FULL PAPER

A new species, *Aphanomyces salsuginosus* sp. nov., isolated from ice fish *Salangichthys microdon*

Daisuke Takuma · Ayako Sano · Shinpei Wada ·
Osamu Kurata · Kishio Hatai

Abstract A species of *Aphanomyces* was isolated from the ice fish *Salangichthys microdon* living in brackish water in Japan. White cotton-like growth was found on the heads and fins of the fish. Hyphae penetrated into the dermal layers, subcutaneous tissues, muscular layers, and cartilaginous tissue of the mandible and maxilla; these hyphae were associated with cellular debris and lesions in host tissue. White fluffy colonies from subcultures of these growths were isolated on glucose–yeast agar plates with 0.5% sodium chloride (NaCl). These isolates consisted of delicate, slightly wavy, and moderately branched hyphae. Zoosporangia were isodiametric with the vegetative hyphae. Oogonia were abundant and approximately 21–33 μm in diameter, with irregular short papillae. Generally they were spherical or subspherical and only rarely pyriform. Individual oogonia usually contained a single oospore, which was spherical and 19–27 μm in diameter, with a large shiny vesicle. Antheridial branches, when present, were usually androgynous; however, they were sometimes monoclinous or diclinous. The optimal growth temperature of the isolates was 20°C, and cultures grew well at low salinity (0–0.5% NaCl). Phylogenic analysis based on the internal transcribed space 1-5.8S-ITS 2 of the ribosomal RNA gene indicates that these isolates will be an as-yet unidentified species of *Aphanomyces*.

D. Takuma · S. Wada · O. Kurata · K. Hatai (✉)
Laboratory of Fish Diseases, Nippon Veterinary and Life
Science University, 1-7-1, Kyonan-Cho, Musashino,
Tokyo 180-8602, Japan
e-mail: khatai0111@nvlu.ac.jp

A. Sano
Research Center for Pathogenic Fungi and Microbial Toxicoses,
Chiba University, Chiba, Japan

Introduction

The genus *Aphanomyces* includes saprotrophs, phytopathogens, and animal pathogens (Scott 1961; Johnson et al. 2002). Recently, the phylogenetic relationship of *Aphanomyces* spp. found on plants, animals, and environmental detritus were clearly classified based on internal transcribed spacer (ITS) regions of ribosomal RNA (rRNA) gene sequences (rDNA) (Diéguez-Uribeondo et al. 2009). The main causal agents of fungal diseases in fishes and shellfishes belong to the genus *Aphanomyces* (Hatai 1989) (Table 1). For example, *A. astaci* Schikora has been reported as the most serious pathogen of European and Australian freshwater crayfish (Unestem 1972). Similarly *A. repetans* nom. nud. and *A. frigidophilus* Kitanch. & Hatai were also reported as pathogens of freshwater crayfish (Royo et al. 2004; Ballesteros et al. 2006).

A. invadans Willoughby, R.J. Roberts & Chinabut (also called *A. piscicida* Hatai) was first reported as the pathogen causing mycotic granulomatosis in ayu *Plecoglossus altivelis* in Japan (Hatai 1980). Later, *A. invadans* was identified as the cause of epizootic ulcerative syndrome (eEUS) in some fishes from Southeast Asian countries (Willoughby and Roberts 1994; Lilley et al. 1998) and in sand whiting *Sillago ciliate* from South Africa (Andrew et al. 2008). Similarly, *A. invadans* was found to cause ulcerative mycosis in Atlantic menhaden *Brevoortia tyrannus* from the USA (Dykstra et al. 1986) and red spot disease (RSD) in sea mullet *Mugil cephalus* and yellowfin bream *Acanthopagrus australis* from Australia (Fraser et al. 1992). In 2002 a group of experts on fungal diseases

Table 1 *Aphanomyces* species isolated from fish and shellfish

Species	Host	Reference
Aphanomyces helicoides	Common carp	Srivastava and Srivastava (1976)
A. laevis	Guppy	Shanor and Saslow (1944)
A. stellatus	Eel	Hoshina et al. (1960)
	Common carp	Shah et al. (1977)
A. astaci	Crayfish	Unestem (1972)
A. repetans	Crayfish	Royo et al. (2004)
A. frigidophilus	Japanese char egg	Kitancharoen and Hatai (1997)
	Crayfish	Ballesteros et al. (2006)
A. invadans (=*A. piscicida*)	Ayu	Hatai (1980)
	Atlantic menharden	Dykstra et al. (1986)
	Sea mullet	Fraser et al. (1992)
	Yellowfin bream	Fraser et al. (1992)
	Sand whiting	Fraser et al. (1992)
Aphanomyces sp.	Soft-shelled turtle	Sinmuk et al. (1996)

of fish from Japan, Australia, USA, India, Philippines, and Thailand concluded that mycotic granulomatosis (MG), RSD, ulcerative mycosis (UM), and eEUS are all the same disease and proposed a new name: epizootic granulomatous aphanomycosis (EGA) (Baldoc et al. 2005). This effort helped bring continuity to the variety of reports on fungal fish disease from around the world in which *A. invadans* had been isolated from the fish suffering from disease. Reports also indicate that *A. laevis* de Bary has been repeatedly isolated from the guppy *Lebistes reticulates* (Shanor and Saslow 1944). Similarly, *A. stellatus* de Bary was isolated from the common carp *Cyprinus carpio* (Shah et al. 1977) and the Japanese eel *Anguilla japonica* (Hoshina et al. 1960). All of these *Aphanomyces* spp. were isolated from fish suffering from pathogenic fungal growth.

In February 2008, we encountered an outbreak of a fungal infection in the ice fish *Salangichtys microdon* in an aquarium at Lake Shinji, Shimane Prefecture, Japan. Ice fish live in blackish water, but water mold infection usually occurs in fresh water. We were interested in the infection because it occurred in blackish water fish, then tried to isolate the water mold. As a result, *Aphanomyces* spp. were only isolated from the fish. In this paper, we describe the causal agent of the infection caused by *Aphanomyces* sp., and compare it to the other animal parasitic *Aphanomyces* spp. according to morphological, physiological, and phylogenetic criteria.

Materials and methods

Isolation

Typically, an infected fish showed small whitish masses with cotton-like appearance at the mouth and/or fins (Fig. 1). Small pieces from these masses were rinsed in sterilized tap water with 0.5% sodium chloride (NaCl). The rinsed pieces were then used to inoculate the center of 90 × 20 mm disposable Petri dishes containing 1% glucose–0.25% yeast agar (GY agar) supplemented with 0.5% NaCl and 500 mg/ml of each ampicillin and streptomycin, Inoculated plates were incubated at 15°C for 4–6 days. The resulting fungal colonies were subsequently cultured on GY agar plates supplemented with 0.5% NaCl. Five fungal colonies were isolated from the specimens: NJM 0801, NJM 0802, NJM 0803, NJM 0804, and NJM 0805 (Table 2).

Histopathological observation

All fish bodies were fixed in 10% phosphate-buffered formalin solution, decalcified in ethylenediaminetetraacetic acid (EDTA) with 5% formalin, and processed for routine histology, as described in Hendrickson (1985). All paraffin sections from the specimens were stained with hematoxylin and eosin (H & E), Gomori's methenamine silver-nitrate, Grocott's variation counterstained with H & E (Grocott-H & E), and Gram stains.

Identification

To grow mycelia, an agar block was cut from the edge of a colony, put into GY broth with 0.5% NaCl, and incubated at 20°C for 2 days. Growing mycelia were rinsed twice in sterilized tap water with 0.5% NaCl and resuspended in sterilized tap water with 0.5% NaCl to induce development

Fig. 1 Ice fish affected with water mold. Lesions of white, cotton-like masses of mycelia on the head, mouth, and/or fins of ice fish. *Bar* 1 cm

Table 2 Isolates from ice fish

Strains	Fish no.	Site of isolation	Accession no.
NJM 0801	1	Rostrum	AB510348
NJM 0802	2	Rostrum	AB510349
NJM 0803	3	Rostrum	AB510350
NJM 0804	4	Natsi fin	AB510351
NJM 0805	5	Rostrum	AB510352

of zoospores and oogonia. The isolated fungi were identified according to Sparrow (1960) and Scott (1961) based on the asexual and sexual structures in the water culture.

Physiological studies

The effect of temperature on vegetative growth was determined using isolates NJM 0801 and NJM 0803. The advancing edge of 6-day-old colonies cultured on GY agar with 0.5% NaCl was cut out with a No. 2 cork borer (approximately 5.5 mm in diameter). Individual agar cores were placed on the center of 90×20-mm Petri dishes containing 20 ml of GY agar with 0.5% NaCl, and the plates were incubated at 5, 10, 15, 20, 25, 30, or 35°C. Colony diameters were estimated as follows: The widest and narrowest diameter of radial growth from the center of each core was measured. Colony diameter was estimated as the average of these two measurements minus the 5.5 mm of the original agar core. Estimates of colony diameter were determined 3, 5, and 7 days after inoculation. The effect of NaCl concentration on vegetative growth was determined using isolates NJM 0801 and NJM 0803. The advancing edge of 7-day-old colonies cultured on GY agar with 0.5% NaCl were cut out with a No. 2 cork borer. Individual agar cores were placed on the center of 90×20-mm Petri dishes containing 20 ml of GY agar and 0, 0.5, 1, 2, 3, or 5% NaCl. All plates were incubated at 20°C. The diameter of each colony was determined using the same method described for studies on the effects of temperature on vegetative growth. These colonies were measured at days 3, 5 and 7. These tests were carried out one time.

Molecular studies

The ITS rDNA was sequenced. DNA was extracted from hyphae cultured at 20°C in GY broth with 0.5% NaCl. Mycelia were dried on tissue paper, and each mycelium was transferred to a separate 1.5-ml microcentrifuge tube. The samples were frozen at −85°C prior to DNA extraction as described by Abliz et al. (2004). ITS rDNA sequences were determined as follows: We used a nested polymerase chain reaction (PCR) system to amplify ITS rDNA using universal primers (rDNA primer sequences for

fungal sequencing can be found at http://www.biology.duke.edu/fungi/mycolab/primers.htm, and Kurtzman and Robnett 1997). PCR reaction mixtures included 2.5 µl of DNA extracted with a Ready-to-Go bead (Amersham Pharmacia Tokyo, Japan), 2.5 µl of 10 pM primers solution (NS-7: 5′-GAG GCA ATA ACA GGT CTG TGA TGC-3′ and NL-2: 5′-CTT GTT CGC TAT CGG TCT C-3′), and 17.5 µl of distilled water. The reaction mixtures were subjected to one cycle of denaturation at 95°C for 4 min, 30 cycles of amplification (94°C for 1 min, 54–55°C for 1 min, and 72°C for 2 min), and a final extension cycle at 72°C for 10 min in a PCR Thermal Cycler MP (TaKaRa, Ohtsu, Japan). First-round PCR products were purified with a commercial kit (QIAquick®, Qiagen Co. Ltd., Tokyo, Japan) and then diluted 10- to 100-fold. The second round of PCR amplification used one of the following combinations of internal primer pairs: NS-7 and ITS-2: 5′-GCT GCG TTC TTC ATC GAT GC-3′, ITS-5: 5′-GGA AGT AAA AGT CGT AAC AAGG-3′, ITS-4: 5′-TCC TCC GCT TAT TGA TAT GC-3′, and ITS-3: 5′-GCA TCG ATG AAG AAC GCA GC-3′. The condition of second-round PCR was performed under the same condition as the first. The second-round PCR products were visualized by electrophoresis in 1.0% agarose in $1 \times$ TBE buffer [0.04 M Tris-boric acid, 0.001 M EDTA (pH 8.0)] followed by ethidium bromide staining. The PCR products were purified directly from PCR reaction mixtures using a PCR purification kit (QIAquick®) and then labeled with BigDye® Terminator Ver. 1.1 (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. The labeled samples were directly sequenced on an ABI PRISM 3100 sequencer (Applied Biosystems) using the same primers used in the second round of PCR amplification.

Phylogenetic analysis

DNA sequences were aligned using GENETEX-WIN genetic information processing software (Software Development, Tokyo, Japan). Sequences were analyzed using the BLAST search of GeneBank (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi), and closely related sequences were identified through analysis of the tree generated by the BLAST program. *Aphanomyces* species with sequence data deposited in GenBank were selected as relatives of the *Aphanomyces* sp. isolated in our studies based on a distance tree view available via a BLAST search (Table 3). Thirty nucleotide sequences selected from the GenBank database and this study were aligned using the computer program Clustal X (Version 1.8) (Jeanmougin et al. 1998), followed by manual adjustments with a text editor.

The aligned data set used in the analysis has been deposited with the TreeBASE under the accession number

Table 3 List of accession numbers of the internal transcribed spacer (ITS) gene of *Aphanomyces* spp. at the GenBank database

Species	Host	GenBank accession no.	Reference
Aphanomyces spp.			
A. frigidophilus	Japanese char egg	AY647192	Unpublished
A. piscicida	Ayu	AY283643	Unpublished
A. piscicida	Striped snakehead	AY455773	Unpublished
A. invadans	No date	AY082908	Unpublished
A. invadans	No date	AY082907	Unpublished
A. astaci	Crayfish	AY310499	Oidtmann et al. (2004)
A. astaci	Crayfish	AY683893	Royo et al. (2004)
A. astac	Crayfish	AY683894	Royo et al. (2004)
A. euteiches	Alfalfa	AY353908	Levenfors and Fatehi (2004)
A. euteiches	Green bean	AY353910	Levenfors and Fatehi (2004)
A. euteiches	Green bean	AY353909	Levenfors and Fatehi (2004)
A. euteiches	Garden pea	AY353902	Levenfors and Fatehi (2004)
A. laevis	No date	AY683885	Royo et al. (2004)
A. laevis	No date	AM947028	Vralstad et al. (2009)
A. laevis	No date	AY310497	Oidtmann et al. (2004)
A. stellatus	Water	AY455774	Unpublished
A. stellatus	Unknown	AM947029	Vralstad et al. (2009)
A. stellatus	Soil	AY283647	Unpublished
A. cladogamus	Spinach	AY353918	Levenfors and Fatehi (2004)
A. cladogamus	Alfalfa	AY353913	Levenfors and Fatehi (2004)
A. cladogamus	Red clover	AY353915	Levenfors and Fatehi (2004)
A. repetans	Crayfish	AY683889	Royo et al. (2004)
A. repetans	Crayfish	AY683892	Royo et al. (2004)
A. repetans	Crayfish	AY683890	Royo et al. (2004)
A. repetans	Crayfish	AY683891	Royo et al. (2004)
Out group			
Pythium ultimum	No date	EU253560	Unpublished

S2676. Phylogenetic analyses were performed with PAUP v4. 0 (beta 10) (Swofford 2001) using a heuristic search for maximum parsimony (MP) trees. Base-pair composition and transition and/or transversion patterns of the data set were estimated by PAUP v4.0 (beta 10). Bootstrap values were calculated over 1,000 replicates to assess branch topology. A phylogenetic tree was selected from 1,000 unrooted trees and was drawn by Tree View PPC (Roderic D. M. Page, Glasgow, Scotland, UK, 1998; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Clades were supported by bootstrap values >80%.

Results

Case

The ice fish, *S. microdon*, were captured from a wild population in Lake Shinji in Shimane Prefecture, Japan, located at northern latitude of 35°25′ and longitude 132°57′ east. These fish belong to the family Salangidae, which have a transparent body with few dark pigments and very few scales, even as adults. The digestive tract is straight, has no gastric gland, and retains larval features, not only in appearance but also in internal organs. Therefore, this ice fish is considered neotenic (Harada et al. 2005). These fish were transferred to the aquarium to be exhibited at the end of January 2008. They were housed in a rearing tank with a closed circulating system, and the water was aerated and heated to maintain a temperature of 15°C. The fish were fed brine shrimp *Artemia* sp. Fish colonies were maintained in exhibits of 20 fish per 300-l tank. Lesions began to appear on some fish at the beginning of February 2008. The affected fish were approximately 10 months posthatching and had developed white cotton-like lesions on the fins and heads. Three moribund and one dead fish with body weights ranging from 1.3 to 2.4 g were collected from the rearing tank.

Fig. 2 Histopathological characteristics of diseased fish. **a** Mycelial growth in the rostrum, lower jaw, and oral cavity (*arrow*). Grocott-hematoxylin and eosin (H&E). **b** Hyphae penetrating deep into tissue are associated with necrosis (*arrow*). Grocott-H&E. **c** Inflammatory cells surround hyphae (*arrow*). Grocott-H&E. **d** Gram-negative short-rod bacteria in the lesion (*arrow*). Gram stains. *Bars* **a** 300 μm; **b** 100 μm; **c, d** 30 μm
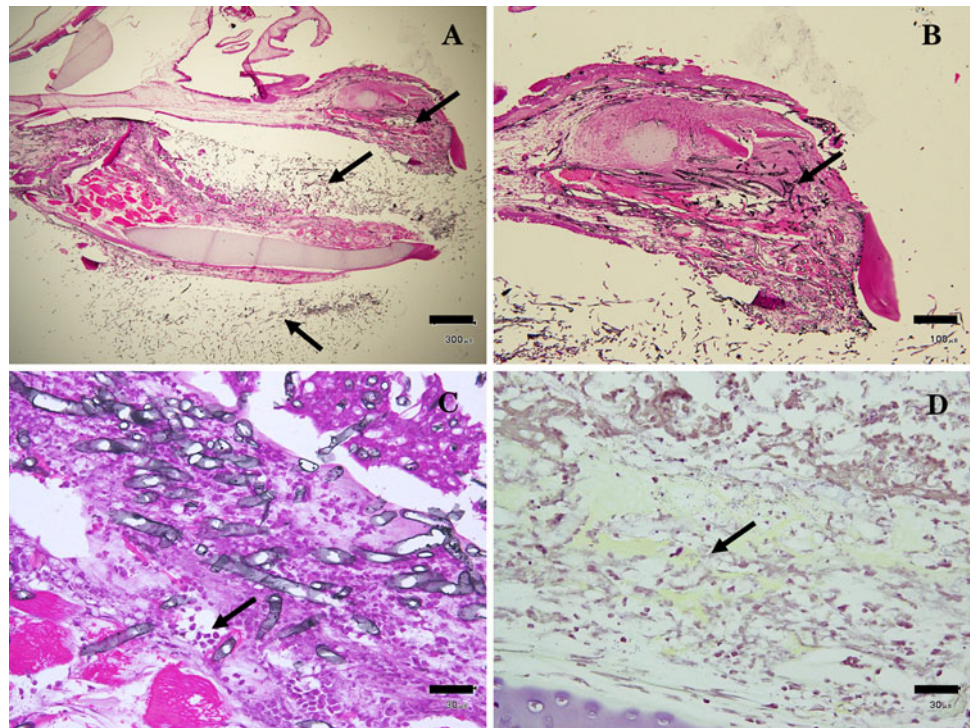

Histopathological observation

Lesions in the affected ice fish contained massive amounts of aseptate fungal hyphae that filled the oral cavity and covered wide areas of the mandible and maxilla (Fig. 2a). Many hyphae penetrated into dermal layers, subcutaneous tissues, and muscular layers and were associated with cellular debris. Hyphae often infiltrated into the cartilaginous tissue of the mandible and maxilla (Fig. 2b). The hyphae that had infiltrated into the host tissue were surrounded by a thin layer of the mononuclear rounded macrophage-like cells (Fig. 2c). Often, numerous Gram-negative short-rod bacteria were associated with the mycotic lesions on the affected fish (Fig. 2d). Multinucleated giant cells were not observed in any of the lesions.

Morphology

Five strains, NJM 0801, 0802, 0803, 0804, and 0805, were isolated from affected ice fish and determined to be identical to one another based on morphological observations. Colonies of all isolated strains developed white flat mycelia when grown on GY agar with 0.5% NaCl. Vegetative mycelia were delicate, about 5–10 μm in width, aseptate, smooth, slightly wavy, and moderately branched (Fig. 3). Zoosporangia were slender with the same diameter as hyphae, namely, isodiametric. Primary zoospores were produced in a single row within zoosporangium and were encysted in a cluster at the top of the zoosporangium.
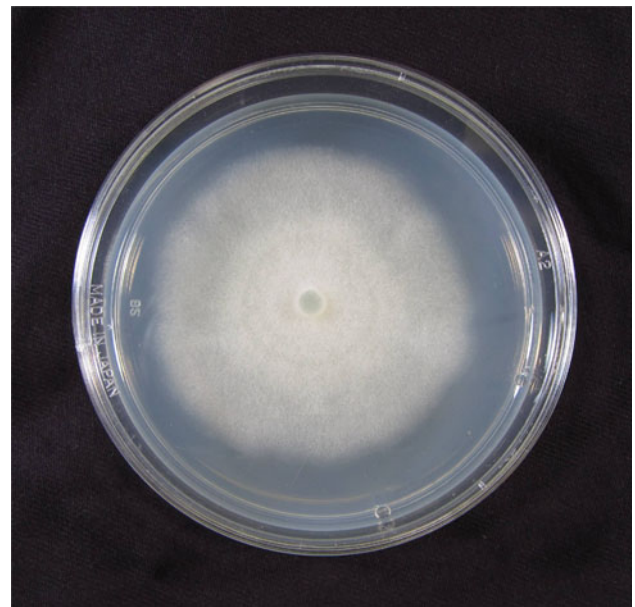

Fig. 3 A colony of the isolate NJM 0801 cultured on glucose–yeast (GY) agar with 0.5% sodium chloride (NaCl) at 20°C

Primary zoospores were about 8–11 μm in diameter. Based on this mode of zoospore formation and other morphological criterion, strains NJM 0801, 0802, 0803, 0804, and 0805 were identified as members of the genus *Aphanomyces* (Figs. 4, 5).

After hyphae were resuspended in sterilized tap water with 0.5% NaCl, sexual reproductive organs were observed

Fig. 4 Morphological
characteristics of *Aphanomyces*
sp. NJM 0801 isolated from ice
fish. **a** Zoospores swimming
away from zoosporangium in a
row. **b** Primary zoospores,
which encysted as cluster at the
orifice. **c** A young oogonium,
which has irregular short
papillated antheridia on the
outer surface. **d** A matured
oogonium with an antheridium,
which has a subcentric oospore.
A matured oogonium with
irregular short papillate. *Bars*
40 μm
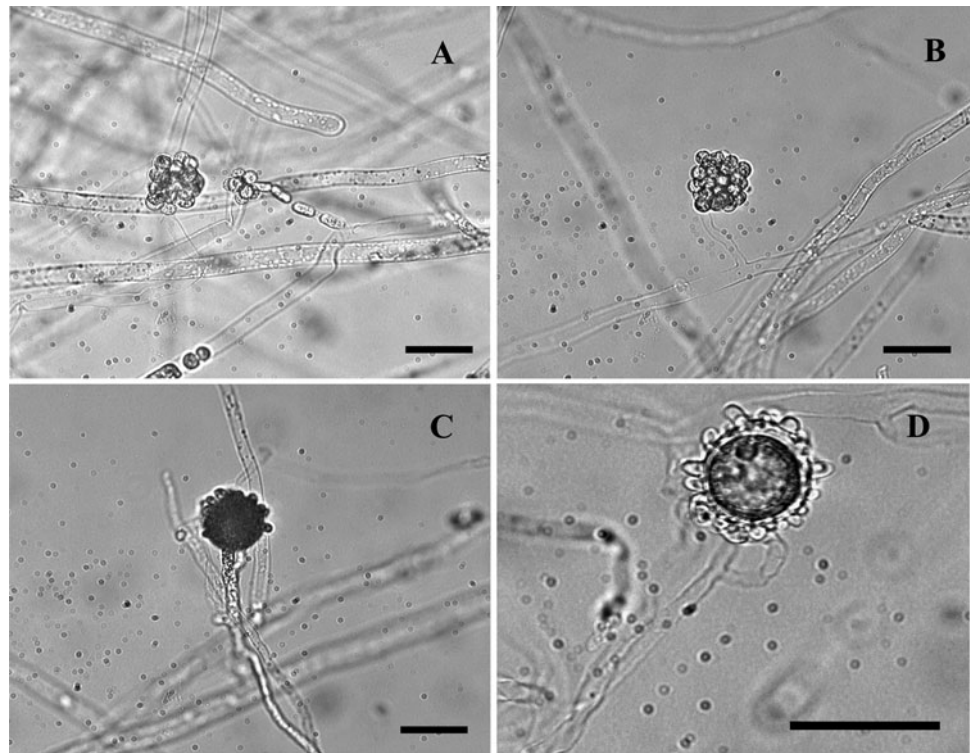
Fig. 5 Morphological
characteristics of *Aphanomyces*
sp. NJM 0801 isolated from ice
fish. **a** Zoospores swimming
away from zoosporangium in a
row. **b** Primary zoospores,
which encysted as cluster at the
orifice. **c** Young oogonia, which
have irregular short papillated
antheridia on the outer surface.
d Matured oogonia with an
antheridium, which have a
subcentric oospore. *Bar* 40 μm
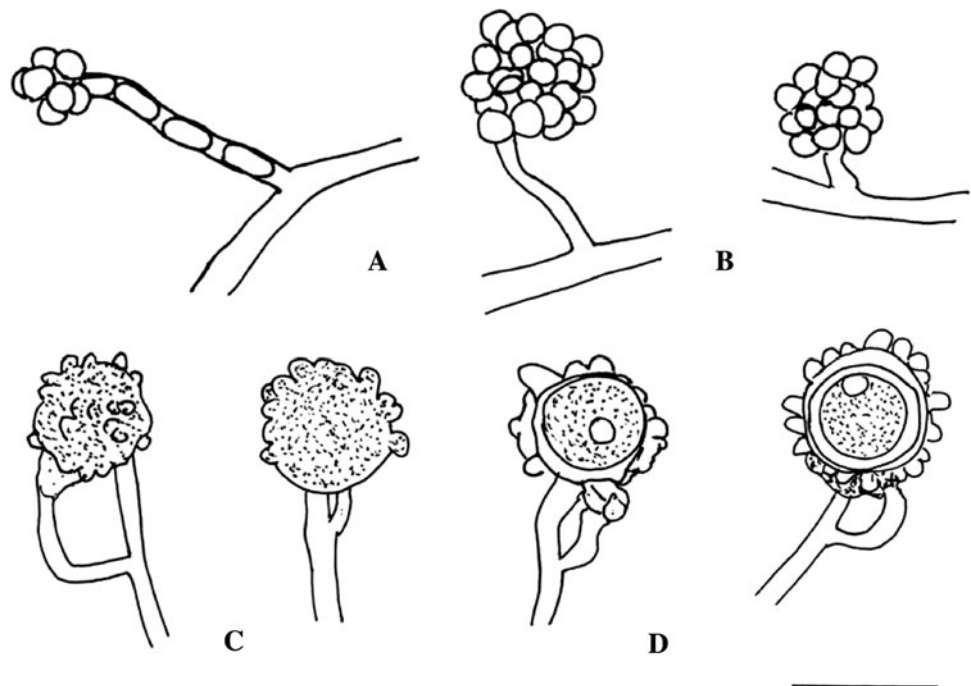
at day 3. Oogonia were abundant, usually spherical or subspherical, and only rarely pyriform; they ranged in diameter from 21 to 33 μm, with irregular short papillae, even in young oogonia. Singly sprouted oospores mostly filled oogonium and were generally spherical, ranging from 19 to 27 μm in diameter. Oospores contained a subcentric refractive globule in the cytoplasm. Antheridal branches, when present, were predominantly androgynous; monoclinous and diclinous antheridial branches occurred infrequently. Oogonial stalks were unbranched. Oogonia with single antheridium predominated; however, there were a few oogonia with double or triple antheridia (Figs. 4, 5).
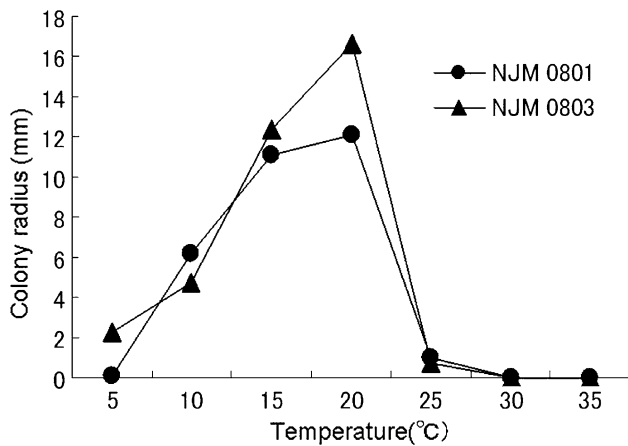
Fig. 6 Effects of temperature on the hyphal growth of isolates NJM 0801 and NJM 0803 cultured on glucose–yeast (GY) agar with 0.5% sodium chloride (NaCl)

Fig. 7 Effects of sodium chloride (NaCl) concentrations on the hyphal growth of isolates NJM 0801 and NJM 0803 cultured at 20°C for 7 days

Physiology

Vegetative growth of isolates NJM 0801 and NJM 0803 was robust at temperatures ranging from 10 to 25°C, with maximal growth at 20°C. At temperatures >25°C, mycelial growth immediately declined, and there was no growth at 30°C (Fig. 6).
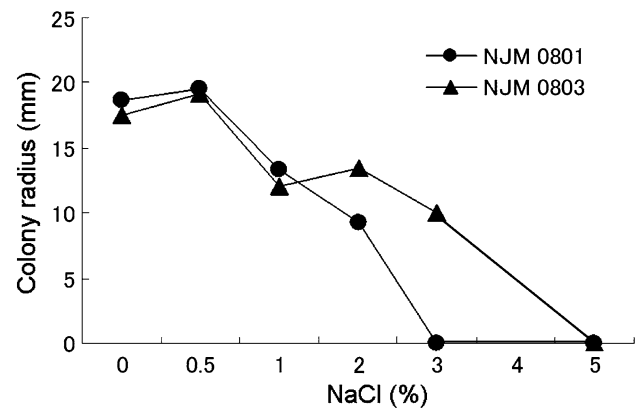
Isolates NJM 0801 and NJM 0803 grew well on GY agar supplemented with 0.5% NaCl, and they grew slowly on the media containing 1.0% or 2.0% NaCl (Fig. 7). Isolates NJM 0801 and NJM 0803 did not grow on GY agar of 3% and 5%, respectively.

Phylogenetic analysis

The sequences were submitted to the DNA database through DDBJ (DNA Data Bank of Japan, Mishima, Shizuoka, Japan). The accession numbers of all five strains, NJM 0801, 0802, 0803, 0804, and 0805, were AB510348, AB510349, AB510350, AB510351, and AB510352, respectively. All five isolates, NJM 0801–0805, formed an independent cluster in the phylogenetic analysis. This cluster had a significant boot strap value at 100% from the related species *A. astaci* (Fig. 8).

Discussion

We confirmed that a February 2008 outbreak of cotton-like lesions in the ice fish was caused by *Aphanomyces* sp. based on clinical, histopathological, morphological, physiological, and molecular biological data. As numerous Gram-negative short rods were observed in these lesions, it was suggested that the outbreak was caused by simultaneous infections of bacteria and *Aphanomyces* sp. Nevertheless, the fish tissues

were severely damaged by the mycelial penetrations, indicating that the primary causal agent for the disease was *Aphanomyces* but not the bacteria. Interestingly the clinical symptoms associated with this outbreak were different from those resulting from *A. invadans*, the causal agent of EGA in menhaden (Dykstra et al. 1986; Noga and Dykstra 1986; Johnson et al. 2004). The outbreak we studied was a water-mold disease, whereas the disease caused by *A. invadans* results in skin ulcers known as eEUS and affects a wide range of freshwater and brackish-water animals (Baldoc et al. 2005). The lesions observed in the ice fish were associated with a minimal granulomatous reaction when inspected using microscopy. Nevertheless, hyphae did penetrate the skin, muscles, and bone, indicating significant damage to the hosts. The most striking characteristic of this disease is mycelial growth into the host tissue. In contrast, *A. invadans* infections formed mycotic granuloma in menhaden (Noga and Dykstra 1986) and ayu (Miyazaki and Egusa 1973; Hatai 1980).

In March, the ice fish were in the terminal phase of their life because of their 1-year life span, and might have been fatigued, had weakened host defense mechanism, were aged, and susceptible to various opportunistic pathogens. Therefore, the ice fish have been infected with new *Aphanomyces* sp. pathogen. Two primary factors, host—parasite specificity and compromised host condition—might be correlated to the simultaneous infection of the *Aphanomyces* sp. as the outbreak.

As *A. stellatus* and *A. laevis* are reported as fish pathogens and produce similar sexual reproductive organs (Johnson et al. 2002), we morphologically compared our isolates with the two species (Table 4). Superficial structures of the spherical oogonia, with irregular short papillate projections of the NJM isolates, resembled those of *A. stellatus*, and this papillate surface of oogonia was different from the smooth surface of *A. laevis*. In contrast, the straight oogonial stalks

Fig. 8 Phylogenetic tree of *Aphanomyces* including isolates NJM 0801–0805. One of the 52 most parsimonious trees obtained from heuristic searches based on 659 base pair of internal transcribed spacer (ITS) 1-5.8S-ITS 2 rDNA sequence. Clusters were supported by bootstrap values >88%. The *bar* indicates ten base differences. Data are shown with accession number and fungal species. EU253560 derived from *Pythium ultimum* was used as an outgroup sequence. *CI* consistency index, *RI* retention index, *RC* rescaled consistency index, *HI* homoplasy index
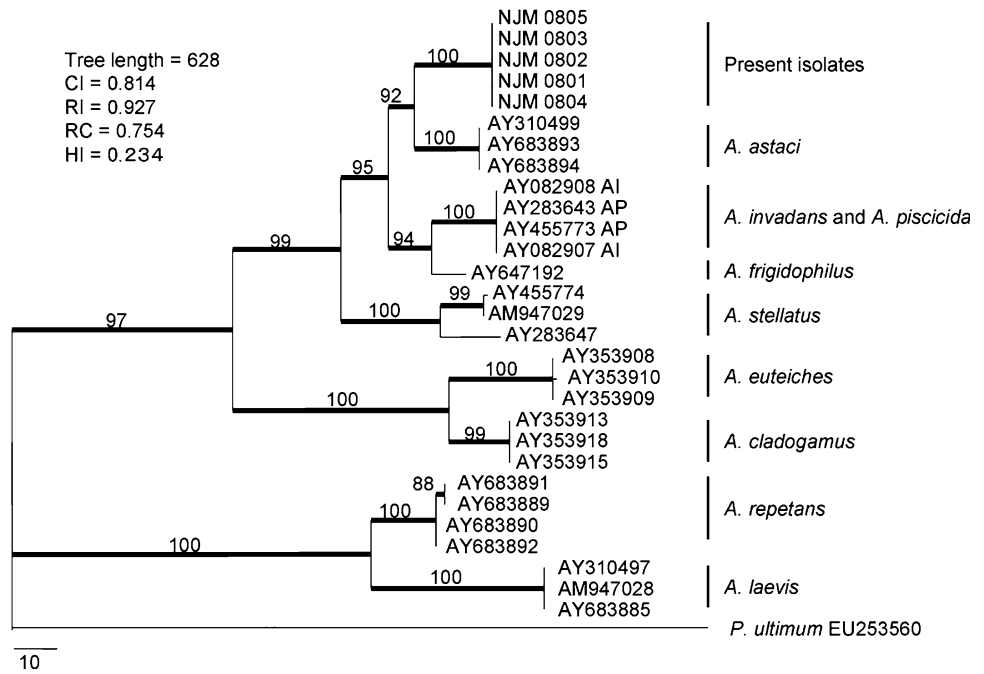
Table 4 Comparison of morphological feature between isolate *Aphanomyces* sp. NJM 0801 and *Aphanomyces laevis* and *A. stellatus* showing closer morphological similarities

	Aphanomyces sp. NJM 0801	*A. laevis*[a]	*A. stellatus*[a]
Oogonia wall ornamentation	Irregular short papillate	Smooth	Irregular short papillate
Basal portion of the oogonium	Broad	Broad	Tapered
Many antheridial branch formations	Androgynous	Monoclinous and diclinous	Monoclinous and diclinous

[a] Johnson et al. (2002)

of the isolates were more similar to the straight stalks of *A. laevis* than to the tapered oogonial stalks of *A. stellatus*. Finally, the antheridial branches of *A. laevis* and *A. stellatus* were more similar to one another than to those of NJM isolates 0801–0805. Specifically, the origin of the antheridial branch of the isolates was different from those of *A. laevis* and *A. stellatus* (Table 4). As *A. astaci* is a pathogen of crayfish, not fish, and does not produce sexual organs, we did not compared it with our isolates.

The optimal growth temperature for NJM isolates 0801 and 0803 was 20°C, and growth rate decreased when the temperature was raised to 25°C. Isolate NJM 0803 was able to grow at 5°C, indicating that the effects of low temperature on growth were mild when compared with the effects of higher temperatures. Furthermore, the growth rates of isolates 0801 and 0803 increased progressively with the incremental increases in temperature from 10°C to 20°C (Fig. 5). Optimal growth temperatures of NJM isolates may have been significant in the onset of the outbreak. The water temperature in the aquarium during the outbreak was 13.4°C, whereas the water temperature of Lake Shinji, when measure on 6 February 2009, was 8.2°C. These

observations suggest that the nursing environments of the ice fish in the aquarium were suitable for aggressive growth of the isolated *Aphanomyces* sp., whereas the temperature of Lake Shinji was too low for aggressive growth. Therefore, no outbreak of this fungal species has ever been recorded in Lake Shinji. Water temperature control may be an effective strategy to prevent fungal outbreaks in ice fish housed in tanks.

Future studies are necessary to determine the natural habitat of the *Aphanomyces* sp. responsible for the disease outbreak in these ice fish. We propose several possibilities: (1) Ice fish were infected with the fungi while in the lake before capture, and the disease state emerged because of some stress. (2) The species were introduced from the sand-filtered lake water used during the exhibition. (3) Fish were infected after feeding on brine shrimp harboring the species. (4) Fungi were introduced from some other unidentified source.

The optimal growth temperatures for other *Aphanomyces* spp. have been established. *A. astaci* grows best at temperatures ranging from 20 to 25°C and halt growth at temperatures of 30°C and above (Unestam 1965). In

Table 5 Comparison of identities between the present isolate *Aphanomyces* sp. NJM 0801 and the other *Aphanomyces* spp.

	Species	Accession no.	bps	1	2	3	4	5	6	7	8	9	10	11	12
1	This isolate	AB510348	659												
2	*A. astaci*	AY683894	663	93.5											
3	*A. cladogamus*	AY353918	655	79.2	80.4										
4	*A. euteiches*	AY353908	651	79.5	80.8	92.8									
5	*A. frigidophilus*	AY647192	650	92.9	93.4	82.5	82.4								
6	*A. invadans*	AY082907	634	91.3	92.4	81.9	81.9	96.5							
7	*A. laevis*	AY683885	657	78.3	78.0	76.5	75.9	79.5	79.4						
8	*A. piscicida*	AY283643	648	90.9	91.4	81.8	81.8	96.2	99.7	79.6					
9	*A. repetans*	AY683889	655	77.0	76.9	75.8	75.6	79.1	77.8	90.6	78.0				
10	*A. repetans*	AY683890	654	76.2	75.7	75.3	75.0	78.1	77.5	90.2	77.6	98.6			
11	*A. stellatus*	AY283647	661	89.8	89.3	80.4	80.3	90.2	89.8	77.6	89.7	77.6	76.5		
12	*A. stellatus*	AY455774	662	89.6	90.1	79.9	79.0	91.1	90.1	77.8	90.1	77.2	76.2	95.1	
13	*P. ultimum*	EU253560	684	62.2	62.4	60.8	60.8	62.1	61.9	61.1	60.7	61.1	59.8	60.7	63.3

contrast, *A. frigidophilus* NJM 9500 has a narrow optimal growth temperature of 25°C, and this fungal species stops growing at temperature immediately above 25°C (Kitancharoen and Hatai 1997). Sinmuk et al. (1996) reported that optimal growth temperature of *A. invadans* NJM 8997 isolated from ayu was also 30°C, whereas *Aphanomyces* sp. NJM 9525 isolated from juvenile soft-shelled turtles, *Pelodiscus senensis*, was the same as that of *A. frigidophilus*. Interestingly, isolates NJM 0801 and 0803 showed maximum growth rates on GY medium supplemented with 0.5% NaCl, suggesting they might be accustomed to brackish water. *A. piscicida* isolated from ayu and *Aphanomyces* sp. isolated from dwarf gourami cannot grow on GY agar with 1.5% NaCl (Hatai et al. 1994). In contrast, the *Aphamnomyces* sp. isolated in this study were able to grow, albeit slowly, on media with 1% and 2% NaCl, and they grew well on 0.5% NaCl, suggesting that the isolates were adapted to blackish conditions. *A. invadans* also grow in brackish water but not at high NaCl concentration. The morphological differences between isolates NJM 0801–0805 and *A. invadans*, including the absence of sexual structure in *A. invadans*, suggest that these are separate species.

According to Diéguez-Uribeondo et al. (2009), Scott (1961), and Johnson et al. (2002), *Aphanomyces* taxa correspond to the host taxa. For example, all *Aphanomyces* spp. that parasitize animals are more closely related to one another than they are to any species that does not parasitize an animal host. The isolates NJM 0801–0805 are limited to fish hosts. According to Scott (1961), Dick (2001), and Johnson et al. (2002), *Aphanomyces* spp. occur in very different ecological niches and include host-specific plant or animal parasites and saprotrophic species growing on decaying animal and plant debris. The host specificities of *Aphanomyces* spp. most closely related to isolates NJM

0801–0805 were as follows: *A. astaci* parasitize crayfish, *A. invadance* and *A. piscida* parasitize fish, *A. frigidophilus* parasitize crayfish and/or fish egg, and *A. stellatus* live on fish and in soil. The sequence identities based on 659 base pairs of the ITS rDNA for isolates NJM 0801–0805 were 93.5% (*A. astaci*), 92.9% (*A. frigidophilus*), 91.3% (*A. invadans*), 89.6% or 89.8% (*A. stellatus*), 79.5% (*A. euteiches*), 79.2% (*A. cladogamus*), 78.3% (*A. laevis*), 77.0% (*A. repetans*), and 62.2% (*Pithium ultimum*) (Table 5). The species was consequently regarded to represent a hitherto undescribed taxon based on morphology, physiology, and molecular biology.

Isolates NJM 0801–0805 formed an independent phylogenetic cluster separated from closely related *Aphanomyces* spp., all of which are isolated from the other sources. The bootstrap value of the cluster consisted of NJM 0801–0805 in this analysis also supported the observation that the isolates might belong to a separate, as-yet unknown, species of *Aphanomyces*. As mentioned above, this isolate was identified as a new species and new name is proposed in the genus *Aphanomyces*—*A. salsuginosus*—which was also confirmed as the pathogen causing the disease outbreak in the ice fish. The new name of *A. salsuginosus* has been registered with the Mycobank under the accession number MB 516781.

Aphanomyces salsuginosus sp. nov. Takuma,
Hatai & A. Sano   Figs. 4, 5

Mycelium aseptatum, subtile, 5–10 μm diametro, laeve, leviter undulatum, modice ramosum; zoosporangia isodiametra diam hyphae aequantia; zoosporae prope orificio emergentes et incystatae, conglobatae in globum; oogonia sphaero vel subsphaerica raro pyriformia, 21–33 μm diametro, oospore singulars, 19–27 μm diametro.

A congeneribus sequentiis nucleotidi distinctus, a specie proxima *A. astraci* 93.5 per centum identitati regionis ITS.

Holotypus: NJM 0801, colonia exsiccata ex cultura ex rostro *Salangichitys microdon* aqualio, Izumo, Shimane Pref., Japoniaia, 18-II-2008 a D. Takuma isolata, in collectione culturae Universitatis Scientiae Veterinariae et Animalis Nipponensis (NJM) conservata.

Type specimen: On rostrum of ice fish *Salangichitys microdon* on 18 Feb. 2008, the aquarium, Izumo-shi, Shimane prefecture, Japan, collected by D. Takuma (ex-type culture NJM 0801 = NBRC 106578). Figures 4 and 5 showing that the strain NJM 0801 is designated as the holptype because there are technical difficulties in preserving the type specimen: i.e., during slide preparation of hyphae with zoospore and sexual reproductive organs, their characteristic structures of this species are easily destroyed.

The vegetative mycelium was delicate, about 5–10 μm in diameter, aseptate, smooth, slightly wavy, moderately branched. Zoosporangia were slender and the same diameter as hyphae, namely, isodiametric; primary encysted zoospores were produced in a single row within zoosporangium and were encysted in a cluster at the top of the zoosporangium. Primary zoospores were about 8–11 μm in diameter. Oogonia were abundant, usually spherical or subspherical, rarely pyriform, from 21 to 33 μm in diameter with irregular short papillate, even in young oogonia. Pits were not found on the oogonial wall. Singly sprouted oospores were dominantly spherical, from 19 to 27 μm in diameter. Oogonial stalks were unbranched. Antheridial cells were simple, vermiform, often irregular. Antheridial branches, when present, were dominantly androgynous, infrequently monoclinous, and diclinous. Single antheridium was dominant; however, there were a few double or triple ones. The sequence identity based on 659 base pairs between the present isolates and *A. astaci*, closest species, was 93.5%.

References

Abliz P, Fukushima K, Takizawa K, Nishimura K (2004) Identification of pathogenic dematiaceous fungi and related taxa based on large subunit ribosomal DNA D1/D2 domain sequence analysis. FEMS Immunol Med Microbiol 40:41–49

Andrew TG, Huchzermeyer KD, Mbeha BC, Nengu SM (2008) Epizootic ulcerative syndrome affecting fish in the Zambezi river system in southern Africa. Vet Rec 163:629–631

Baldoc FC, Blazer V, Callinan R, Hatai K, Karunasagar I, Mohan CV, Bondad-Reantaso MG (2005) Outcomes of a short expert consultation on epizootic ulcerative syndrome (EUS): reexamination of causal factors, case definition and nomenclature. In: Walker P, Bondad-Reantaso MG (eds) Diseases in Asian aquaculture V. Fish Health Section, Asian Fisheries Society, Manila, pp 555–585

Ballesteros I, Martín MP, Diéguez-Uribeondo J (2006) First isolation of *Aphanomyces frigidophilus* (Saprolegniales) in Europe. Mycotaxon 95:335–340

Dick MW (2001) The saprolegniales. In: Straminipilous fungi: systematics of the Peronosporomycetes including accounts of the marine Straminipilous Protists, the Plasmodiophorids and similar organisms. Kluwer Academic Publisher. Dordrecht, pp 160–165

Diéguez-Uribeondo J, García MA, Cerenius L, Kozubíková E, Ballesteros I, Windels C, Weiland J, Kator H, Söderhäll K, Martín MP (2009) Phylogenetic relationships among plant and animal parasites, and saprotrophs in *Aphanomyces* (Oomycetes). Fungal Genet Biol 46:365–376

Dykstra MJ, Noga EJ, Levine JF, Moye DW (1986) Characterization of the *Aphanomyces* species involved with ulcerative mycosis (UM) in menhaden. Mycologia 78:664–672

Fraser GC, Millar SD, Calder LM (1992) *Aphanomyces* species associated with red spot disease: an ulcerative disease of estuarine fish from eastern Australia. J Fish Dis 15:173–181

Harada Y, Kuwamura K, Kinoshita I, Tanaka M, Tagawa M (2005) Histological observation of the pituitary-thyroid axis of a neotenic fish (the ice fish, *Salangichtys microdon*). Fish Sci 71:115–121

Hatai K (1980) Studies on pathogenic agents of saprolegniasis in fresh water fishes. Spec Rep Nagasaki Pref Inst Fish 8:1–95

Hatai K (1989) Fungal pathogens/parasites of aquatic animals. In: Austin B, Austin DA (eds) Methods for the microbiological examination of fish and shellfish. Ellis Horwood Ltd., West Susssex, pp 240–272

Hatai K, Nakamura K, Rha SA, Yuasa K, Wada S (1994) *Aphanomyces* infection in Dwarf Gourami (*Colisa lalia*). Fish Pathol 29:95–99

Hendrickson DA (1985) Reagents and stains. In: Lennette EH (ed) Manual of clinical microbiology, 4th edn. American Society for Microbiology, Washington, pp 1093–1107

Hoshina T, Sano T, Sunayama M (1960) Studies on the saprolegniasis of eel. J Tokyo Univ Fish 47:59–79

Jeanmougin F, Thomson JD, Gouy M, Higgins DG, Gibson TJ (1998) Multiple sequence alignment with Clustal X. Trend Biochem Sci 23:403–405

Johnson TW Jr, Seymour RL, Padgett DE (2002) Biology and the systematics of the Saprolegniaceae. http://dl.uncw.edu/digilib/biology/fungi/taxonomy%20and%20systematics/padgett%20book/

Johnson RA, Zabrecky J, Kiryu Y, Shields JD (2004) Infection experiments with *Aphanomyces invadans* in four species of estuarine fish. J Fish Dis 27:287–295

Kitancharoen N, Hatai K (1997) *Aphanomyces frigidophilus* sp nov. from eggs of Japanese char, Salvelinus leucomaens. Mycoscience. Mycoscience 38:135–140

Kurtzman CP, Robnett CJ (1997) Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 50 end of the large-subunit (26S) ribosomal DNA gene. J Clin Microbiol 35:1216–1223

Levenfors JP, Fatehi J (2004) Molecular characterization of *Aphanomyces* species associated with legumes. Mycol Res 108:682–689

Lilley JH, Callinan RB, Chinabut S, Kanchanakhan S, MacRae IH, Phillips MJ (1998) Epizootic ulcerative syndrome (EUS) technical handbook. Aquatic Animal Health Research Institute, Department of fisheries, Bangkok

Miyazaki T, Egusa S (1973) Studies on mycotic granulomatosis in fresh water fishes-II Mycotic granulomatosis in ayu, Plecoglossus. Fish Pathol 7:25–133

Noga EJ, Dykstra MJ (1986) Oomycete fungi associated with ulcerative mycosis in menhaden, *Brevoortia tyrannus* (Latrobe). J Fish Dis 9:47–53

Oidtmann B, Schaefers N, Cerenius L, Soderhall K, Hoffmann RW (2004) Detection of genomic DNA of the crayfish plague fungus *Aphanomyces astaci* (Oomycete) in clinical samples by PCR. Vet Microbiol 100:269–282

Royo F, Andersson G, Bangyeekhun E, Muzquiz JL, Soderhall K, Cerenius L (2004) Physiological and genetic characterisation of some new *Aphanomyces* strains isolated from freshwater crayfish. Vet Microbiol 104:103–112

Scott WW (1961) A monograph of the genus *Aphanomyces*. Virginia Agri Exp Stat Tech Bull 151

Shah KL, Jha BC, Jhingran AG (1977) Observations on some aquatic phycomycetes pathogenic to eggs and fry of freshwater fish and prawn. Aquaculture 12:141–147

Shanor L, Saslow HB (1944) *Aphanomyces* as a fish parasite. Mycologia 36:413–415

Sinmuk S, Suda H, Hatai K (1996) *Aphanomyces* infection in juvenile soft-shelled turtle, *Pelodiscus sinensis*, imported from Singapore. Mycoscience 37:249–254

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

Srivastava GC, Srivastava RC (1976) A note on the destruction of the eggs of *Cyprinus carpio* var. Communis by the members of Saprolegniaceae. Sci Cult 42:612–614

Swofford DL (2001) PAUP phylogenetic analysis using parsimony (and other methods) (version 4.0) Sinauer Associates, Sunderland

Unestam T (1965) Studies on the crayfish plague fungus *Aphanomyces astaci* Part I. Some factors affecting growth in vitro. Physiol Plant 18:483–505

Unestem T (1972) On the host range and origin of the crayfish plague fungus. Rep Inst Freshw Res Drottningholm 52:192–198

Vralstad T, Knutsen AK, Tengs T, Holst-Jensen A (2009) A quantitative TaqMan[R] MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci*. Vet Microbiol 137:145–155

Willoughby LG, Robert RJ (1994) Improved methodology for isolation of the *Aphanomyces* fungal pathogen of epizootic ulcerative syndrome (EUS) in Asian fish. J Fish Dis 17:237–248