

Full Length Research Paper

Aphanomyces frigidophilus, fungus-like organisms isolated from water of springs in Białystok, Poland

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Accepted 17 October, 2013

Investigations into occurrence of fungus *Aphanomyces frigidophilus* in water of springs Dojlidy Górne, Jaroszkówka and Pietrasze within the town Białystok in Podlasie Province, Poland were conducted in Winter, Spring, Summer and Autumn of the year 2005. Samples were processed in the laboratory by routine methods commonly used to isolate these organisms. Bait method with the use of hemp seeds *Cannabis sativa*, small pieces of snake skin *Natrix natrix* and exuviae of shrimp *Gammarus* sp. as bait was applied to isolate the fungus *Aphanomyces frigidophilus* from the springs. The isolate was maintained on Potato Dextrose Agar PDA and stored in the culture collection of the Real Jardín Botánico CSIC Madrid, Spain. *Aphanomyces frigidophilus* occurred in 18 [(6)16.7% in Winter, 3(8.3%) in Spring, 2(5.6%) in Summer, 7(19.4%) in Autumn, 2005] of the examined water samples. In Spring Dojlidy Górne it was very common and was found in all research seasons. The isolate was characterized by studding sequencing the internal transcribed spacer of nuclear DNA (ITS1+5.8S+ITS2). The results indicated the sequence comparisons of two ITS nuclear DNA for species identification: *Aphanomyces frigidophilus* 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence and 28S ribosomal RNA gene, partial sequence. The results indicated the sequence of our isolate corresponded to the species *Aphanomyces frigidophilus* (AY647192, version AY647192.1; GI: 48766837).

Key words: *Aphanomyces frigidophilus*, snake skin of *Natrix natrix* and exuviae of shrimp *Gammarus* sp.

INTRODUCTION

Water mould constitute a common group of organisms found in a variety of water ecosystems. Some of them are animal or human parasites. In favorable conditions, water mold acting as saprobionts can assume pathogenic properties, being a potential source of infection (Dick, 2001; Czeczuga et al., 2004a; b; Kiziewicz and Kurzatowska, 2004; Kirk et al., 2008). In spring of rivers, a lot of representatives of water moulds of the class Oomycetes are met. The phylogenetic relationship of

Oomycetes (watermolds) to fungi has been debated for many years. Oomycota (oomycota means egg fungus) or Oomycetes have been for a long time recognized as significantly different from the organisms classified as the Phylum Oomycetes in the Kingdom Fungi (True fungi) (Alexopoulos et al., 1996). Scientific studies have shown that some organisms may look like fungi yet are not really members of the Kingdom fungi. A cladistic classification based on modern insights supports a relatively close

Abbreviations: PDA, Potato Dextrose Agar; ITS, internal transcribed spacer; PCR, polymerase chain reaction; NCBI, National Centre of Biotechnology Information.

relationship between Oomycetes with photosynthetic organisms such as brown algae and diatoms, within the heterokonts. The Oomycetes have been differently classified by numerous taxonomists, for instance they have been classified as heterokont organisms in the Kingdom Stramenopila, Phylum Heterokonta, Class Peronosporomycetes (Alexopoulos et al., 1996; Dick, 2001).

De Bary in 1860 (de Bary, 1860) established a new genus *Aphanomyces* to include a number of saprotrophic and parasitic plants observed during this period. The genus *Aphanomyces* comprising approximately 35-40 species is a smaller and less frequently encountered genera of the Saprolegniales order, Oomycetes Class (Scott, 1961; Uribeondo et al., 2009). A few species are parasites and responsible for economical important diseases affecting agriculture and aquaculture crops as well as wildlife populations of freshwater animals (Papavizas and Ayers, 1974; Söderhall and Cerenius, 1999).

The main task of the present study was to assess distribution of fungus-like organisms *Aphanomyces frigidophilus* from water of three limnocratic springs situated in Białystok, Podlasie Province, Poland.

MATERIALS AND METHODS

Study area

Investigations into occurrence of fungus *A. frigidophilus* in water of springs Dojlidy Górne, Jaroszkówka and Pietrasze within the town Białystok were conducted in Winter, Spring, Summer and Autumn of the year 2005. Spring Dojlidy Górne (53°06'N, 23°12'E) of Biała River, located in the eastern part of Białystok, Poland. Limnocratic type, an artificial basin: area 0.380 km², max. width 0.65 m, depth 0.12 m, discharge 2.4 dm³ s⁻¹, surroundings without trees. Spring Jaroszkówka (53°10'N, 23°11'E) of Jaroszkówka River, located in the north part of Białystok, Poland. Limnocratic type, an artificial basin: area 0.340 km², max. width 0.60 m, depth 0.12 m, discharge 2.4 dm³ s⁻¹, surroundings without herbaceous and trees. The spring is surrounded by cultivated fields. The bed is covered with sand. Spring Pietrasze (53°10'N, 23°9'E) of River Biała, located in the north part of Białystok, Poland, limnocratic type, an artificial basin: area 0.290 km², max. width 0.60 m, depth 0.12 m, discharge 2.5 dm³ s⁻¹, surroundings without herbaceous and trees.

Microbial analyses

Microbial analyses was made in the laboratory, The Real Jardín Botánico CSIC, Madrid, Spain of the year 2006. For the microbial analysis of fungi samples of water were collected from each sampling site of research springs of rivers. The water collected from the respective reservoirs was poured in sterile conditions into plastic bottles, and placed in the laboratory. All containers were incubated at 13 ±2°C, with access to daylight resembling natural conditions and following the recommended instructions (Seymour and Fuller, 1987). Water limnocratic type of springs in Poland have almost constant temperature throughout the year. The water temperature the research springs was about 9°C throughout the period of the study. pH of springs of water was neutral close to the acid.

Samples were processed in the laboratory by routine methods commonly used to isolate these organisms. Bait method with the use hemp seeds *Cannabis sativa* L., small pieces of snake skin

Natrix natrix and exuviae of shrimp *Gammarus* sp. as bait was applied to isolate the fungus *A. frigidophilus* from the springs. Water samples (100 ml) of each site were homogenized and four aliquots of 25 ml were placed in Petri dishes of 9 cm diameter with sterile baits. Dishes were stored in the laboratory at room temperature 13 ±2°C for 4-5 days (Seymour and Fuller, 1987). The colonized fragments of hemp-seeds, shrimp and snake skin were transferred to new Petri dishes which contained sterilized, filtered spring or distilled water and crystalline penicillin (2000 units per litre of water) to inhibit bacterial growth. Dishes were microscopically examined weekly for up to three weeks in order to identify the genus or species. The isolate was maintained on Potato Dextrose Agar (PDA) (Merck Cat. No. 1.10130.0500, Merck KCaA 64271 Damstadt, Germany) (Unestam, 1965) and stored in the culture collection of the Real Jardín Botánico CSIC, Madrid, Spain. Morphological characters of asexual structures and measurements were made microscopically on material mounted in water. Fungi were successively observed under an optic microscope Olympus BX 51 (100, 400 and 1000x magnification). The respective stages of the fungal development were evaluated by means of an ocular micrometer. Several microscopic preparations were made from each sample.

Isolates and internal transcribed spacer (ITS) sequences

Sequences were obtained from pure cultures, ITS-polymerase chain reaction (PCR)-based specific tests from GenBank representing a total of 12 *Aphanomyces* spp. All the isolates were growing on PDA (Merck Cat. No. 1.10130.0500, Merck KCaA 64271 Damstadt, Germany) (Unestam, 1965) for 3-5 days at room temperature as described by Cerenius et al., (1987). Mycellia pellets were washed with sterile water collected in 1.5 ml microcentrifuge tubes and stored at 20°C before DNA extractions. The origin of the isolates and their reference numbers were coded with the initials SAP and are maintained in the Oomycetes culture collection of the Real Jardín Botánico CSIC, Madrid. In this study, was used the following sequences as reference for species *A. frigidophilus* Kitanch, and Hatai, AY647192.

DNA extraction and PCR amplification

For DNA extraction, mycelium was grown as drop cultures (Cerenius and Söderhall, 1985) and from them, genomic DNA was extracted using an Easy Nucleic Acid® (EZNA) (Fungal DNA Miniprep Kit (Omega Biotek, Doraville, USA) as described in the study of Martín and García-Figueres (1999). DNA fragments containing internal transcribed spacers ITS1 and ITS2 including 5.8S gene of the nuclear DNA was amplified with primer pairs ITS5/ITS4 (White et al., 1990) primers as described in Martín et al., (2004). Nucleotide BLASTN searches with option Standard nucleotide BLAST and BLASTN 2.6 were used to compare the sequence obtained against the sequences in the National Centre of Biotechnology Information (NCBI) nucleotide databases.

Hypal growth rates and repeated zoospore emergence

Selected isolates of parasitic and saprotrophic/opportunistic species were inoculated in Potato Dextrose Agar PDA (Unestam, 1965) and incubated at 20°C. Colony diameter was measured every 24 h during maximum period of 3 days. Briefly, mycelia were grown in PDA drop cultures for 3 days at 20°C. To trigger sporulation, mycelia were washed three times with sterile water and incubated in petri dishes containing sterile water for 14 at 15°C to allow zoospore release. The release of new zoospores was observed under the microscope after incubating the cyst suspension at 15°C for 150 min.

Table 1. Distribution and seasonal occurrence of aquatic fungus-like organisms *Aphanomyces frigidophilus* in 36 samples from three different sites of springs in Białystok (n =3).

Water reservoirs	Number of collected water samples	Seasonal occurrence/number of water samples where fungus was found				
		Winter	Spring	Summer	Autumn	Together
Dojlidy Górne	12	3	2	1	3	9
Jaroszówka	12	2	1		2	5
Pietrasze	12	1		1	2	4
Total number of samples	36	6	3	2	7	18
Percentage %	100	16.7	8.3	5.6	19.4	50.0

Production of sexual structures

Aphanomyces isolates were cultivated on sterile substrates including hemp seeds (*C. sativa* L.), exuviae of *Gammarus* sp., and snake skin *N.atrix* in petri dishes containing autoclaves, sterilized water (one bait per dish) and incubated in the dark at 20°C. Dishes were examined periodically (for 3 weeks) for evidence of growth with a stereo microscope and to verify the presence or absence of sexual structures. All isolates were characterized and identified according to the study of Scott (1961). Taxonomic identifications were made according to the study of Scott (1961), Batko (1975), Pystina (1994) and Seymour and Fuller (1987).

RESULTS

The study conducted in springs located in Białystok, Poland showed the occurrence of fungus-like organisms *A. frigidophilus* Kitanch and Hatai in samples of water reservoirs. As shown in Table 1, *Ap. frigidophilus* occurred in all three springs of examined waters. It occurred in 18 [(6)16.7%] in Winter, 3(8.3%) in Spring, 2(5.6%) in Summer, 7(19.4%) in Autumn, 2005] of the examined 36 water samples. Compared to Spring Jaroszówka and Pietrasze in Spring Dojlidy Górne it was very common and was found in all research seasons. The results indicated the sequence comparisons of two ITS nuclear DNA for species identification: *Ap. frigidophilus* 18S ribosomal RNA gene, partial sequence, internal transcribed spacer 1, 5.8 S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence and 28S ribosomal RNA gene, partial sequence. The results indicated the sequence of our isolate corresponded to the species *Ap. frigidophilus* (AY647192, version AY647192.1; GI: 48766837).

DISCUSSION

An optical microscope was used for microscopic observations with total magnifications of 100, 400 and 1000 times. Scientific classification: *Ap. frigidophilus* Kitanch. and Hatai 1997 belonging to the Domain Eukarya, Supergroup/Subkingdom Chromalveolata,

Kingdom Chromista=Stramenopila, Phylum Heterokontophyta, Class Oomycota, Order Saprolegniales, Family Saprolegniaceae, Genus *Aphanomyces* (Kirk et al., 2008). Genus *Aphanomyces* is water and soil saprotrophs of plants and animals. Facultative parasites of algae, aquatic zoosporic fungi from genus *Achlya*. There are around 35-40 described *Aphanomyces* species which occur in very different ecological niches ranging from specialized plant or animal parasites with a narrow host range, to saprotrophic species growing on decaying animal and plant debris (Scott, 1961; Dick, 2001; Johnson et al., 2002; Uribeondo et al., 2009). Hyphae are usually thin, highly branched, 3-8 µm in diameter, sometimes ragouts (*Ap. frigidophilus*). Zoosporangia morphologically identical in each species. Zoospores 7-9

µm in diameter. Oogonia predominantly single, 18-33 µm in diameter, with delicate (*Aphanomyces laevis*), wrinkled (*Aphanomyces irregularis*) or granular (*Ap. frigidophilus*) wall, spherical with conical spine-like projection, placed on short, lateral hyphae (*Aphanomyces stellatus*). Cytospores 7-10 µm in diameter (*Ap. laevis*). Oospores subcentric (*Ap. frigidophilus*), with thick wall (*Ap. laevis*), 15-29 µm in diameter, predominantly single or 2 in number, filling the oogonium. Antheridia - one (*Ap. stellatus*) or more around one oogonium (*Ap. laevis*, *Ap. stellatus*), declinous or monoclinal, rarely androgynous (*Ap. laevis*, *Ap. stellatus*) (Johnson et al., 2002).

Ap. frigidophilus has the hyphae with rounded hyphal tips, hyphae thin in 5-7 µm in diameter, aseptate, sporangia long with a single row. Primary zoospores near the orifice emerged in this manner, whereas those emerging later became elongate with rounded ends during the passage through zoosporangium. Secondary zoospores were reniform, laterally biflagellate. Oogonia were abundant on short oogonial stalk, 16-25 µm diameter. Young oogonia with rough-ended outer contours, elongated oogonia between hyphae. Mature oogonium showing single subcentric oospore 14-22 µm diameter, with a large shiny vesicle surrounded by fine granules, the outer contours have short papillate or irregular (Kitancharoen and Hatai, 1997).

However, fungi in the genus *Aphanomyces* have been

occasionally reported on fish eggs. *Ap. laevis* is only species of this genus that has been reported on eggs of rainbow trout *Onchorhynchus mykiss* (Scott and O' Bier, 1962) and vendace *Coregonus albula* (Czeczuga and Woronowicz, 1993), although an unidentified *Aphanomyces* was also reported on rainbow trout (Scott and O' Bier, 1962). The first *A. frigidophilus* has been reported only from Japan. *Ap. frigidophilus* was detected on the eggs of Japanese char *Salvelinus leucomaenis* from Tochigi Prefectural Fisheries Experimental Station, Utsonomiya, Japan (Kitanchaoren and Hatai, 1997, 1998). The first in Poland and in Europe *Ap. frigidophilus* have been described only on the basic morphological studies by Czeczuga et al., (2004a; b) in fish eggs of European Whitefish *Coregonus lavaretus holsatus* obtained from Lake Wdzydze in Kaszuby (Poland). The next in Europe *Ap. frigidophilus* was described on crayfish cuticle *Austropotamobius pallipes* by Ballesteros et al. (2006), too. Dead crayfish were collected in river Tajuña, Guadalajara (Spain). The fungus was grown on fish eggs of European Whitefish in water of Cypiesk Spring and Akcent Pond in Białystok, Poland. Worth a special note was the finding *Ap. frigidophilus*, new to Polish freshwaters (Czeczuga et al., 2004a; b). The fungus was growing also on the eggs of sea trout *Salmo trutta* m. *trutta* in running water from River Biała, Krasna and Supraśl near Białystok (Poland) (Czeczuga et al., 2005). *Ap. frigidophilus* was the most common straminipilous organisms found on the eggs of Atlantic salmon *Salmo salar*. The investigated eggs were collected from 60 females of Atlantic salmon caught during their spawning migration in Darłowo town on the River Wieprza (wild form), and Świbno town on the River Vistula (wild form), and from those bred in fresh water in hatcheries at Miastko town (farmed form). *Ap. frigidophilus* was found growing on the eggs of Atlantic salmon from 14 females from research rivers such as Wieprza and Vistula and from hatcheries in Miastko (Czeczuga et al., 2011).

Molecular phylogenetic relationships among 12 species of *Aphanomyces* de Bary were analyzed based on 108 ITS sequence s of nuclear DNA by Uribeondo et al., (2009). Within *Aphanomyces* clade, three main lineages were found: plant parasitic, animal parasitic and saprotrophic or oportunic parasitic. The animal parasitic lineage had low support and comprised sequences from *Aphanomyces* spp. that are parasites of animals, or have been isolated from animals, and isolates of the saprotrophic species *Ap. stellatus*. This lineage contained the reference sequences for *Aphanomyces astaci*, *Ap. frigidophilus*, *Ap. invadans*, *Ap. piscicida* and *Ap. stellatus*. This lineage comprised species *Aphanomyces* that thrive in freshwater or estuarine aquatic environments, and included all species parasitic to animals, the animal associated species *Ap. frigidophilus*, and saprotrophic species *Ap. stellatus*. The *Ap. frigidophilus* clade comprised the sequences of refe-

rence for *A. frigidophilus* (AY647192). Isolates originated from Japan and Spain with a wide host range that included salmonids (Japan) and freshwater crayfish (Spain) (Kitanchaoren and Hatai, 1997; Ballesteros et al., 2006; Uribeondo et al., 2009). Although, isolates of this species have always been obtained from diseased aquatic animals, it is uncertain whether this species is a parasitic or opportunistic (Uribeondo et al., 2009).

The results indicated the sequence comparisons of two ITS nuclear DNA for species identification. The results indicated the sequence of our isolate corresponded to the species *Ap. frigidophilus* (AY647192, version AY647192.1; GI: 48766837). GenBank sequence AY647192.1 corresponding to strain NJM9500 of *Ap. frigidophilus* directly submitted by Phadee et al. (2004). Thus, this study represents the first isolation of *Ap. frigidophilus* in fresh waters in Poland. This parasitic fungus considered among one hundred worst invasive species (Global Invasive Species Database, 2005) and is responsible for the decline of the indigenous European freshwater animals not only to crayfish, and also fish which are currently endangered in Europe and specially in Poland (Czeczuga et al., 2004a; b, 2005; Ballesteros et al., 2006; Uribeondo et al., 2009).

Conclusion

The study conducted in springs of rivers located in Białystok, Poland showed the occurrence of fungus-like organisms *Aphanomyces frigidophilus* Kitanch. and Hatai in samples of water reservoirs. The results indicated the sequence comparisons of two ITS nuclear DNA for species identification. The results indicated the sequence of our isolate corresponded to the species *Ap. frigidophilus*. Thus, this study represents the first isolation (on the basic molecular studies) of *Ap. frigidophilus* in fresh waters in Poland.

ACKNOWLEDGMENTS

This study was supported by the European Union Program SYNTHESYS ES-TAF.

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