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First isolation of *Aphanomyces frigidophilus* (*Saprolegniales*) in Europe

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Abstract—*Aphanomyces frigidophilus* was isolated and described for the first time in Europe. The isolate was characterized by studying its ability to undergo repeated zoospore emergence, to parasitize crayfish, and to produce chitinase constitutively, and by sequencing the internal transcribed spacer of nuclear ribosomal DNA (ITS1+5.8S+ITS2). The physiological properties studied differed from those of the "crayfish plague" parasite, *A. astaci*, but they were similar to those of saprobiotic *Aphanomyces* species. The ITS nrDNA sequence obtained from this isolate corresponded to *A. frigidophilus*.

Key words—*Aphanomyces astaci*, crayfish, conservation, rDNA, taxonomy

Introduction

The genus *Aphanomyces* de Bary belongs to the order *Saprolegniales* (Oomycetes) and comprises ca 30 species. Many of the species of this group have a saprobiotic mode of life, living on decayed animals and plant remains. A few species are detrimental parasites and responsible for economically important diseases affecting agriculture and aquaculture crops, as well as wildlife populations of freshwater animals (Papavizas & Ayers 1974, Söderhäll & Cerenius 1999). *Aphanomyces frigidophilus* Kitanch. & Hatai is a recently described species, which so far has only been found in salmonids eggs in Japan (Kitancharoen & Hatai 1997, 1998). Specimens resembling *A. frigidophilus* have been described in fish eggs in Poland (Czeczuga et al. 2004a, b, 2005). However, no isolations have been done to confirm the presence of this species in Europe or to study the molecular relatedness of European strains to the Japanese reference isolate of *A. frigidophilus*.

The taxonomy of *Aphanomyces* is largely based on the morphological characters of their sexual structures. However, the main taxonomic problems when describing species of this genus are: (i) that no reference isolates or cultures exist for several of the described species, and (ii) that many isolates, especially those of animal parasitic species are sterile;

consequently, species identification is largely based on their ability to parasitize their host, and a number of physiological properties.

The recent application of molecular tools to the genus *Aphanomyces* has helped identifying sterile isolates either in culture or clinical samples (Oidtmann et al. 2002, 2004; Phadee et al. 2004, Royo et al. 2004, Vandersea et al. 2006) and even defining new species (Royo et al. 2004). In this article, we have studied the internal transcribed spacer of nuclear ribosomal DNA sequences (ITS nrDNA) to identify a sterile isolate obtained from a mass mortality of indigenous crayfish, *Austropotamobius pallipes* (Lereboullet 1858) that occurred in the Central Iberian Peninsula region. Because the isolate exhibited different physiological properties from the crayfish plague fungus, *A. astaci* Schikora, it was decided to explore this isolate more carefully and obtain ITS sequence data, the results of which are described in this contribution.

Materials and Methods

Dead crayfish, *Austropotamobius pallipes*, were collected in the river Tajuña, Guadalajara (Spain). The isolation procedure was done from pieces of sub-abdominal cuticle taken from the crayfish as described by Cerenius et al. (1988). The isolate was maintained on PG1-agar (Unestam 1965) and stored under the strain name SAP233 in the culture collection of the Real Jardín Botanico de Madrid. Morphological characters of asexual structures and measurements were made microscopically on material mounted in water. Light micrographs were captured using a QImaging Micropublisher digital camera (QImaging, Burnaby, BC, Canada) mounted on an Olympus BX51 compound microscope as described in Diéguez-Uribeondo et al. (2003).

Physiological properties: repeated zoospore emergence characteristic of parasitic *Aphanomyces species*, and constitutive production of chitinase, characteristic of *A. astaci*, were studied according to methods described by Cerenius & Söderhäll (1985), and Andersson & Cerenius (2002), respectively. Production of sexual structures was studied by growing the isolate in corn meal agar, hemp seed or snake skin. In corn meal agar the isolate was paired with representative strains of the four genotypes of *A. astaci* (Huang et al. 1994, Diéguez-Uribeondo et al. 1995). The cultures were maintained and regularly checked for the production of sexual structures during a one month period.

The ability to infect crayfish was tested by following the method described in Cerenius et al. (1988). After finishing the experiment pieces of sub-abdominal cuticle were examined under the microscope for the presence of hyphae.

For DNA extraction, mycelium was grown as drop cultures (Cerenius & Söderhäll 1985), and from them, genomic DNA was extracted using an E.Z.N.A.-Fungi DNA miniprep kit (Omega Biotek, Doraville, USA). DNA fragments containing internal transcribed spacers ITS1 and ITS2 including 5.8S, were amplified with primer pair ITS5/ITS4 (White et al. 1990) primers as described in Martín et al. (2004). Nucleotide BLASTN searches with option Standard nucleotide BLAST of BLASTN 2.6 were used to compare the sequence obtained against the sequences in the National Center of Biotechnology Information (NCBI) nucleotide databases. The new consensus sequence has been deposited in the EMLB database with the accession Number 281399.

Results

The isolate obtained exhibited thin hyphae with rounded hyphal tips, and with a hyphal diameter that ranged from 5 to 7 µm (Fig. 1a). The isolate produced sporangia with a single row of primary spores. The primary spores were eventually released and encysted at the hyphal tip forming spore-balls characteristic for the genus *Aphanomyces* (Fig. 1b). No oogonia or antheridia were seen in either individual cultures or in co-culture with the other isolates or with representive strains of four genetic groups of *A. astaci*. Thus the strains appeared to be sterile and lack sexual reproduction.

The encysted zoospores did not undergo repeated zoospore emergence and instead germinated. No chitinase activity was detected in the culture filtrates of the new isolate, while reference *A. astaci* strains produced high level of extracellular chitinase (Fig. 1 c, d, e). Crayfish challenged with zoospores of the *Aphanomyce*s sp. isolate did not die under the experimental conditions. However, addition of zoospores of the reference isolates of *A. astaci* always caused 100% mortality of crayfish.

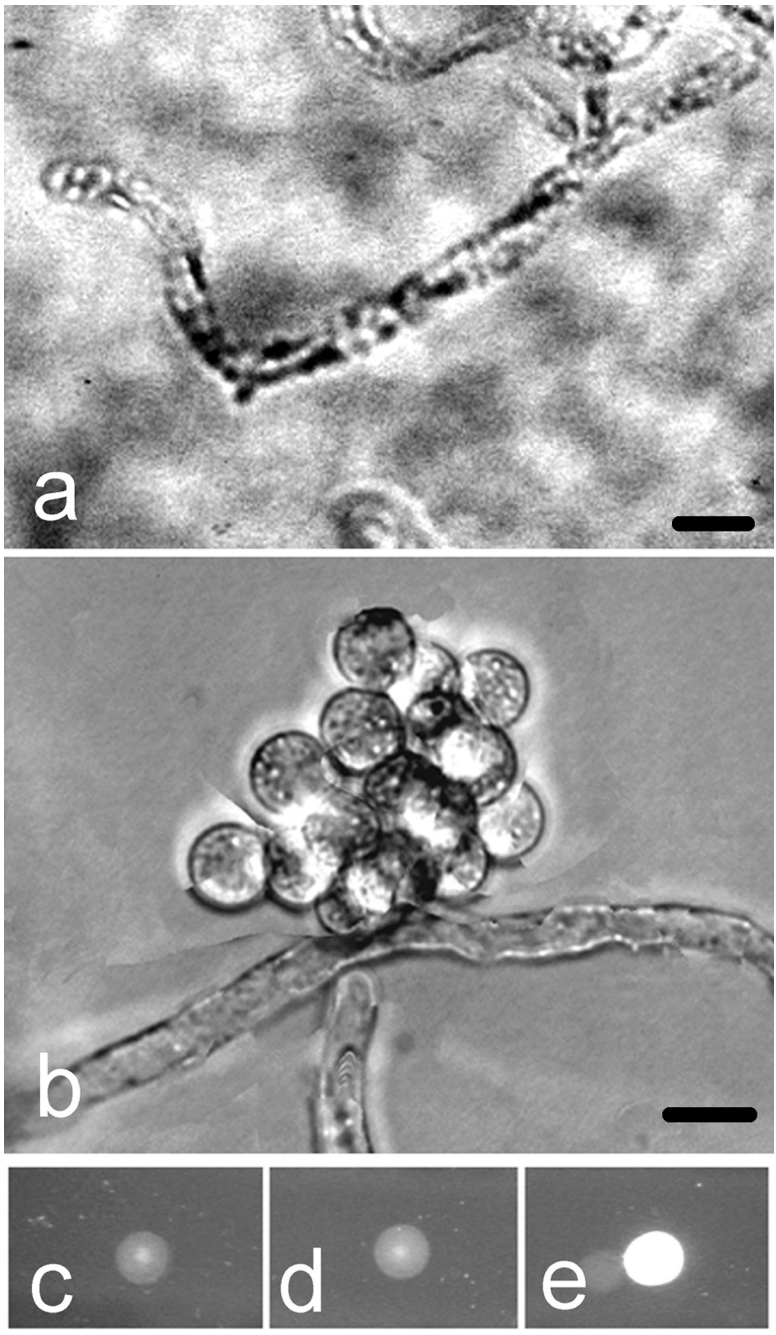
The blast search of the sequence of the isolate showed 99% similarity (the sequence differed in one base for ITS1 and four bases in ITS2 out of the 673 bases compared) to Genbank sequence AY647192 corresponding to strain NJM9500 of *A. frigidophilus* directly submitted by Phadee et al. (2004), and 95% (the sequence differed in 22 bases for ITS1 and 10 bases in ITS2 out of the 614 bases compared to Genbank sequences of *A. astaci*.

Discussion

Previous studies have shown that analyses of the ITS nrDNA represents a useful tool for differentiating individual saprolegniaceous species (Molina et al. 1995, Leclerc 2000, Oidtmann et al. 2004, Phadee et al. 2004). Due to the lack of sexual structures of the studied isolate, we carried out Genbank sequence comparisons of the two ITS nrDNA for species identification. The results indicated the sequence of our isolate corresponded to the species, *Aphanomyces frigidophilus*. Thus, this study represents the first isolation of *A. frigidophilus* in Europe and the first description of this species growing in a different substrate from salmonid eggs.

Interestingly, this isolate was growing in crayfish cuticle and associated to a mass mortality of indigenous European species of freshwater crayfish. These episodes are generally caused by the "crayfish plague" parasite *Aphanomyces astaci*. This parasite is considered among the 100 worst invasive species (Global Invasive Species Database 2005) and is responsible for the dramatic decline of the indigenous European freshwater crayfish species, which are currently endangered in Europe and at risk of extinction in the Iberian Peninsula (Diéguez-Uribeondo et al. 1997a, b, Söderhäll & Cerenius 1999). Current attempts to develop and improve techniques for rapid and accurate identification of this economically important parasitic species need to take into account the existence of closely related species, such as *A. frigidophilus*, in Europe.

Regarding the possible parasitic abilities of *A. frigidophilus*, our results indicate that its physiological properties are characteristic of a saprobiotic and/or opportunistic pathogen. Thus, the isolate exhibited a low percentage of secondary cysts undergoing repeated zoospore emergence, and, therefore, it lacks a character that appears to be


a
b
c
d
e

related to parasitism in *Aphanomyces* species (Cerenius & Söderhäll, 1985). The failure to kill crayfish challenged with zoospores and to produce chitinase constitutively, are also characters of the specialized crayfish parasite, *A. astaci* (Cerenius et al. 1988; Söderhäll & Cerenius 1999, Andersson & Cerenius 2004).

Aphanomyces frigidophilus and *A. astaci* seem to be closely related species which may occur in the same host with different abilities to colonize it. Further studies on phylogenetic relationships among *Aphanomyces* species need to be carried out in order to more accurately establish species limits. Finally, the results of this work emphasized the need to carry out isolations for a correct identification and characterization of saprolegniaceous species.

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Literature Cited

Andersson MG, Cerenius L. 2002. Analysis of chitinase expression in the crayfish plague fungus *Aphanomyces astaci*. Diseases of Aquatic Organisms **51**: 139-147.

Cerenius L, Söderhäll K, 1985. Repeated zoospore emergence as a possible adaptation to parasitism in *Aphanomyces*. Experimental Mycology **9**: 259-263.

Cerenius L, Söderhäll K, Persson M, Ajaxon R. 1988. The crayfish plague fungus, *Aphanomyces astaci* – diagnosis, isolation, and pathobiology. Freshwater Crayfish **7**: 131-144.

Czeczuga B, Kiziewicz B, Muszynska E. 2004a. Presence of zoospore fungus species on the eggs of whitefish from Lake Goldopiwo, Mazury Region. Medycyna Weterynaryjna **60**: 379-383.

Czeczuga B, Kiziewicz B, Godlewska A. 2004b. Zoosporic fungi growing on eggs of *Coregonus lavaretus holsatus* Thienemann, 1916 from Lake Wdzydze in Kaszuby. Polish Journal of Environmental Studies **13**: 355-359 2.

Czeczuga B, Bartel R, Kiziewicz B, Godlewska A, Muszynska E. 2005. Zoosporic fungi growing on the eggs of sea trout (*Salmo trutta* m. *trutta* L.) in river water of varied trophicity. Polish Journal of Environmental Studies **14**: 295-303.

Diéguez-Uribeondo J, Huang TS, Cerenius L, Söderhäll K. 1995. Physiological adaptation of an *Aphanomyces astaci* strain isolated from the freshwater crayfish *Procambarus clarkii*. Mycological Research **99**: 574-578.

Diéguez-Uribeondo J, Rueda-Díez A, Castién E, Bascones JC. 1997a. A plan of restoration for the native freshwater crayfish species, *Austropotamobius pallipes*, in Navarra. Bulletin Francais de la Peche et de la Pisciculture **347**: 625-637.

Fig. 1 *Aphanomyces frigidophilus*. **a)** Hypha with a rounded tip growing within the cuticle of the freshwater crayfish *Austropotamobius pallipes*. **b)** "Spore balls" characteristic of the genus *Aphanomyces* (Bars 10 μm). **c–e)** Chitinase assay for production of chitinase constitutively during growth: **c)** negative control, without fungus; **d)** *A. frigidophilus* (negative); **e**) *A. astaci* (positive).

Diéguez-Uribeondo J, Temiño C, Múzquiz JL. 1997b. The crayfish plague fungus, *Aphanomyces astaci* in Spain. Bulletin Francais de la Peche et de la Pisciculture **347**: 753-763.

Diéguez-Uribeondo J, Förster H, Adaskaveg JE. 2003. Digital image analysis of internal light spots of appressoria of *Colletotrichum acutatum*. Phytopathology **93**: 923-930.

Global Invasive Species Database. 2005. Information Infrastructure (NBII) and Invasive Species Specialist Group (ISSG). *Aphanomyces astaci*. Available at: <http://www.issg.org/database/species/ecology.asp?si=107&fr=1&sts=sss>

Huang TS, Cerenius L, Söderhäll K. 1994. Analysis of the genetic diversity in crayfish plague fungus, *Aphanomyces astaci,* by random amplification of polymorphic DNA assay. Aquaculture **26**:1-10.

Kitancharoen N, Hatai K. 1997. *Aphanomyces frigidophilus* sp. nov. from eggs of Japanese char, *Salvelinus leucomaenis*. Mycoscience 38: 135-140.

Kitancharoen N, Hatai K. 1998. Some biochemical characteristics of fungi isolated from salmonid eggs. Mycoscience **39**: 249-255.

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.

Martín MP, Raidl S, Tellería MT. 2004. Molecular analysis confirm the relationship between *Stephanospora caroticolor* and *Lidtneria trachyspora*. Mycotaxon **90**: 133-140.

Molina FI., Jong SC., MA, G. 1995. Molecular characterization and identification of *Saprolegnia* by restriction analysis of genes coding for ribosomal RNA. Antonie van Leeuwenhoek **68**: 65-74.

Oidtmann B, Bausewein S, Hölzle L, Hoffmann R, Wittenbrink M. 2002. Identification of the crayfish plague fungus *Aphanomyces astaci* by polymerase chain reaction and restriction enzyme analysis. Veterinary Microbiology **85**: 183–194.

Oidtmann B, Schaefer N, Cerenius L, Söderhäll K, Hoffmann RW. 2004. Detection of genomic DNA of the crayfish plague fungus *Aphanomyces astaci* (Oomycete) in clinical samples by PCR. Veterinary Microbiology **100**: 269-282.

Papavizas GC, Ayers A. 1974. *Aphanomyces* species and their root diseases in pea and sugar beet. U.S. Department of Agriculture Technical Bulletin no **1485**.

Phadee P, Kurata O, Hatai K, Irono I, Aoki T. 2004. Detection and identification of the fish-pathogenic *Aphanomyces piscicida* using polymerase chain reaction (PCR) with species-specific primers. Journal of Aquatic Animal Helath **16**: 220-230.

Royo F, Andersson MG, Bangyeekhun E, Muzquiz JL, Söderhäll K, Cerenius L. 2004. Physiological and genetic characterisation of some new *Aphanomyces* strains isolated from freshwater crayfish. Veterinary Microbiology **104**: 103-112.

Söderhäll K, Cerenius L. 1999. The crayfish plague fungus: history and recent advances. Freshwater Crayfish **12**: 11-35.

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 invadans* in ulcerative mycotic fish lesions Applied and Environmental Microbiology **72**: 1551-1557.

Unestam T. 1965. Studies on the crayfish plague fungus, *Aphanomyces astaci* I. Some factors affecting growth in vitro. Physiologia Plantarum **18**: 483-505.

White TJ, Bruns T, Lee S, Taylor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR Protocols. A guide to methods and applications (eds. Innis MA, Gelfand DH, Sninsky JJ, White TJ.), pp. 315-322. Academic Press Inc., San Diego, CA.