *Hyphochytrium catenoides*. *Hyphochytrium catenoides* was defined as an outgroup in the tree. Estimated ti/tv ratio is 1.339391. Bootstrap values (%) correspond to ML (100 replicates) and NJ (1000 replicates), respectively
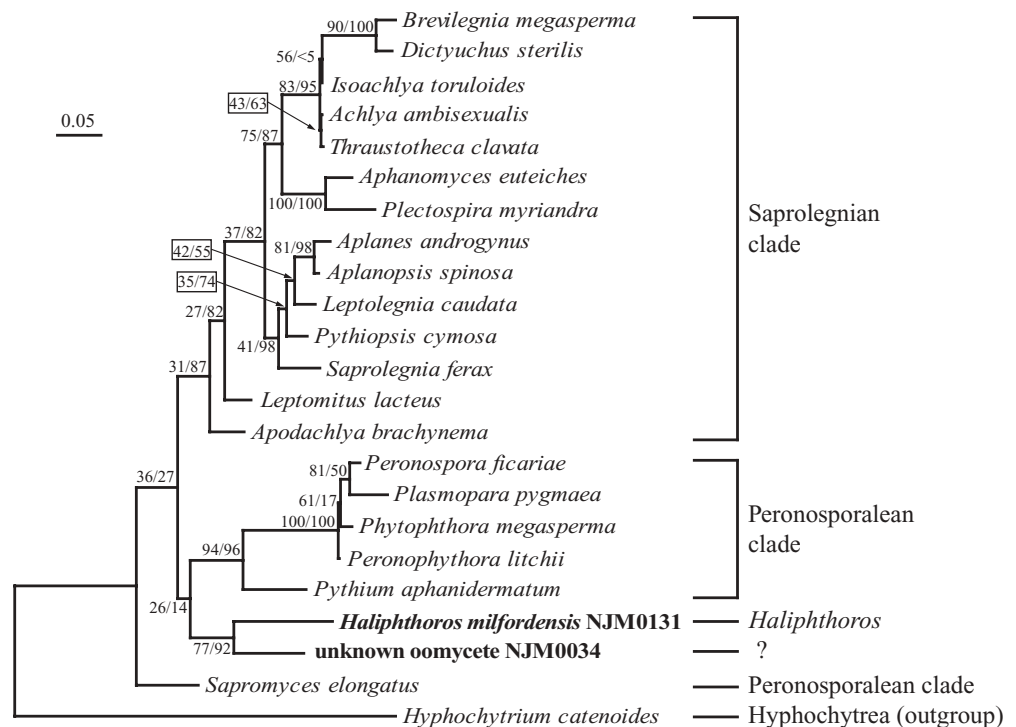
537 nucleotides were used for analysis. The ML tree inferred from the LSU rRNA gene (Fig. 2) shows that both NJM0131 and NJM0034 isolates form a monophyletic clade, which is a sister-group to the clade containing the orders Peronosporales and Pythiales (peronosporalean clade). *Sapromyces elongatus* (Cornu) Thaxt. (Rhipidiales) is located at the most basal position of the class Oomycetes but is not supported by a high bootstrap value (36%/27% of ML/NJ bootstrap values). The branching order between the four large monophyletic groups (Saprolegniales-Leptomitales group, Peronosporales-Pythiales group, NJM0131-NJM0034 group, and the Rhipidialean group represented by the single species *S. elongatus*) tends to change depending on the molecular analysis settings, but the clustering of each monophyletic group is nevertheless always consistently observed.

COII amino acid tree phylogeny

Of the partial cox2 gene sequences of NJM0034 and NJM0131, 180 amino acids (540 bp) and 178 amino acids (535 bp), respectively, were determined in this study; 20 of 194 aligned sites were excluded as gaps, and 174 sites were used for analysis. In the ML tree inferred from COII amino acid sequences (Fig. 3), *H. milfordensis* NJM0131 clusters with two *Haliphthoros* isolates (*H. milfordensis* NJM9434 and *H. philippinensis* SANK 15178), and its branching is supported by a high bootstrap value (98%/100% of ML/NJ bootstrap values). *Haliphthoros milfordensis* NJM0131, however, is clustered with *H. philippinensis* SANK 15178 but not with *H. milfordensis* NJM9434. Three *Haliphthoros* isolates are sister to *Halocrusticida (Halo.) okinawaensis* (K. Nakam. et Hatai) K. Nakam. et Hatai in the tree.

NJM0034 is separated from the clade including three *Haliphthoros* isolates and *Halo. okinawaensis,* and is also separated from the two main oomycete clades, but these branchings are not well supported (28%/12% of ML/NJ bootstrap values for the separation from the clade including three *Haliphthoros* isolates and *Halo. okinawaensis*; 38%/26% of ML/NJ bootstrap values for the separation from the two main oomycete clades). The same result was also given by the ML tree inferred from the cox2 gene DNA sequence (data not shown), but this data set had quite a low transition/transversion (ti/tv) ratio (0.463959). For this reason, the COII amino acid data set is shown for analysis in this study.

Combined tree phylogeny

In the tree-combining sequence data from all three genes (Fig. 4), NJM0034 forms a monophyletic clade together with three *Haliphthoros* isolates and *Halo. okinawaensis*, and its clade is branched before separation of the main saprolegnian and peronosporalean clades. *Eurychasma dicksonii* is clearly separated from *Haliphthoros* and *Halocrusticida*. *Sapromyces elongatus* forms a monophyletic clade together with the oomycete monophyletic species of the peronosporalean clade.

COII "indels" in the amino acid alignment

Hudspeth et al. (2000) and their further studies (Cook et al. 2001) identified a number of distinctive "indels" (insertion–deletion sequences) in the Oomycetes from the align-

Fig. 3. Maximum-likelihood tree (174 sites) based on 29 COII amino acid sequences of unknown oomycete NJM0034 and *Haliphthoros milfordensis* NJM0131 (in *boldface*), the organisms in the class Oomycetes, *Hyphochytrium catenoides*, *Cyanidium caldarium*, and *Prototheca wickerhamii*. *Cyanidium caldarium* and *P. wickerhamii* were defined as an outgroup in the tree. Bootstrap values (%) correspond to ML (500 replicates) and NJ (2000 replicates), respectively
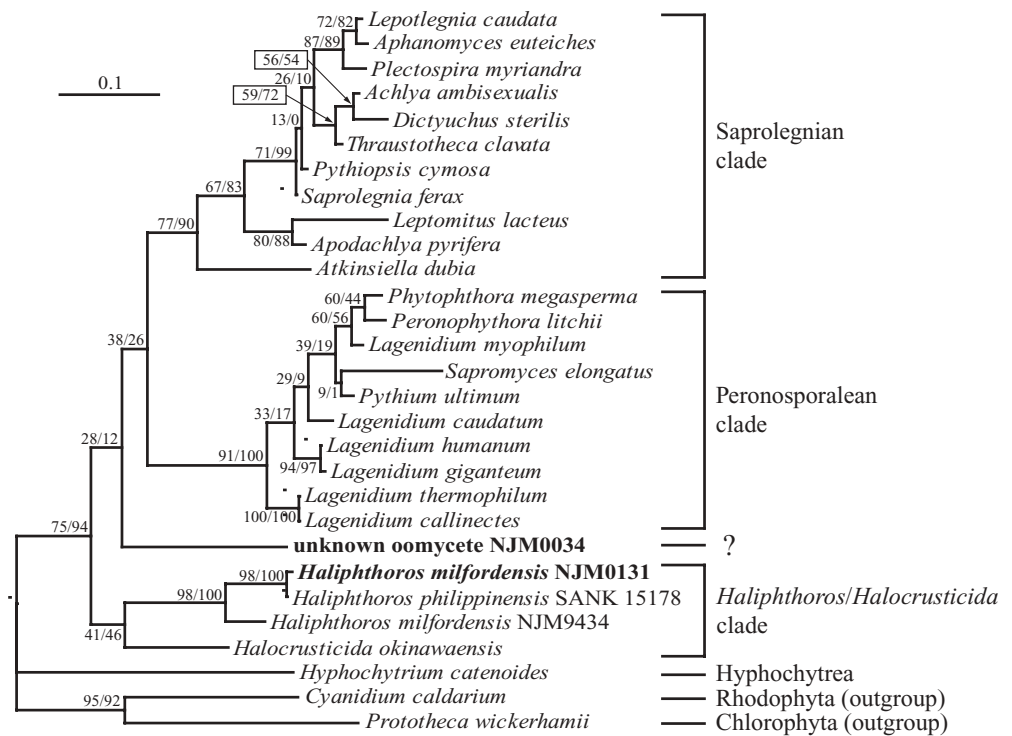
0.1

72/82 *Lepotlegnia caudata*
87/89 *Aphanomyces euteiches*
Plectospira myriandra
56/54
26/10 *Achlya ambisexualis*
59/72
13/0 *Dictyuchus sterilis*
Thraustotheca clavata
71/99 *Pythiopsis cymosa*
67/83 *Saprolegnia ferax*
Leptomitus lacteus
77/90
80/88 *Apodachlya pyrifera*
Atkinsiella dubia

Saprolegnian clade

60/44 *Phytophthora megasperma*
60/56 *Peronophythora litchii*
39/19 *Lagenidium myophilum*
Sapromyces elongatus
29/9
9/1 *Pythium ultimum*
33/17 *Lagenidium caudatum*
38/26 *Lagenidium humanum*
91/100 94/97 *Lagenidium giganteum*
Lagenidium thermophilum
100/100 *Lagenidium callinectes*

Peronosporalean clade

unknown oomycete NJM0034 ?

28/12
Haliphthoros milfordensis NJM0131
98/100
98/100 *Haliphthoros philippinensis* SANK 15178
41/46 *Haliphthoros milfordensis* NJM9434
Halocrusticida okinawaensis

Haliphthoros/Halocrusticida clade

75/94 *Hyphochytrium catenoides* Hyphochytrea
Cyanidium caldarium Rhodophyta (outgroup)
95/92 *Prototheca wickerhamii* Chlorophyta (outgroup)

Fig. 4. Maximum-likelihood tree (3630 sites) based on the combined three genes alignments [SSU rRNA, LSU rRNA, and cox2 genes; 42 operational taxonomic units (OTUs)], including the data of unknown oomycete NJM0034 and *Haliphthoros milfordensis* NJM0131 (in *boldface*). The cox2 gene sequences of *Prototheca wickerhamii* and *Cyanidium caldarium* were excluded from this analysis. Two hypochytrids (*Hyphochytrium catenoides* and *Rhizidiomyces apophysatus*) were defined as an outgroup. All gaps were treated as the ignored sites for pairwise comparisons. Estimated ti/tv ratio is 0.938950
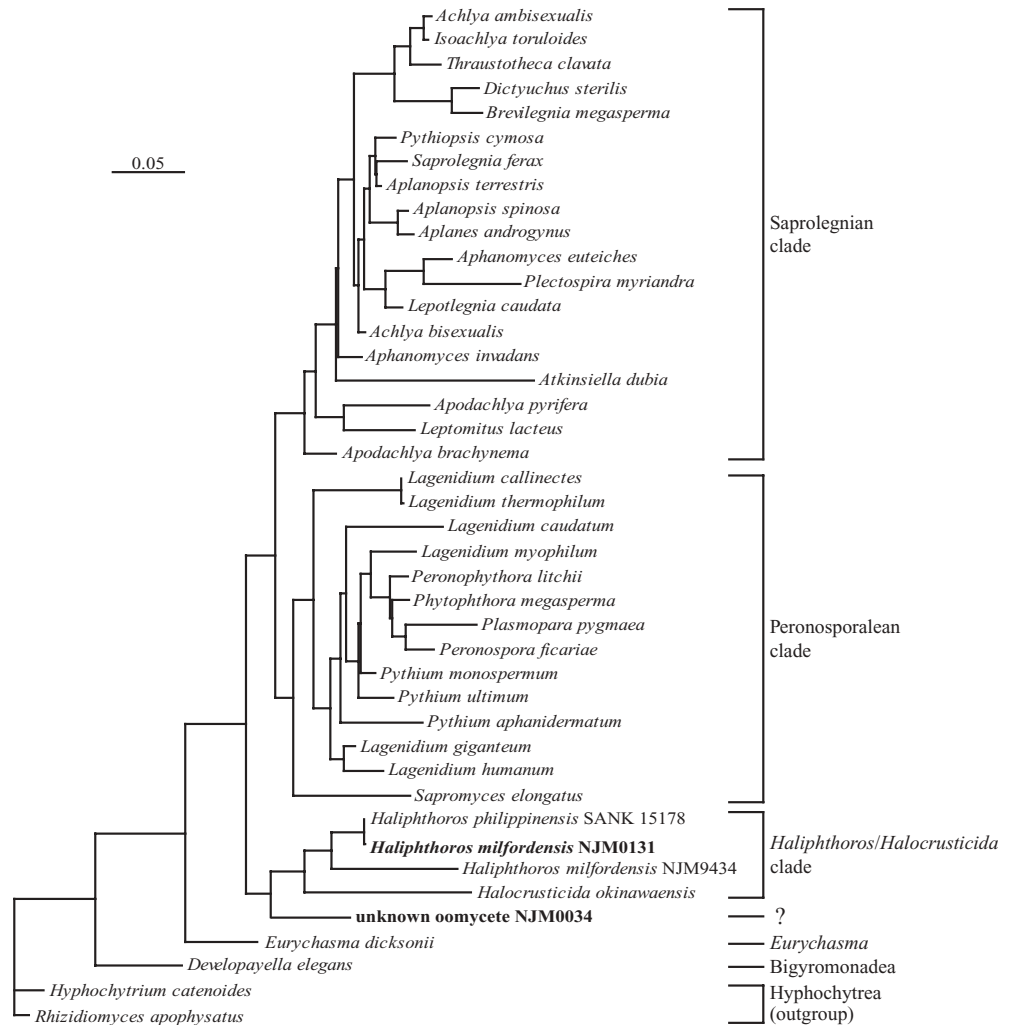
0.05

Achlya ambisexualis
Isoachlya toruloides
Thraustotheca clavata
Dictyuchus sterilis
Brevilegnia megasperma
Pythiopsis cymosa
Saprolegnia ferax
Aplanopsis terrestris
Aplanopsis spinosa
Aplanes androgynus
Aphanomyces euteiches
Plectospira myriandra
Lepotlegnia caudata
Achlya bisexualis
Aphanomyces invadans
Atkinsiella dubia
Apodachlya pyrifera
Leptomitus lacteus
Apodachlya brachynema

Saprolegnian clade

Lagenidium callinectes
Lagenidium thermophilum
Lagenidium caudatum
Lagenidium myophilum
Peronophythora litchii
Phytophthora megasperma
Plasmopara pygmaea
Peronospora ficariae
Pythium monospermum
Pythium ultimum
Pythium aphanidermatum
Lagenidium giganteum
Lagenidium humanum
Sapromyces elongatus

Peronosporalean clade

Haliphthoros philippinensis SANK 15178
Haliphthoros milfordensis NJM0131
Haliphthoros milfordensis NJM9434
Halocrusticida okinawaensis

Haliphthoros/Halocrusticida clade

unknown oomycete NJM0034 ?

Eurychasma dicksonii *Eurychasma*
Developayella elegans Bigyromonadea
Hyphochytrium catenoides Hyphochytrea (outgroup)
Rhizidiomyces apophysatus

Table 2. The parts of the alignment of partial COII amino acid sequences of the class Oomycetes

Oomycete order	Species	Amino acid sequences positioned at 38–43	Amino acid sequences positioned at 105–112
Saprolegniales	*Achlya ambisexualis*	N E N T N K	DA_____VE
Saprolegniales	*Aphanomyces euteiches*	N E N T N K	DA_____VE
Saprolegniales	*Dictyuchus sterilis*	N E N T N K	DA_____VE
Saprolegniales	*Leptolegnia caudata*	N E N T N K	DA_____VE
Saprolegniales	*Plectospira myriandra*	N E N T N K	DA_____VE
Saprolegniales	*Pythiopsis cymosa*	N E N T N K	DA_____VE
Saprolegniales	*Saprolegnia ferax*	N E N T N K	DA_____VE
Saprolegniales	*Thraustotheca clavata*	N E N T N K	DA_____VE
Saprolegniales	*Atkinsiella dubia*	T E _ _ N K	DNFSL_NN
Saprolegniales	*Haliphthoros milfordensis*	V E _ _ N K	DNLNSFEE
Saprolegniales	*Halihthoros philippinensis*	T N _ _ N K	DNIDNINE
Saprolegniales	*Halocrusticida okinawaensis*	N E D V K P	DNTDL_DD
Unknown	**NJM0034**	**N Q Q K N P**	**DDFIN_LE**
Saprolegniales	**NJM0131**	**T N _ _ N K**	**DNIDNINE**
Leptomitales	*Apodachlya pyrifera*	D E D K H K	DSYTD_TN
Leptomitales	*Leptomitus lacteus*	D E D K Q K	DSYTD_TN
Pythiales	*Lagenidium callinectes*	D E K K N P	DNLEY_AD
Pythiales	*Lagenidium caudatum*	D E K K N P	DNLEF_AD
Pythiales	*Lagenidium giganteum*	D E K K N P	DNLEY_AD
Pythiales	*Lagenidium humanum*	D E K K N P	DNLEY_AD
Pythiales	*Lagenidium myophilum*	D E K K N K	DNLEF_AD
Pythiales	*Lagenidium thermophilum*	D E K K N P	DNLEY_AD
Pythiales	*Pythium ultimum*	D E K K N K	DNLEF_SD
Peronosporales	*Phytophthora megasperma*	D E K K N K	DNLEF_SD
Peronosporales	*Peronophythora litchii*	D E K K N K	DNLEF_SD
Rhipidiales	*Sapromyces elongatus*	D E K K N K	DNLEF_SD
Non-oomycete lineage	*Hyphochytum catenoides*	N S K Q N P	DYNTK_EK
Non-oomycete lineage	*Cyanidium caldarium*	N S K T N P	DYVNE_EN
Non-oomycete lineage	*Prototheca wickerhamii*	H Y T R N P	DYSLA_DD

Two sequences determined in this study are shown in boldface; _ means deletion
Source: Original table from Cook et al. (2001)

ment data of COII amino acid sequences (summarized in Table 2) that were useful in discriminating between some of the major orders with this class. This indel (positioned at 105–112) is not generally found in the order Saprolegniales, except for some of the marine holocarpic species. The order Leptomitales is characterized by a YTD indel sequence, and the peronosporalean clade by LEF/LEY sequences. According to the COII alignment data of Cook et al. (2001), the four marine holocarpic oomycete parasites possess distinctive additional tri/tetra peptide indels as follows: *Atkinsiella dubia* (D. Atkins) Vishniac, FSL, *H. milfordensis* NJM9434, LNSF, *H. philippinensis* SANK 15178, IDNI, and *Halo. okinawaensis*, TDL (see Table 2). NJM0034 also possesses an additional "tripeptide" indel, FIN. *Haliphthoros milfordensis* NJM0131 also possesses a "tetrapeptide" additional indel, IDNI. Other distinguishable indels described by Cook et al. (see Table 2) are dipeptide deletions positioned at the 38th to 43rd amino acids. In this region, two amino acid deletions are observed in *A. dubia*, *H. milfordensis* NJM9434, and *H. philippinensis* SANK 15178 but not in *Halo. okinawaensis*. In our data, this deletion is observed in *H. milfordensis* NJM0131 but is not observed in NJM0034 (see Table 2). Comparison of the COII amino acid sequences substitutions among both isolates clearly shows the molecular phylogenetic affinity of *H. milfordensis* NJM0131 with *H. philippinensis* SANK 15178. The DNA base substitution

between the cox2 gene DNA sequences of *H. milfordensis* NJM0131 and *H. philippinensis* SANK 15178 is only 1 of 535 bases, which corresponds to 1 of 178 amino acid substitutions in the COII amino acid sequences, whereas the DNA base substitutions between *H. milfordensis* NJM0131 and *H. milfordensis* NJM9434 in the cox2 DNA sequences are 70 of 535 bases (21 of 178 amino acids).

Discussion

Classification of the oomycete organisms

The classification of the class Oomycetes has been widely debated and discussed (Sparrow 1960, 1973, 1976; Dick et al. 1984; Dick 1995, 1998, 2001). Although the most recent detailed revision of the class is that of Dick (2001), which gave a new name for the class, as "Peronosporomycetes," this revision was based entirely on morphological criteria rather than molecular analysis. His revised scheme is considerably more complex, particularly in relation to many of the marine genera described in this study. For instance, he transferred five marine species of the genus *Halocrusticida* (all of which were formerly assigned to the genus *Atkinsiella*) to his newly erected genus *Halodaphnea* M.W. Dick, and one insect pathogenic species (*Halocrusticida ento-*

mophaga (W.W. Martin) K. Nakam. et Hatai) to the genus *Crypticola* Humber et al. in the new family Crypticolaceae (Dick 1998), and included them within a group of nematode pathogens within a newly erected order, the Myzocytiopsidales (Dick 2001). However, the somewhat similar *Haliphthoros* was left unassigned to any order. Without underlying molecular data, many of these revisions appear confusing and untenable; therefore, we have fundamentally adopted (in Table 1) the earlier classification nomenclature used in earlier schemes by Dick et al. (1984). In these classification schemes, *Haliphthoros* and *Atkinsiella* were both placed in the order Saprolegniales. We have included the more recently described genus *Halocrusticida* is in the order as well because it was erected as a new genus to accommodate six species formerly placed in the genus *Atkinsiella* (Nakamura and Hatai 1995). *Leptomitus* C. Agardh is in the order Leptomitales, *Sapromyces* Fritsch is in the order Rhipidiales, and *Peronospora* Corda and related peronosporalean taxa are in the order Peronosporales. *Pythium* Pringsh. and *Lagenidium* Schenk, which were formerly in the order Peronosporales and Lagenidiales, respectively (Ainsworth et al. 1973), are placed in the order Pythiales (Dick et al. 1984). For convenience, the orders Saprolegniales and Leptomitales are grouped together under the "saprolegnian clade" and the orders Rhipidiales, Peronosporales, and Pythiales form the "peronosporalean clade." These map on the concept of Saprolegnian and Peronosporalean "galaxies" as defined by Sparrow (1976) and are equivalent to the subclasses Saprolegniomycetidae and Peronosporomycetidae proposed by Dick et al. (1984).

Overview of the molecular trees in this study

In the LSU rRNA gene tree (see Fig. 2), both NJM0034 and NJM0131 isolates formed a monophyletic clade together within the Oomycetes, but rather surprisingly appearing as a basal of the peronosporalean clade. The one member of the Rhipidiales so far sequenced, *Sapromyces*, formed a separate earliest diverging clade on the LSU rRNA gene tree, which may reflect the limited taxon sample available for this gene. However, in both the SSU rRNA gene and COII amino acid trees, the two marine isolates being investigated in this study diverged before separation of both the main saprolegnian and peronosporalean clades (see Figs. 1, 3), as demonstrated by Cook et al (2001). Furthermore, when data for all three genes were combined (see Fig. 4), the tree also showed that our two isolates clustered with other marine genera in a monophyletic assemblage, which branched before separation of the two main clades. Only *Eurychasma dicksonii*, a parasite of marine seaweeds, diverged before the marine animal parasite clade (Fig. 4). NJM0034 and NJM0131 isolates appeared separately in the marine animal parasite clade (Fig. 4). Unfortunately, sequence data for all three genes have been determined for very few oomycete organisms at present, and consequently our alignment data for this combined tree include quite a number of "missing data sites" that were not used for pairwise comparison.

The phylogenetic position of isolate NJM0034

The relative phylogenetic position of the nonsporulating isolate NJM0034 compared with the isolate of *H. milfordensis* NJM0131, which had been identified using morphological criteria, was found to vary depending on the different genes (and taxon samples) used. NJM0034 forms a monophyletic clade together with NJM0131 in the LSU rRNA gene tree (see Fig. 2) whereas NJM0034 is not clustered with *Haliphthoros*/*Halocrusticida* species, nor does it form a sister-group with them in the more taxon rich SSU rRNA gene tree, the COII amino acid tree, and the combined tree (see Figs. 1, 3, 4). In the COII amino acid tree (Fig. 3), the grouping of three *Haliphthoros* isolates is supported by a high bootstrap value (98%/100% of ML/NJ bootstrap values). Although the grouping of the three *Haliphthoros* isolates and *Halo. okinawaensis* is supported by a somewhat low bootstrap value (41%/46% of ML/NJ bootstrap values) in the amino acid tree, it is supported by high bootstrap values in the cox2 gene DNA tree (77%/93% ML/NJ bootstrap value; data not shown). In this tree, the unidentifiable NJM0034 branches out as the sister-group of *Haliphthoros*/*Halocrusticida* species and is therefore likely to be a hitherto unknown taxon. These results suggest that NJM0034 is phylogenetically separate from the genus *Haliphthoros*. Indel analyses (see Table 2) also indicate that NJM0034 is not clustered with any known *Haliphthoros* species. Fortunately, other nonsporulating *Haliphthoros*-like isolates have already been isolated (Hatai et al., unpublished data) and will warrant further study using molecular characters.

Molecular phylogeny of the genus *Haliphthoros*

Our COII amino acid tree (see Fig. 3) and the analyses of the indels (see Table 2) show that *H. milfordensis* NJM0131 clusters with *H. philippinensis* SANK 15178 (type strain of this species), not with *H. milfordensis* NJM9434. Hatai et al. (1980) separated *H. philippinensis* from *H. milfordensis* based upon the following features: (i) *H. milfordensis* releases zoospores only from the orifice of the discharge tube, whereas zoospore release in *H. philippinensis* occurs both via discharge tube and openings in the sporangium wall; (ii) zoosporogenesis in *H. philippinensis* is initiated more quickly upon transfer to seawater compared to *H. milfordensis*; (iii) the zoospores of *H. philippinensis* are polymorphic; and (iv) the thallus fragments into dense segments more readily in *H. philippinensis*. In this study both NJM0131 and NJM9434 isolates were considered to be "typical" *H. milfordensis* in that they released their zoospores only from the orifice of the discharge tube (Nakamura and Hatai 1995; Chukanhom et al. 2003). However, our COII amino acid tree data showed that two "typical" *H. milfordensis* isolates did not form a monophyletic clade. One of our two *H. milfordensis* isolates (NJM0131) actually clusters with *H. philippinensis*. Rather surprisingly, the branch length separating NJM0131 and NJM9434 is not short and is comparable to the branch lengths that separate many of the genera in the main saprolegnian and pero-

nosporalean clades. This result suggests that the genus *Haliphthoros* as currently defined contains a much more diverse range of organisms than previously thought. There appear to be several cryptic species and/or genera within this *Haliphthoros* clade. At least, our data suggest that a taxonomical reinvestigation into the species descriptors within this genus must be considered. Future DNA sequencing of more *Haliphthoros* isolates will contribute to clarify the phylogenetic diversity and affinity of this genus, and taxonomic investigation of this genus based on the combined thallus/zoosporangium morphology and molecular phylogeny is clearly required.

References

Ainsworth GC, Sparrow FK, Sussman AS (1973) The fungi, an advanced treatise, vol IV B. Academic Press, New York

Alderman DJ (1982) Fungal disease of aquatic animals. In: Roberts RJ (ed) Microbial diseases of fish. Special publication 9. Academic Press, London, pp 189–242

Chukanhom K, Borisutpeth P, Khoa LV, Hatai K (2003) *Haliphthoros milfordensis* isolated from black tiger prawn larvae (*Panaeus monodon*) in Vietnam. Mycoscience 44:123–127

Cook KL, Hudspeth DSS, Hudspeth MES (2001) A cox2 phylogeny of representative marine peronosporomycetes (Oomycetes). Nova Hedwigia Beih 122:231–243

Dick MW (1995) Sexual reproduction in the Peronosporomycetes (chromistan fungi). Can J Bot 73(suppl 1):S712–S724

Dick MW (1998) The species and systematic position of *Crypticola* in the Peronosporomycetes, and a new names for *Halocrusticida* and species therein. Mycol Res 102:1062–1066

Dick MW (2001) Straminipilous fungi: systematics of the peronosporomycetes, including accounts of the marine straminipilous protests, the plasmodiophorids, and similar organisms. Kluwer, Dordrecht

Dick MW, Wong PTW, Clark G (1984) The identify of the oomycete causing "Kikuyu Yellows," with a reclassification of the downy mildews. Bot J Linn Soc 89:171–197

Dick MW, Vick MC, Gibbings JG, Hedderson TA, Lopez Lastra CC (1999) 18S rDNA for species of *Leptolegnia* and other Peronosporomycetes: justification of the subclass taxa Saprolegniomycetidae and Peronosporomycetidae and division of the Saprolegniaceae sensu lato into the Leptolegniaceae and Saprolegniaceae. Mycol Res 103:1119–1125

Diggles BK (2001) A mycosis of juvenile spiny lobster, *Jasus edwardsii* (Hutton, 1875) caused by *Haliphthoros* sp., and possible methods of chemical control. J Fish Dis 24:99–110

Felsenstein J (1989) PHYLIP: phylogeny inference package (version 3.2). Cladistics 5:164–166

Forster H, Coffey MD, Elwood H, Sogin ML (1990) Sequence analysis of the small subunit ribosomal RNAs of three zoosporic fungi and implications for fungal evolution. Mycologia 82:306–312

Gunderson JH, Elwood H, Ingold A, Kindle K, Sogin ML (1987) Phylogenetic relationships between chlorophytes, chrysophytes, andoomycetes. Proc Natl Acad Sci U S A 84:5823–5827

Hasegawa M, Kishino H, Yano T (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J Mol Evol 22:160–174

Hatai K (1989) Fungal pathogens and parasites of aquatic animals. In: Austin B, Austin DA (eds) Methods for the microbiological examination of fish and shellfish. Horwood, London, pp 250–258

Hatai K, Bian BZ, Baticados MCL, Egusa S (1980) Studies on the fungal diseases in crustaceans. II. *Haliphthoros philippinensis* sp.

nov. isolated from cultivated larvae of the jumbo tiger prawn (*Penaeus monodon*). Trans Mycol Soc Jpn 21:47–55

Hatai K, Rhoobunjongde W, Wada S (1992) *Haliphthoros milfordensis* isolated from gills of juvenile kuruma prawn (*Penaeus japonicus*) with black gill disease. Trans Mycol Soc Jpn 33:185–192

Hatai K, Roza D, Nakayama T (2000) Identification of lower fungi isolated from larvae of mangrove crab, *Scylla serrata*, in Indonesia. Mycoscience 41:565–572

Hausner G, Belkhiri A, Klassen GR (2000) Phylogenetic analysis of the small subunit ribosomal RNA gene of the hyphochytrid *Rhizidiomyces apophysatus*. Can J Bot 78:124–128

Hudspeth DSS, Nadler SA, Hudspeth MES (2000) A cox II molecular phylogeny of the Peronosporomycetes. Mycologia 92:674–684

Jones DT, Taylor WR, Thornton JM (1992) A new approach to protein fold recognition. Nature (Lond) 358:86–89

Küpper FC, Maier I, Müller DG, Loiseaux-de Goer S, Guillou L (2006) Phylogenetic affinities of two eukaryotic pathogens of marine macroalgae, *Eurychasma dicksonii* (Wright) Magnus and *Chytridium polysiphoniae* Cohn. Cryptogamie Algol 27:165–184

Leclerc MC, Guillot J, Deville M (2000) Taxonomic and phylogenetic analysis of Saprolegniaceae (Oomycetes) inferred from LSU rDNA and ITS sequence comparisons. Antonie Van Leeuwenhoek 77:369–377

Leipe DD, Tong SM, Goggin CL, Slemenda SB, Pieniazek NJ, Sogin ML (1996) 16S-like rDNA sequences from *Developayella elegans*, *Labyrinthuloides haliotidis*, and *Proteromonas lacertae* confirm that the stramenopiles are a primarily heterotrophic group. Eur J Protistol 32:449–458

Lightner DV (1981) Fungal diseases of marine crustacea. In: Davidson EW (ed) Pathogenesis of invertebrate microbial diseases. Allanheld, Osmun, NJ, pp 451–484

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321–4325

Nakamura K, Hatai K (1995) Three species of Lagenidiales isolated from the eggs and zoeae of the marine crab *Portunus pelagicus*. Mycoscience 36:87–95

Nakayama T, Watanabe S, Mitsui K, Uchida H, Inouye I (1996) The phylogenetic relationship between the Chlamydomonadales and Chlorococcales inferred from 18S rDNA sequence data. Phycol Res 44:47–55

Petersen AB, Rosendahl S (2000) Phylogeny of the Peronosporomycetes (Oomycota) based on partial sequences of the large ribosomal subunit (LSU rDNA). Mycol Res 104:1295–1303

Riethmüller A, Weiss M, Oberwinkler F (1999) Phylogenetic studies of Saprolegniomycetidae and related groups based on nuclear large subunit ribosomal DNA sequences. Can J Bot 77:1790–1800

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sparrow FK (1960) Aquatic Phycomycetes, 2nd edn. University of Michigan Press, Ann Arbor

Sparrow FK (1973) Lagenidiales. In: Ainsworth GC, Sparrow FK, Sussman AS (eds) The fungi, an advanced treatise, vol IV B. Academic Press, New York, pp 159–163

Sparrow FK (1976) The present status of classification in biflagellate fungi. In: Gareth-Jones EB (ed) Recent advances in aquatic mycology, 2nd edn. Elek Science, London, pp 213–222

Swofford DL (2003) PAUP*: phylogenetic analysis using parsimony (*and other methods), version 4.0b10. Sinauer, Sunderland, MA

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Van der Auwera G, Chapelle S, De Wachter R (1994) Structure of the large ribosomal subunit RNA of *Phytophthora megasperma*, and phylogeny of the oomycetes. FEBS Lett 338:133–136

Van der Auwera G, De Baere R, Van de Peer Y, De Rijk P, Van den Broeck I, De Wachter R (1995) The phylogeny of the Hyphochytriomycota as deduced from ribosomal RNA sequences of *Hyphochytrium catenoides*. Mol Biol Evol 12:671–678

Vandersea MW, Litaker RW, Yonnish B, Sosa E, Landsberg JH, Pullinger C, Moon-Butzin P, Green J, Morris JA, Kator H, Noga EJ, Tester PA (2006) Molecular assays for detecting *Aphanomyces inva-*

dans in ulcerative mycotic fish lesions. Appl Environ Microbiol 72:1551–1557

Viehmann S, Richard O, Boyen C, Zetsche K (1996) Genes for two subunits of succinate dehydrogenase form a cluster on the mitochondrial genome of Rhodophyta. Curr Genet 29:199–201

Vishniac HS (1958) A new marine phycomycete. Mycologia 50:66–79

Wolff G, Kuck U (1990) The structural analysis of the mitochondrial SSU rRNA implies a close phylogenetic relationship between mitochondria from plants and from the heterotrophic alga *Prototheca wickerhamii*. Curr Genet 17:347–351

Wuyts J, Perriere G, Van de Peer Y (2004) The European ribosomal RNA database. Nucleic Acids Res 32:D101–D103