Mycoscience (2011) 52:119–131
DOI 10.1007/s10267-010-0082-3

FULL PAPER

Aphanomyces sinensis sp. nov., isolated from juvenile soft-shelled turtle, *Pelodiscus sinensis*, in Japan

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Received: 18 February 2010 / Accepted: 15 October 2010 / Published online: 16 November 2010

Abstract A species of *Aphanomyces* was isolated from juvenile soft-shelled turtles, *Pelodiscus sinensis*, cultured in Japan. Typically, an infected turtle showed small whitish maculae on the carapace. Many hyphae were observed in the epidermis. The hyphae were isolated using glucose–yeast (GY) agar plates. The morphological characteristics were very similar to those of *Aphanomyces laevis*, but a clear nuclear spot was observed in the center of the oospore in the strains isolated from the soft-shelled turtles. The optimal growth temperature for the isolates was 25–30°C and the optimum pH was 6–9. Experimental infection tests with isolates produced small whitish maculae on the carapace, and soft-shelled turtles artificially infected with the zoospores showed high mortality, especially in the high-dose group. Phylogenetic analysis based on the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) indicated that the isolates from the soft-shelled turtles were unidentified species of *Aphanomyces*. As a result, the strain was described as a new species, *Aphanomyces sinensis*.

Keywords Artificial infection · Fungal infection · Histopathology · Identification · Oomycetes

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Introduction

The genus *Aphanomyces* includes saprotrophs, phytopathogens, and animal pathogens (Scott 1961; Johnson et al. 2002). Diéguez-Uribeondo et al. (2009) classified the phylogenetic relationship of *Aphanomyces* spp. based on internal transcribed spacer (ITS) regions of ribosomal RNA gene (rDNA) sequences. Most fungal disease pathogens of fishes and shellfish belong to the genus *Aphanomyces* (Hatai 1989) (Table 1).

Aphanomyces piscicida Hatai was first reported as a pathogen causing mycotic granulomatosis in ayu *Plecoglossus altivelis* Temminck & Schlegel from Japan (Hatai 1980). Later, *A. invadans* Willoughby, Roberts & Chniabut was reported as the causative agent of epizootic ulcerative syndrome (EUS) in Thailand (Willoughby et al. 1998). Furthermore, some diseases caused by the same fungus were found 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 United States (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 of fish from Japan, Australia, the United States, India, the Philippines, and Thailand concluded that mycotic granulomatosis (MG), RSD, ulcerative mycosis (UM), and EUS are all the same disease and proposed a new name—epizootic granulomatous aphanomycosis (EGA) (Baldock 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 fish suffering from the disease.

Table 1 *Aphanomyces* spp. 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	Unestam (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)

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.

Mycosis has also been reported in chelonians. Egusa (1970) has reported a fungal infection of cultured soft-shelled turtles, caused by *Mucor* sp. The disease first occurred in Ooita Prefecture, Japan, in 1969. Ulcerative epidermitis associated with a *Mucor* sp. infection has been described in the United States in soft-shelled turtles *Trionyx ferox* (Schneider) (Jacobson et al. 1980). *Aspergillus* sp. was isolated from the Musk turtle *Sternotherus adoratus* (Frye and Dutra 1974), and *Fusarium solani* Sacc. was isolated from the Loggerhead turtle *Caretta caretta* (Cabanes et al. 1997). *Fusarium semitectum* Berk. & Ravenel was isolated from Texas tortoises *Gopherus berlandieri* (Rose et al. 2001), and *Paecilomyces lilacinus* Samson was isolated from Chinese soft-shelled turtles (*Trionyx sinens*) (Li et al. 2008). In 1996, Sinmuk et al. reported *Aphanomyces* infection in juvenile soft-shelled turtles imported from Singapore.

In 2007–2009, outbreaks of fungal disease were found in juvenile cultured soft-shelled turtles in Japan. In the present study we describe this new fungal disease and compare the causal fungus with other animal parasitic *Aphanomyces*, using morphological, physiological, and phylogenetic criteria.

Materials and methods

Fungal isolation

Lesions began to appear on some turtles 10–20 days after their arrival, from local hatcheries, at the Laboratory of Fish Diseases, Nippon Veterinary and Life Science University. Small pieces from the carapace were rinsed in sterilized tap water (STW) and placed on glucose–yeast (GY) agar (1% glucose, 0.25% yeast extract, and 1.5% agar containing 500 mg/ml of both ampicillin and streptomycin in 90 × 20 mm disposable Petri dishes. Inoculated plates were incubated at 25°C for 4–6 days.

Histopathological observation

All turtle bodies were fixed in 10% phosphate-buffered formalin solution, decalcified in ethylene diamine tetra-acetic acid (EDTA) with 5% formalin and processed for routine histology, as described by Hendrickson (1985). 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), Uvitex 2B counterstained with H&E (Uvitex 2B-H&E, Wada et al. 2003), and Gram stains.

Morphology

Mycelia were grown from an agar block cut from the edge of a colony, immersed in GY broth, and incubated at 25°C for 3 days. Growing mycelia were rinsed twice in STW and re-suspended in STW to induce the development of zoospores. Sexual reproduction was induced using hemp seed culture; the hemp seeds with fungi were first cultured at 25°C for 2 days, and then they were incubated at 20°C (Seymour 1970).

The isolated fungi were identified from the characteristics of zoospore production and sexual reproduction according to Sparrow (1960) and Scott (1961).

Effect of temperature on fungal growth

The effect of temperature on vegetative growth was determined using isolates NJM 0719, NJM 0817, and NJM 0818. Agar blocks (approximately 5.5 mm in diameter) were taken from the advancing edge of 5-day-old colonies cultured on GY agar and placed in the center of 90 × 20 mm Petri dishes containing 20 ml of GY agar, and incubated at 15, 20, 25, 30, 35, or 40°C.

The diameters of the colonies were estimated from the average of the widest and narrowest radial measurements minus the 5.5 mm of the original agar block. Estimates of colony diameter were determined 3, 5, and 7 days after inoculation.

Effect of pH on fungal growth

The effect of pH on vegetative growth was determined using isolates NJM 0719, NJM 0817, and NJM 0818. The pH of sterile GY broth was adjusted to various values, in the range of 2–13, with 5 N NaOH or 5 N HCl. The broth was filtered through 0.2 μm cellulose acetate (Advantic; Tokyo Roshi Kaisha, Tokyo, Japan) into a test tube. The control was unadjusted GY broth (pH 6.6). After 5 days, an agar plug with mycelia from each isolate was placed into 10 ml of the GY broth. All test tubes were incubated at 25°C. Hyphal growth in the test tube was measured on days 3, 5, and 7.

Pathogenicity

Zoospore suspensions of the isolates NJM 0719 and NJM 0817 were prepared as described above. Fifty healthy soft-shelled turtles (40.5 mm average carapace length and 12.0 g average body weight) were divided into five equal groups. The zoospore concentrations in NJM 0719 were 2,000 and 200 zoospores/ml in high-dose and low-dose suspensions, respectively, and those in NJM 0817 were 1,700 and 170 zoospores/ml, respectively; STW was used as a negative control. Turtles were immersed in the zoospore suspensions or STW (500 ml in each) in 2 l beakers for 5 h. After immersion, the inoculated animals were transferred to tanks filled with tap water for 14 days. The tank water was changed daily and the turtles were fed every 4 days with commercial food. The water temperature in each tank was maintained at 20°C. After 14 days, the surviving turtles were euthanized with diethylether and autopsied. Small pieces of skin stripped from the carapaces were examined under light microscopy to detect fungal elements. Initially, the fungi were re-isolated from the lesions on carapaces, then, whole turtles were fixed in 10% phosphate-buffered formalin solution for histopathological examination with H&E, Grocott-H&E, and Uvitex 2B-H&E. A total of 8 freshly dead turtles (four from the high-dose suspensions with NJM 0719, two from the high-dose suspensions with NJM 0817, and two from the low-dose suspensions with NJM 0817) were also collected for histopathological examination of infected carapaces.

Phylogenetic relationship

The ITS 1 and 2 including the 5.8S rRNA gene region (ITS rDNA) were sequenced. DNA was extracted from hyphae cultured at 25°C in GY broth. Sampled mycelia were dried on tissue paper and transferred to separate 1.5 ml microcentrifuge tubes. The samples were frozen at −85°C prior to DNA extraction (Abliz et al. 2004).

Mycelial fragments of approximately 50 mg were suspended in 600 μl of extraction buffer (200 mM Tris–HCl, pH 7.5, 25 mM EDTA, 0.3% w/v sodium dodecyl sulfate, 250 mM NaCl). The mixture was vortexed for 15 s, incubated at 100°C for 15 min, kept on ice for 60 min, and then centrifuged at 14,000×*g* for 15 min. The supernatants were transferred to new tubes and extraction was performed with phenol–chloroform–isoamyl alcohol (25:24:1). Each sample DNA was precipitated with cold isopropanol (−20°C), dried, and resuspended in 100 μl distilled water.

We used a nested PCR for the determination of the ITS rDNA sequence, using universal primers (rDNA primer sequences at http://www.biology.duke.edu/fungi/mycolab/primers.htm; and Kurtzman and Robnett 1997). The first-round PCR was as follows: We mixed 2.5 μl of the DNA extract with a Readyto-Go bead (Amersham Pharmacia Tokyo, Japan), 2.5 μl of 10 pM of the primers 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 mixture was subjected to 1 cycle of denaturation at 95°C for 4 min, 30 cycles of amplification at 94°C for 1 min, 54 or 55°C for 1 min, and 72°C for 2 min, and a final extension cycle at 72°C for 10 min with a PCR Thermal Cycler MP (TaKaRa, Ohtsu, Japan).

Then the first-round PCR products were purified with a commercial kit (QIAquick®; Qiagen, Tokyo, Japan) and diluted 10- to 100-fold. The second round of PCR amplifications was performed using 3 combinations of fungal universal primers; 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′) and ITS-4 (5′-TCC TCC GCT TAT TGA TAT GC-3′), and ITS-3 (5′-GCA TCG ATG AAG AAC GCA GC-3′), and NL-2. The condition of the second-round PCR was the same condition as that of the first one regardless of the primer combinations.

The second-round PCR products were visualized by electrophoresis in 1.0% agarose in 1× 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 with the QIAquick® kit and labeled with BigDye® Terminator Ver. 1.1 (Applied Biosystems, Foster City, CA, USA), following the manufacturer's protocol, using 6 primers; NS-7, ITS-2, ITS-5, ITS-4, ITS-3, and NL-2. The conditions of labeling PCR were: 0.25 min at 96°C, 0.5 min at 55°C, and 4 min at 60°C for 25 cycles, followed by a 4°C soak. The labeled samples were directly sequenced on an ABI PRISM 3100 sequencer (Applied Biosystems).

Twenty-five nucleotide sequences from *Aphanomyces* spp. were selected on the basis of a BLAST search of the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi), a sequence from *Pithium ultinum* (GenBank accession number EU253560) was used as an outgroup sequence, and the present 5 sequences shown in Table 2 were aligned using the computer program CLUSTALX (Version 1.8) (Jeanmougin et al. 1998) followed by manual adjustments with a text editor.

Phylogenetic analyses were performed with a computer program; PAUP v4.0 (beta 10) (Swofford 2001), using a heuristic search for maximum parsimony (MP) trees. We determined base-pair composition and transition and/or transversion patterns of the dataset. Bootstrap values were calculated over 1,000 replicates to assess branch topology. The maximum numbers of trees was adjusted to 1,000 trees at the initial setting. Briefly, 662 base pairs of the ITS rDNA were aligned. The consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasy index (HI) were obtained.

Results

Isolation

From 2007 to 2009, a number of juvenile soft-shelled turtles, *Pelodiscus sinensis*, 34.7–63.2 mm in carapace length, kept at local hatcheries in Fukuoka and Oita prefectures, Japan, were transferred to the Laboratory of Fish Diseases, Nippon Veterinary and Life Science University. Although healthy individuals were selected at the local hatcheries, the turtles developed whitish lesions on the carapaces and died 10–20 days after arrival. The original water temperature of the culturing pond was 30°C, whereas the water temperature of the rearing tanks in the laboratory was 20°C. Typically, an infected turtle showed a small whitish macula on the carapace (Fig. 1).

The resulting fungal colonies were transferred onto new GY agar plates. From 2007 to 2009, five fungal colonies were isolated from infected turtles. The strains were designated: NJM 0719, NJM 0817, NJM 0818, NJM 0901, and NJM 0902. Details of the isolates are shown in Table 3.

Histopathological observation

In infected turtles, many sparsely branched aseptate hyphae were observed in the carapace epidermis. However; these hyphae did not penetrate into the dermal layers (Fig. 2). Numerous Gram-negative short rods were often observed in the mycotic lesions on the affected epidermis of carapaces. The hyphae and bacteria were not observed in the internal organs.

Morphology

Colonies of all isolated strains developed white, flat mycelia when grown on GY agar (Fig. 3). Vegetative hyphae were delicate, about 3–12 μm in diameter, aseptate, smooth, slightly wavy, and moderately branched. Zoosporangia were slender and the same diameter as the hyphae; that is, isodiametric. Primary zoospores were produced in a single row within the zoosporangium and were encysted in a cluster at the orifice of the zoosporangium (Figs. 4a–c, 5a–c). The primary zoospores were about 8–12 μm in diameter. Secondary zoospores were reniform and laterally biflagellate.

Based on this mode of zoospore formation and other morphological criteria, strains NJM 0719, NJM 0817, NJM 0818, NJM 0901, and NJM 0902 were identified as members of the genus *Aphanomyces* (Figs. 4, 5).

NJM 0719 and NJM 0901 strains produced sexual reproductive structures in hemp seed culture. The oogonia of isolate NJM 0719 were abundant, usually spherical or sub-spherical, and only rarely pyriform; their diameters ranged from 18 to 25 μm, and even the immature oogonia had a smooth surface (Figs. 4d, e, 5d, e). Singly sprouted oospores were mostly filled with an oogonium, and were generally spherical, ranging from 15 to 22 μm in diameter. The internal structure of oospores was dominantly centric to sub-centric with a large shiny vesicle with antheridia (Figs. 4f, 5f). A nuclear spot, which was often clear, was observed in the center of the oospore (Figs. 4g, 5g, h). Oogonial stalks were unbranched. Antheridial branches, when present, were predominantly diclinous, and infrequently monoclinous and androgynous (Fig. 5f–h). Oogonia with double or triple antheridia predominated. Antheridial cells were simple and vermiform, and often irregular.

Effect of temperature on fungal growth

The vegetative growth of isolates NJM 0719, NJM 0817, and NJM 0818 was robust at 20–30°C, with maximal growth at 25–30°C. At temperatures above 35°C, mycelial growth declined and growth ceased at 40°C (Table 4).

Table 2 List of accession numbers of the internal transcribed spacer (ITS) of *Aphanomyces* spp. at the GenBank database

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

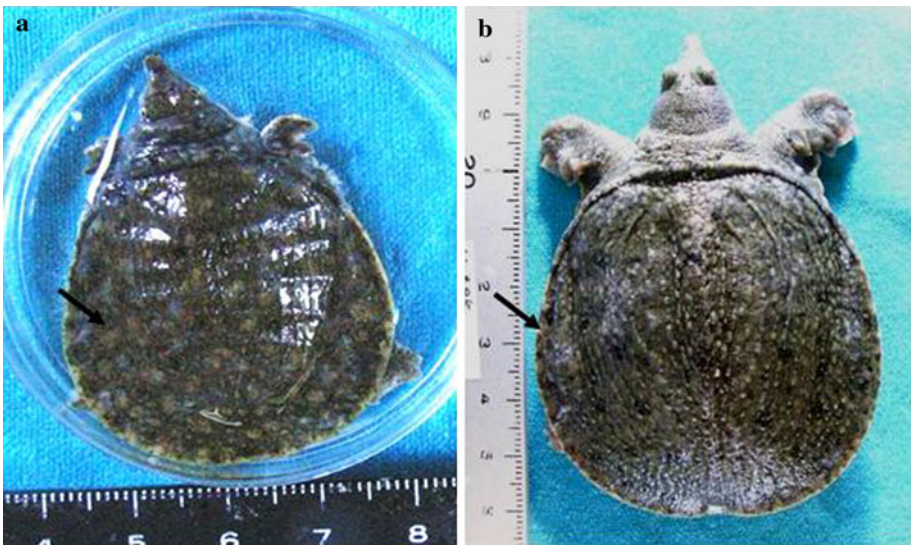

Fig. 1 Diseased juvenile soft-shelled turtle showing small whitish macula on the carapace (*arrow*). **a** Turtle that was immersed in NJM 0719 isolate. **b** Turtle that was immersed in NJM 0817 isolate

Table 3 Isolates isolated from soft-shelled turtles

Isolation no.	Isolation date	Location	Accession no.
NJM 0719	8 Nov 2007	Fukuoka	AB531971
NJM 0817	10 Oct 2008	Oita	AB531972
NJM 0818	10 Oct 2008	Oita	AB531973
NJM 0901	5 Jan 2009	Oita	AB531974
NJM 0902	5 Jan 2009	Oita	AB531975

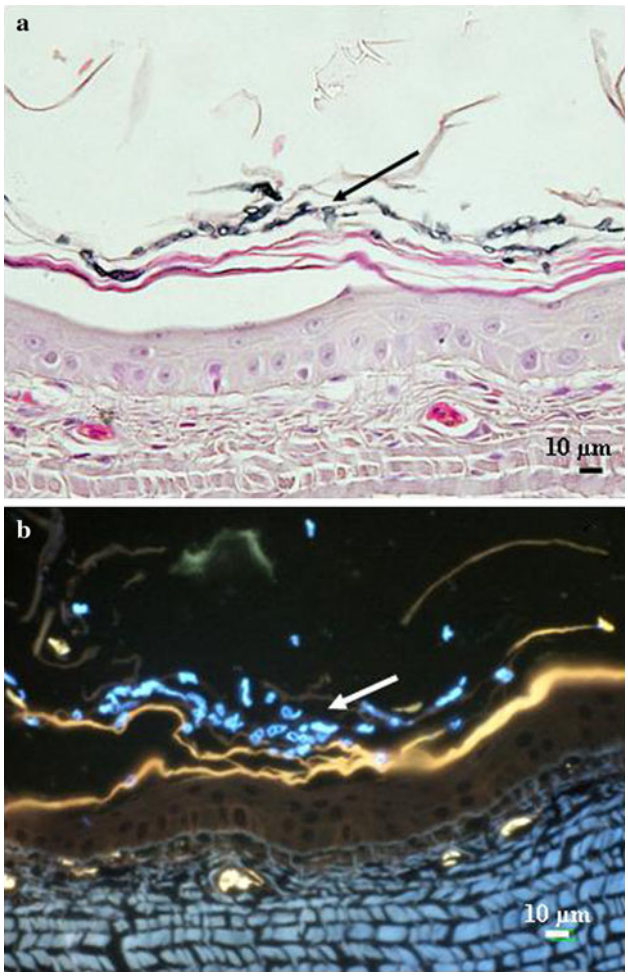

Fig. 2 Histopathological characteristics of the diseased turtle. Many hyphae were observed in the epidermis of the carapace (*arrows*). **a** Grocott-H&E. **b** Uvitex 2B–H&E. *Bar*10 μm

Effect of pH on fungal growth

The optimum pH for the vegetative growth of isolates NJM 0719, NJM 0817, and NJM 0818 in GY broth was pH 6–9 and some growth occurred between pH 5–11 (Table 5).

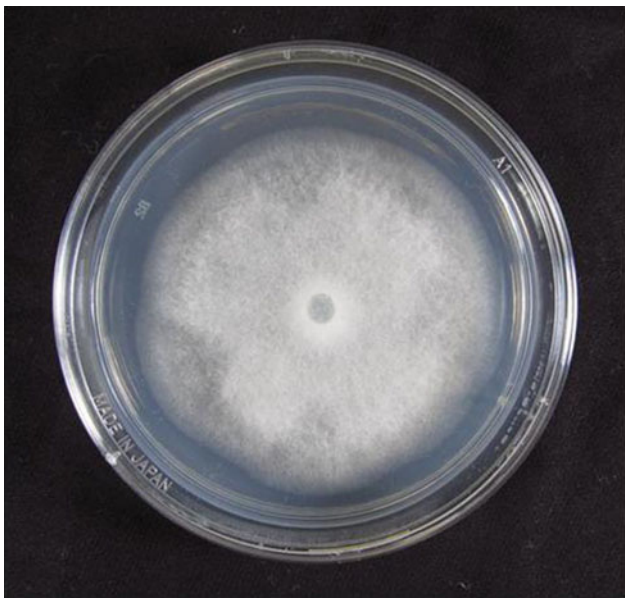

Fig. 3 A colony of the NJM 0719 isolate cultured on glucose–yeast (GY) agar at 20°C for 5 days

Pathogenicity

All turtles in the inoculated groups developed small whitish maculae on the carapaces (Fig. 6). Though the small whitish maculae were also observed after 4 days' immersion, no deaths were recorded in the control group. No fungal and/or bacterial elements were found in the carapaces of the control group. In all turtles exposed to fungal zoospores, we observed many sparsely branched aseptate hyphae by microscopic observation of the carapace skins, and re-isolation of the inoculated fungus from the skin lesions showed high success rates (Table 6). The turtles in groups exposed to fungi became anorectic; anorexia was especially apparent in the high-dose groups.

In both high-dose groups, we observed white maculae on the carapaces of all turtles 4 days after immersion, and mortalities were recorded 6 days after immersion. In the high-dose NJM 0719 group, all turtles (10) had died by 11 days after immersion. Nine turtles died by 12 days after immersion in the high-dose NJM 0817 group (Table 7). All of the turtles in the low-dose NJM 0719 and NJM 0817 groups showed white maculae on the carapaces at 7 and 5 days after inoculation, respectively. By the end of the experiment, one turtle in the low-dose NJM 0719 group had died and four turtles in the low-dose NJM 0817 group had died (Table 7).

Many sparsely branched aseptate hyphae were observed in the cuticle and epidermis of the carapace (Fig. 7a, b) in surviving and dead turtles. In 6 out of 26 turtles examined (three from the high-dose NJM 0719 group, one from the low-dose NJM 0719 group, and two from the low-dose

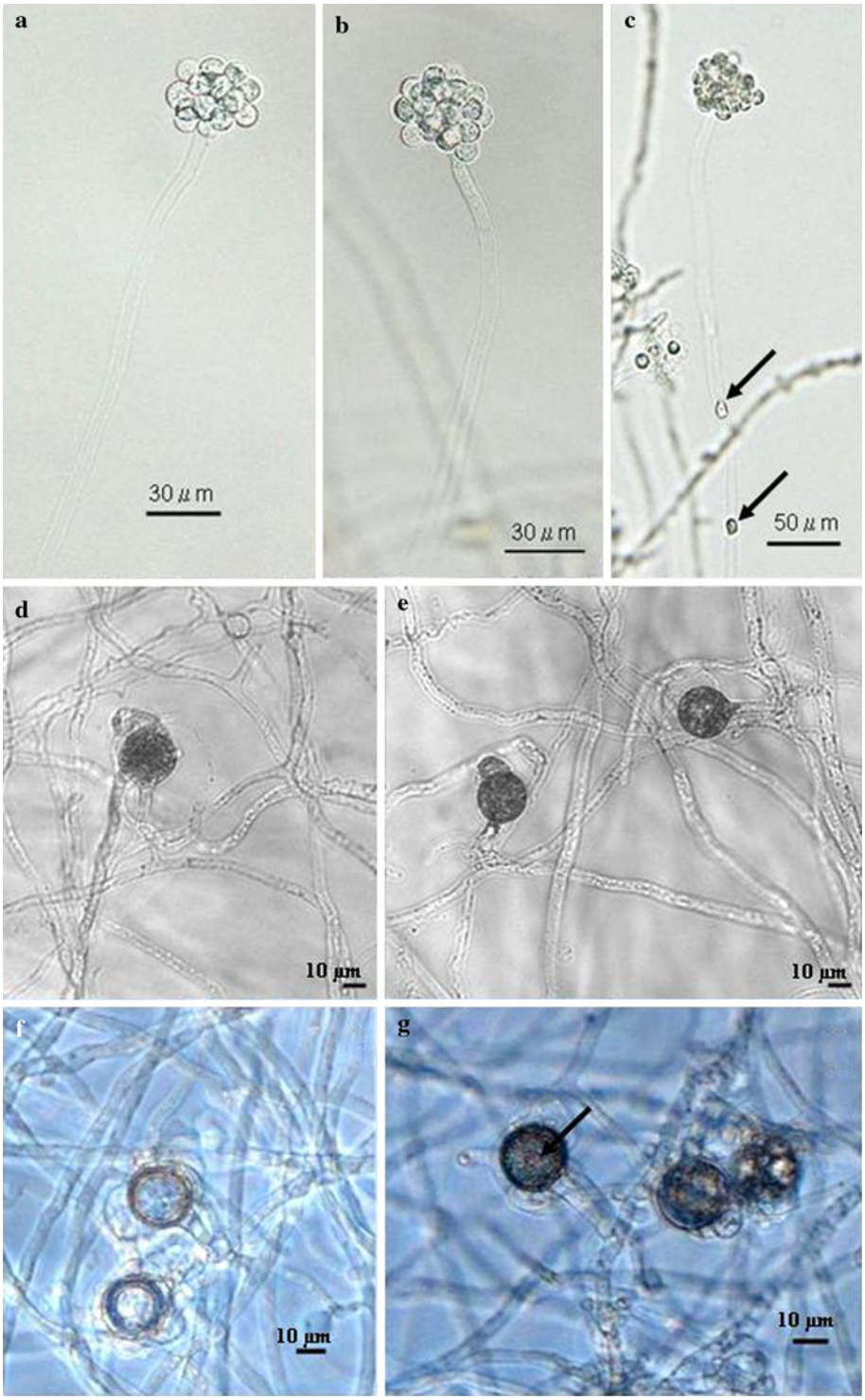

Fig. 4 Morphological characteristics of *Aphanomyces* sp. NJM 0719 isolated from soft-shelled turtle. **a**, **b** Primary zoospores, which were encysted as clusters at the orifice of the zoosporangium. **c** Zoospores swimming away from the zoosporangium in a row (*arrows*). **d**, **e** An immature oogonium with a smooth outer surface. **f** A mature oogonium with an antheridium; the oogonium has a centric oospore. **g** A mature oogonium with an antheridium; there is a clear nuclear spot in the center of the centric oospore (*arrow*). *Bars* **a**, **b** 30 μm; **c** 50 μm; **d**–**g** 10 μm

NJM 0817group), hyphae also penetrated the dermal layers (Fig. 7c, d).

Phylogenetic relationship

The sequences were submitted to the DNA Data Bank of Japan (DDBJ; Mishima, Shizuoka, Japan). The accession numbers of the five strains, NJM 0719, NJM 0817, NJM 0818, NJM 0901, and NJM 0902 were AB531971, AB531972, AB531973, AB531974, and AB531975, respectively.

The sequence identities, based on 662 base pairs of ITS rDNA for isolates NJM 0719, NJM 0817, NJM 0818, NJM 0901, and NJM 0902 were 80.9 and/or 80.1 (*A. stellatus*),

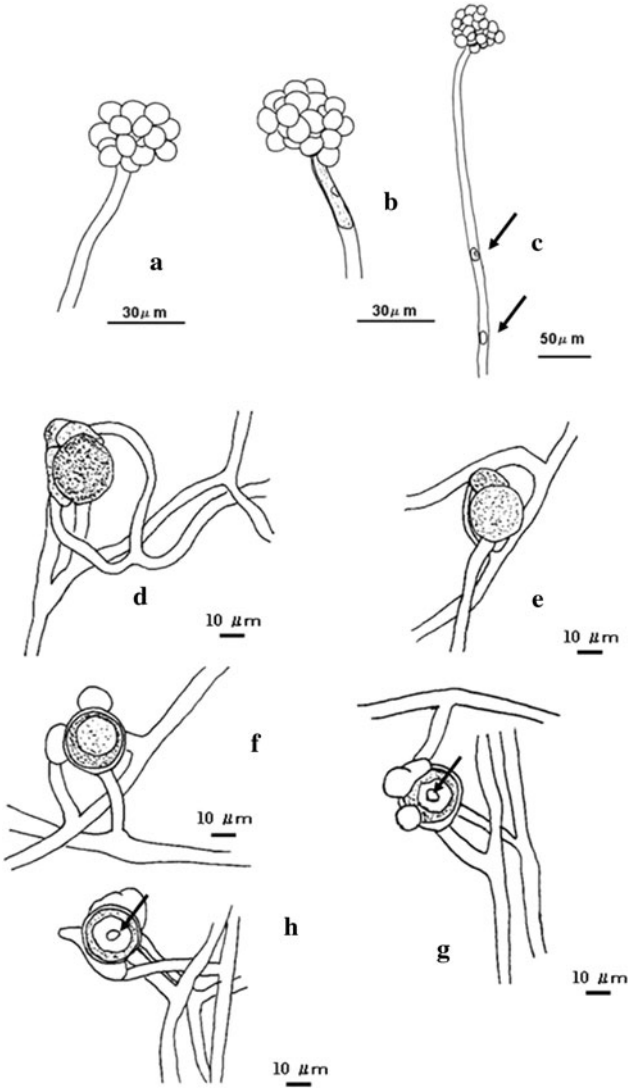

Fig. 5 Morphological characteristics of *Aphanomyces* sp. NJM 0719 isolated from soft-shelled turtle. **a**, **b** Primary zoospores, which were encysted as clusters at the orifice of zoosporangium. **c** Zoospores swimming away from the zoosporangium in a row (*arrows*). **d**, **e** An immature oogonium, with a smooth outer surface. **f** A mature oogonium with an antheridium; the oogonium has a subcentric oospore. **g**, **h** A mature oogonium with an antheridium; there is a clear nuclear spot in the center of a centric oospore (*arrows*). *Bars* **a**, **b** 30 μm; **c** 50 μm; **d**–**h** 10 μm

80.3 (*A. frigidophilus*), 79.5 (*A. astaci*), 79.2 (*A. invadans*), 77.6 (*A. cladogamus*), 76.7 (*A. laevis*), 76.5 (*A. euteiches*), 75.8 (*A. repetans*), and 58.3% (*Pithium ultimum*) (Table 8).

The present 5 isolates; NJM 0719, NJM 0817, NJM 0818, NJM 0901, and NJM 0902 derived from soft-shelled turtles showed identical sequences at the ITS rDNA.

We obtained 27 unrooted trees after analysis, selected one phylogenetic tree showing the features most identical to the consensus, and drew with Tree View PPC (Roderic D. M. Page, Glasgow, Scotland, UK, 1998; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The tree length, CI, RI, RC, and HI values were 696, 0.796, 0.928, 0.739, and 0.204, respectively.

Table 4 Effect of temperature on vegetative growth

Strains	Day	Temperature (°C)					
		15	20	25	30	35	40
NJM 0719	3	5.3	10.3	15.6	14.0	5.0	0
	5	18.5	18.5	24.2	22.8	11.0	0
	7	14.1	25.6	31.2	28.5	16.3	0
NJM 0817	3	5.7	12.4	17.9	17.2	7.4	0
	5	9.4	18.4	24.5	24.0	12.1	0
	7	13.6	24.1	30.8	31.4	18.7	0
NJM 0818	3	5.7	12.4	17.9	18.4	8.4	0
	5	9.5	18.9	24.8	26.8	14.6	0
	7	13.6	24.7	29.0	32.6	19.2	0

The present isolates, NJM 0719, NJM 0817, NJM 0818, NJM 0901, and NJM 0902 formed an independent cluster in the phylogenetic analysis. There were 8 clusters in the phylogenetic tree, supported by a bootstrap value (Fig. 8). The aligned dataset used in the analysis has been deposited with TreeBASE under the accession number S10706.

Discussion

The results of the present study suggest that the mortalities in 2007–2009 of the soft-shelled turtle, *Pelodiscus sinensis*, were associated with an oomycete of a new species belonging to the genus *Aphanomyces*. Numerous Gram-negative short rods were also observed in these lesions, suggesting that the lesions were caused by simultaneous infections of bacteria and fungi. However, it was likely that we satisfied Koch's postulates in the artificial infection tests and we suggest the primary causative agent for the disease was *Aphanomyces* sp.

Many aquatic species exchange respiratory gases through the integument. When submerged, soft-shelled turtles may obtain up to 70% of their oxygen uptake through the leathery shell (Mader 2006). Therefore, hyphal growth by *Aphanomyces* infection in the major part of the leathery shell could cause lethal deterioration in cutaneous respiration. While we suspect that the infected turtles died of hypoxia caused by the fungal infection on the carapaces, the pathogenesis of the present disease remains to be determined by other patho-physiological experiments.

Murray (2006) reported stressors within the captive environment might suppress the immune system and thus be conducive to fungal infection in captive reptiles. A thermal environment below the lower preferred optimal temperature zone appeared to be critically important in the development of fungal pneumonia in a captive American

Table 5 Effect of pH on vegetative growth

Strains	Days	pH												
		2	3	4	5	6	7	8	9	10	11	12	13	Control[a]
NJM 0719	3	0.0	0.0	0.0	49.8	76.8	90.7	100[b]	86.9	52.9	0.0	0.0	0.0	91.5
	5	0.0	0.0	0.0	40.0	68.7	70.2	100	68.5	43.6	13.8	0.0	0.0	69.6
	7	0.0	0.0	0.0	57.5	100	96.7	100	88.0	62.2	15.6	0.0	0.0	92.0
NJM 0817	3	0.0	0.0	0.0	31.7	70.6	100	67.2	76.2	30.9	0.0	0.0	0.0	67.9
	5	0.0	0.0	0.0	24.9	100	82.5	100	64.5	28.5	0.0	0.0	0.0	59.5
	7	0.0	0.0	0.0	33.8	100	100	100	84.7	41.5	18.0	0.0	0.0	100
NJM 0818	3	0.0	0.0	0.0	32.8	55.8	100	69.0	65.3	35.8	0.0	0.0	0.0	56.6
	5	0.0	0.0	0.0	19.8	100	100	100	100	33.8	0.0	0.0	0.0	100
	7	0.0	0.0	0.0	34.2	100	100	100	100	72.0	19.1	0.0	0.0	100

[a] Unadjusted GY broth

[b] The rate is the maximum growth (100%)

Fig. 6 Infected juvenile soft-shelled turtle in the group that was immersed in low-dose NJM 0719 zoospore suspension; a small whitish macula is seen on the carapace (*arrow*).

Table 6 Re-isolation from experimental turtles

Group	High-dose	Low-dose
NJM 0719	100[a]	60
NJM 0817	100	80
Control	0	

[a] Re-isolation success rate (%)

alligator, by interfering with normal reptile immunologic activity (Fromtling et al. 1979). We consider the spontaneous cases of fungal disease associated with

Table 7 Mortality of experimental turtles

Group	High-dose	Low-dose
NJM 0719	100[a]	10
NJM 0817	90	40
Control	0	

[a] Mortality at 14 days (%) after exposure to zoospore suspension

Aphanomyces sp. in juvenile soft-shelled turtles were triggered by strong stress caused by the sudden drop of the rearing temperature from 30 to 20°C. It is likely that the turtles were already infected with the fungi in the original culturing pond and the disease emerged following the increased stress from the transfer and the low temperature in the laboratory tanks.

The spherical oogonia of NJM 0719 were similar to those of *Aphanomyces laevis*. The superficial structures resembled those of *A. laevis*. When antheridial branches were present, the origin of the antheridial branches in the isolates from soft-shelled turtles was mainly diclinous, rarely monoclinous, but not androgynous. On the other hand, the antheridial branches of *A. laevis* were predominantly diclinous and monoclinous, and rarely androgynous (Johnson et al. 2002). Namely, the origin of the antheridial branches of the isolates from soft-shelled turtles showed distinct differences from the origin of the branches in *A. laevis*. A nuclear spot was clearly observed in each oospore of the isolate NJM 0719, but this was not observed in strains IA 1370 and IA 1530 of *A. laevis*. Both strains were distinguished in this point.

Optimal growth temperatures for the isolates in the present study matched water temperature of the farm, in which the turtles were kept. This differs from the optimal growth temperatures established for other *Aphanomyces* spp.: *A. astaci* Schikora 20–25°C (Unestam 1965),

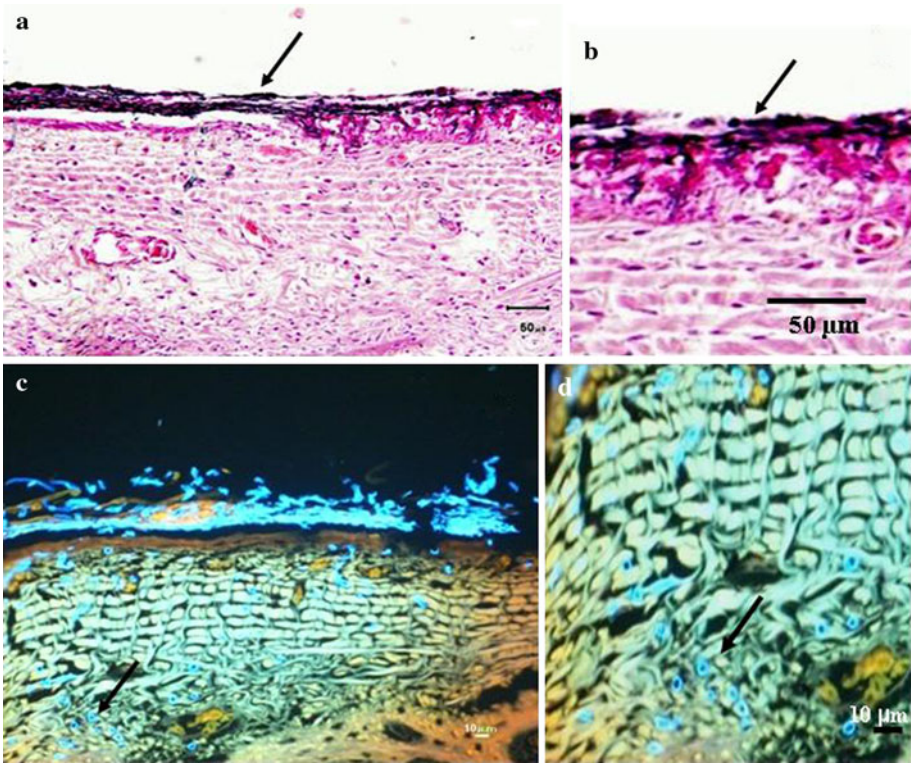

Fig. 7 Histopathological characteristics of an infected turtle in the group that was immersed in low-dose NJM 0719 zoospore suspension . **a** Many hyphae were observed in the cuticle of the carapace (*arrow*). Grocott-H&E. **b** High magnification of **a**. **c** Hyphae have infiltrated into the dermal layers (*arrow*). Uvitex 2B–H&E. **d** High magnification of **c**. *Bars* **a**, **b** 50 μm; **c**, **d** 10 μm

Table 8 Identities compared with the present isolate, *Aphanomyces* sp. NJM 0719 (accession no. AB531971)

Species	Accession no.	bps	PI	AA	AC	AE	AF	AI	AL	AP	AR	AS1	AS2
Present isolate (PI)		662											
A. astaci (AA)	AY683894	663	79.5										
A. cladogamus (AC)	AY353918	655	77.3	80.4									
A. euteiches (AE)	AY353908	651	76.5	80.8	92.8								
A. frigidophilus (AF)	AY647192	650	80.3	93.4	82.5	82.4							
A. invadans (AI)	AY082907	634	79.2	92.4	81.9	81.9	96.5						
A. laevis (AL)	AY683885	657	76.7	78.0	76.5	75.9	79.5	79.4					
A. piscicida (AP)	AY283643	648	79.2	91.4	81.8	81.8	96.2	99.7	79.6				
A. repetans (AR)	AY683889	655	75.8	76.9	75.8	75.6	79.1	77.8	90.6	78.0			
A. stellatus (AS1)	AY283647	661	80.9	89.3	80.4	80.3	90.2	89.8	77.6	89.7	77.6		
A. stellatus (AS2)	AY455774	662	80.1	90.1	79.9	79.0	91.1	90.1	77.8	90.1	77.2	95.1	
P. ultimum (PU)	EU253560	684	58.3	62.4	60.8	60.8	62.1	61.9	61.1	60.7	61.1	60.7	63.3

A. frigidophilus Kitanch. & Hatai 25°C (Kitancharoen and Hatai 1997), and *Aphanomyces* sp. NJM 9525 30–35°C (Sinmuk et al. 1996). The vegetative growth of isolates NJM 0719, NJM 0817, and NJM 0818 in GY broth in the pH range of 5–11 was similar to that of *A. frigidophilus* (Kitancharoen and Hatai 1997).

The isolates NJM 0719, NJM 0817, NJM 0818, NJM 0901, and NJM 0902 were limited to turtle hosts. *Aphanomyces* spp. occur in different ecological niches and include host-specific plant or animal parasites and saprotrophic species growing on decaying animal and plant debris (Scott 1961; Johnson et al. 2002). The host specificities of *Aphanomyces* species closest to the isolates NJM 0719, NJM 0817, NJM 0818, NJM 0901, and NJM 0902 were: *A. astaci*—crayfish, *A. invadans* and *A. piscicida*—fish, *A. frigidophilus*—crayfish and/or fish eggs, and *A. stellatus*, which lives on fish and in soil.

The nucleotide sequences of the ITS regions of rDNA are now used for the identification of species and genus levels of oomycetes (Bruns et al. 1991; Cooke et al. 2000; Constantinescu and Fatehi 2002). The strains isolated from the soft-shelled turtles in the present study could not be clearly identified as a new species, but Diéguez-Uribeondo et al. (2009) analyzed the phylogenetic relationship of

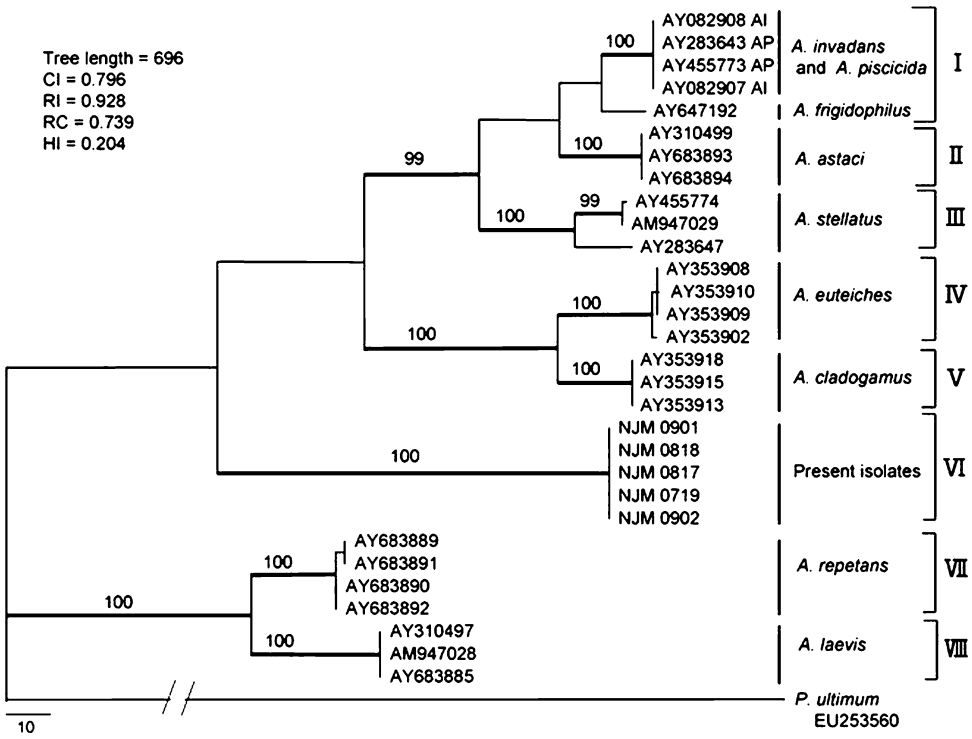

Fig. 8 Phylogenetic tree of *Aphanomyces* including isolates NJM 0719, NJM 0817, NJM 0818, NJM 0901, and NJM 0902. One of twenty-seven most-parsimonious trees obtained from heuristic searches based on 662 base pairs of the internal transcribed spacer (ITS) 1-5.8S-ITS 2 rDNA sequence. Clusters were supported by bootstrap values above 75%. The *bars* indicate 10 base differences. Data are shown with accession numbers 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

Aphanomyces spp. based on the ITS regions of rDNA, and classified them into 10 clusters. However, the present isolates were not included in these clusters. As a result, it was thought that, based on the molecular phylogenetic analysis, the present isolates could be classified as a new species.

Finally, the present isolates derived from soft-shelled turtles were identified as a new *Aphanomyces* species, based on their morphological, physiological, and phylogenetic status and species-specific virulence to the reptile. The new name of *A. sinensis* has been registered with Mycobank under the accession number MB 518613.

Aphanomyces sinensis sp. nov. Takuma, Hatai & Sano
Figs. 4 and 5

Mycelium aseptatum, subtile, 3–12 μm diametro, leave, leviter undulatum, modice ramosum; zoosporangia isodiametra, diametrum hyphae aequantia; zoosporae conglobatae prope orificium incystatae; zoosporae secundae reniformes, lateraliter biflagellatae. Oogonia plerumque sphaerica raro pyriformia, 18–25 μm diametro, stipitata, stipitibus non ramosis, oosporis singularibus, 15–22 μm diamtro. A congeneribus sequentiis nucleotidi distinctus, a specie proxima *Aphanomycete frigidophilo* 80.3 per centum identitati regionis ITS.

Type On the carapace of the soft-shelled turtle, *Pelodiscus sinensis* on 8 November 2007, Oita Prefecture, Japan, collected by D. Takuma (authentic culture NJM 0719 NBRC = 106581). Figure 5, showing the strain NJM 0719, is designated as the holotype, because there were technical difficulties in preserving the type specimen: i.e., in the slide preparation of hyphae with zoospore and sexual reproductive organs, the characteristic structures of this species are easily destroyed.

Vegetative hyphae are delicate, about 3–12 μm in diameter, aseptate, smooth, slightly wavy, and moderately branched. Zoosporangia are slender and have the same diameter as the hyphae; namely, being isodiametric. Primary zoospore production is in a single row within the zoosporangium and the zoospores are encysted in a cluster at the top of the zoosporangium. Primary zoospores are about 8–12 μm in diameter. Secondary zoospores are reniform and laterally biflagellate. Oogonia were abundant, usually spherical or subspherical, and only rarely pyriform; they were 18–25 μm in diameter and stipitate; the stipes were not branched and even young oogonia had a smooth surface. Singly sprouted oospores mostly filled the oogonium, and were generally spherical, ranging from 15 to 22 μm in diameter. The internal structure of oospores was dominantly centric or subcentric with a large shiny vesicle. Oospores often had a clear nuclear spot. Oogonial stalks were unbranched. Antheridial branches, when present, were predominantly diclinous and infrequently monoclinous, but not androgynous. Oogonia had double or triple antheridia. Antheridial cells were simple and vermiform, and often irregular. A sequence identity of 80.3% was obtained for a 662-base-pair region between the present isolates and the most closely related species, *A. frigidophilus.* But the

identity with *A.laevis*, which was morphologically the most closely related species, was 76.7%.

Acknowledgments We would like to give our great thanks for the technical support of Ms. Mihoko Kobayashi and Ms. Shinako Suzuki from our department and Ms. Kyoko Yarita from Chiba University. This study was supported in part by the Special Research Fund for Emerging and Re-emerging Infections of the Japanese Ministry of Health, Welfare and Labor (Grant No. H-18-Shinkou-8. and No. H-21-Shinkou-4) for Dr. Sano.

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

Baldock 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

Bruns TD, White TJ, Taylor JW (1991) Fungal molecular systematics. Annu Rev Ecol Syst 22:525–564

Cabanes FJ, Alonso JM, Castella G, Alegre F, Domingo M, Pont S (1997) Cutaneous hyalohyphomycosis caused by *Fusarium solani* in a Loggerhead sea turtle (*Caretta caretta* L.). J Clin Microbiol 35:3343–3345

Constantinescu O, Fatehi J (2002) *Peronospora*-like fungi (Chromista, Peronosporales) parasitic on Brassicaceae and related hosts. Nova Hedwigia 74:291–338

Cooke DEL, Drenth A, Duncan JM, Wagels G, Brasier CM (2000) A molecular phylogeny of Phytophthora and related oomycetes. Fungal Genet Biol 30:17–32

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

Egusa S (1970) On a *Mucor* infection of cultured soft-shelled turtle. Fish Pathol 5:43–44 (In Japanese)

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

Fromtling RA, Kosanke SD, Jensen JM (1979) Fatal *Beauveria bassiana* infection in a captive American alligator. J Am Vet Med Assoc 175:934

Frye FL, Dutra FR (1974) Mycotic granulomata involving the forefeet of a turtle. Vet Med Small Anim Clin 69:1554–1556

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, West Susssex, pp 240–272

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

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

Jacobson ER, Calderwood MB, Clubb SL (1980) Mucormycosis in hatching Florida softshell turtles. J Am Vet Assoc 117:835–837

Jeanmougin F, Thomson JD, Gouy M, Higgins DG, Gibson TJ (1998) Multiple sequence alignment with Clustal X. Trends 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/

Kitancharoen N, Hatai K (1997) *Aphanomyces frigidophilu*s sp. nov. from eggs of Japanese char, *Salvelinus leucomaens*. 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

Li X, Zhang C, Fang W, Lin F (2008) White-spot disease of Chinese soft-shelled turtles (*Trionyx sinens*) caused by *Paecilomyces lilacinus*. J Zhejiang Univ Sci B 9:578–581

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

Mader DR (2006) Cardiopulmonary anatomy and physiology. In: Mader DR (ed) Reptile medicine and surgery. WB Saunders, Philadelphia, pp 124–134

Murray MJ (2006) Pneumonia and normal respiratory function. In: Mader DR (ed) Reptile medicine and surgery. WB Saunders, Philadelphia, pp 396–405

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

Rose FL, Koke J, Koehn R, Smith D (2001) Identification of the etiological agent for necrotizing scute disease in the Texas tortoise. J Wildl Dis 37:223–228

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*. Va Agric Exp Stat Tech Bull 151:1–95

Seymour RL (1970) The genus *Saplolegnia*. Nova Hedwigia 19:1–124

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. University of 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

Unestam 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® MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci*. Vet Microbiol 137:146–155

Wada S, Yorisada Y, Kurata O, Hatai K (2003) Histological detection of aquatic fungi by Uvitex 2B, a fluorescent dye. Fish Pathol 38:49–52

Willoughby LG, Roberts 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

Willoughby LG, Roberts RJ, Chinabut S (1998) *Aphanomyces invaderis* sp. nov., the fungal pathogen of freshwater tropical fish affected by epizootic ulcerative syndrome. J Fish Dis 18:273–275