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Water molds *Saprolegnia diclina* (FLO) isolated from eggs of *Carassius carassius* L. in Białystok Rivers, Poland

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Investigations of occurrence of *Saprolegnia diclina* from four limnocrenic springs of rivers within the Białystok where eggs of *Carassius carassius* were the bait were done. Identification of isolates was accomplished on the basis of their vegetative, asexual reproduction, generative organs and by studying the sequencing of the internal transcribed spacer of nuclear ribosomal DNA (ITS1+5.8S+ITS2). *S. diclina* occurred in 29 (60.4%) [10(20.1%) in spring, 4 (8.3%) in summer, 11 (23.0%) in autumn and 4 (8.3%) in winter, 2005] of the 48 examined water samples. The results indicate the sequence comparisons of two ITS nuclear DNA for species identification: *S. diclina.* The results indicate that the sequence of our isolate correspond to *S. diclina*. It is very important that this study represents the first isolation (on the basis of molecular features) of *S. diclina* in fresh waters in Poland.

Key words: *Saprolegnia diclina,* detection, nuclear DNA, eggs, *Carassius carassius*, springs, Poland.

INTRODUCTION

Water moulds 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 mould acts as saprobionts and can assume pathogenic properties, being a potential source of infection (Dick, 2001; Czeczuga et al., 2004a, b; Kiziewicz and Kurzątkowska, 2004; Kirk et al., 2008). In springs of rivers, a lot of representatives of water moulds of the class Oomycetes from Saprolegniaceae family are present (Kiziewicz, 2012).

Identification of family Saprolegniaceae traditionally has relied on the observation of morphological features. Genera of the Saprolegniaceae have been differentiated by their method of zoospore release (Daugherty et al., 1998). Species identification has been more challenging because it requires the presence of the sexual structures, the oogonia and the antheridia. More recently, molecular identification has been accomplished with selected Saprolegniaceae by usage of the internal transcribed spacer (ITS) and 5.8S regions of ribosomal DNA (rDNA) (Leclerc et al., 2000). The most complete molecular phylogeny of this family to date identified 10 genera and 40 species through analyses of ITS and the large ribosomal subunit (LSU) (Leclerc et al., 2000; Petrisko et al., 2008).

There have been relatively few published field investigations of water moulds from family Saprolegniaceae diversity and ecology (Johnson et al., 2002).

The main task of the present study was to make the first complete characterization of a strain of *S. diclina* by using morphological and molecular features and assessing *S. diclina* growth on the crucian carp *Carassius carassius* as bait from four limnocrenic springs of rivers

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Table 1. Distribution and seasonal occurrence of aquatic fungus *Saprolegnia diclina* in 48 samples from four different sites of springs in Białystok (n =3).

Water reservoir		Seasonal occurrence/Number of water samples where fungus was found				
(Name of water reservoir –spring)	Number of collected water samples	Spring	Summer	Autumn	Winter	Together
Dojlidy Górne	12	2	1	3	1	7
Jaroszówka	12	3	1	2	1	7
Cypisek	12	3	1	3	1	8
Pietrasze	12	2	1	3	1	7
Total number of samples	48	10	4	11	4	29
Percentage	100	20.1	8.3	23.0	8.3	60.4

situated in Białystok.

MATERIALS AND METHODS

Study area

We conducted investigations about the occurrence of fungus *S. diclina* in water in several springs situated in Dojlidy Górne, Jaroszówka, Cypisek and Pietrasze at Białystok town during the spring, summer, autumn and winter of 2005.

Spring Dojlidy Górne (53°06'N, 23°12'E) and Spring Pietrasze (53°10'N, 23°9'E) in Biała River are located at the eastern and north part of Białystok. Spring Jaroszówka (53°10'N, 23°11'E) and spring Cypisek (53°10'N, 23°11'E) belong to Jaroszówka River and are located in the north part of Białystok.

These springs are characterized as Limnokrenic types, with an artificial basin: area from 0.380 to 0.290 km^2, width 0.65 m, depth 0.12 m, discharge from 2.4 to 2.5 dm^3 s^{-1}, and surroundings characterized by cultivated fields, herbaceous vegetation, trees and buildings.

Microbial analyses

Isolation of the fungus

Microbial analyses were made in the laboratory of the Department of General Biology, Medical University of Białystok, Poland and in the laboratory of the Real Jardin Botanico de Madrid, Spain from 2005-2006. For the microbial analysis of fungi, samples of water were collected from each site described above.

Samples were processed in the laboratory by routine methods commonly used to isolate these organisms. Bait method by using eggs of the crucian carp *C. carassius* and hemp seeds *Cannabis sativa* L. was applied to isolate *S. diclina* from the springs. Water samples (100 ml) from 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 (20-23°C) for 4-5 days (Seymour and Fuller, 1987). The colonized fragments of crucian carp eggs and hemp seeds, were transferred to new Petri dishes which contained sterilized, filtered spring or distilled water and crystalline penicillin C (100 mg L^{-1}) to inhibit bacterial growth. Dishes were microscopically examined weekly for up to three weeks in order to identify water moulds at the level of genus or species. The isolate was placed onto agar medium - PG1. To prevent bacterial growth, penicillin C was added to the agar to a final concentration of 100 mg L^{-1}. A piecem of infected bait was placed on the top of the agar and into a previously placed glass ring 3 cm diameter to protect the growing fungus-like organisms (FLO) from bacteria. The isolates were maintained on agar medium - PG1 and stored in the culture collection of the Real Jardín Botánico de Madrit, Spain. Morphological characters of asexual and sexual structures and measurements were made microscopically on material mounted in water. FLO were successively observed under an optic microscope Olympus BX 51 (100 and 400x magnification). All isolates were characterized and identified according to Unestam (1965), Batko (1975), Seymour and Fuller (1987) and Alexopoulos et al. (1996).

DNA extraction and PCR amplification

For DNA extraction, mycelium was grown as a drop cultures (Cerenius and Söderhall, 1985) and from them, genomic DNA was extracted using an E.Z.N.A.–Fungal DNA Miniprep Kit (Omega Biotek, Doraville, USA) as described by 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 by Martin 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 from the National Centre of Biotechnology Information (NCBI) nucleotide databases.

The new consensus sequence has been deposited in the EMBL data –base under accession number 1289 (*Saprolegnia diclina*).

RESULTS AND DISCUSSION

In the present study, *S. diclina* was isolated from water belonging to several springs in the area of Białystok, Poland and using crucian carp eggs and hemp seeds as bait. The study showed the occurrence of fungus-like organisms (FLO) *S. diclina* in samples of different water reservoirs. Identification of isolates was accomplished on the basis of their vegetative, asexual reproduction, generative organs and by studying the sequencing of the internal transcribed spacer of nuclear ribosomal DNA (ITS1+5.8S+ITS2).

As shown in Table 1, *S. diclina* occurred in all the four springs of examined in 29 (60.4%) of the 48 examined water samples [10 (20.1%) in spring, 4 (8.3%) in summer, 11 (23.0%) in autumn and 4 (8.3%) in winter, 2005]. The

isolate was characterized by studying the sequencing of the internal transcribed spacer of nuclear ribosomal DNA (ITS1+5.8S+ITS2). The results indicate the sequence comparisons of two ITS nuclear DNA for species identification: *S. diclina* 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. Results indicate that our isolate corresponded to the species *S. diclina* Humhrey ATCC 90215 (AY455775, version AY455775.1; GI: 38156477) Oomycetes or Oomycota, which are heterokont fungus-like organisms (FLO) (phylogenetically not true fungi) in the Kingdom Chromista/Stramenopila (Alexopoulos et al., 1996; Dick, 2001; Kirk et al., 2008).

Species of the genus *Saprolegnia* are generally considered saprobes that live on decayed plant or animal debris. *Saprolegnia* is often found in polluted and marshy waters. However, this genus also includes parasites of freshwater animals and their eggs, and some of these are responsible for economically important diseases by affecting farmed and wildlife populations of aquatic animals (Söderhäll et al., 1991; Kiesecker et al., 2001; van West, 2006; Fernández-Benèitez et al., 2008). Some of species from Saprolegniaceae such as *Saprolegnia* sp. and several different genera are parasites of arthropods, crustaceans, mosquito larvae, eggs of freshwater fish and eggs, and tadpoles of amphibians at their embryonic or larval stages and also they were described as parasites of reptiles (Seymour, 1984; Westman, 1991; Vennerstrom et al., 1998; Kitancharoen et al., 1995; Hulvey et al., 2007; Fernández-Benéitez et al., 2008; Eli et al., 2011).

Mycotic infections associated with family Saprolegniaceae are widely reported in freshwater fish; however are rarely found in brackish water (Czeczuga and Woronowicz, 1993; Hussein and Hatai, 2002).The disease saprolegniasis is caused by freshwater fungi usually from the genus *Saprolegnia* which is generally considered as opportunistic pathogens for fish and their eggs (Bruno and Wood, 1999; Bangyeekhun et al., 2003; Kiziewicz et al., 2011). Saprolegniasis is a continuing problem for aquatic animal populations. *Saprolegnia ferax, S. diclina* and *S. parasitica* are known to be pathogens to cold water fishes. Any species of fish that are intensively cultured and captured are at risk of suffering fungal diseases (Czeczuga, 1994; Blazer et al., 2002). Among them, *Saprolegnia ferax* and *S. parasitica* are the most dangerous, causing the death of whole populations of many fish species in certain water basing as is the case of breeding populations of Pacific salmon (Czeczuga and Muszyńska, 1996) and the Atlantic salmon *Salmo salar* in Great Britain and Poland (Stuart and Fuller, 1968; Czeczuga et al., 2011). Some studies revealed mortality around 70-90% of incubated spawns of acipenserids (Dudka et al., 1989). The most common species involved in disease outbreaks of saprolegniales is *S. diclina*. It can occur in salmonids (Kitancharoen and Hatai, 1998; Fregeneda-Grandes et al., 2007). In the present study, we were successful in isolating *S. diclina* from water of springs - located in Białystok, Poland using eggs of crucian carp as bait. Chukanhom and Hatai (2004) identified fungal infections of common carp (*Cyprinus carpio*) eggs in Thailand. The fungus-like organisms from this genus are regarded primarily as a saprothroph - an organism that lives on dead organic matter, but during infection; it proved to be the most efficient at infecting fish eggs. They grow and penetrate into the cell wall reducing water flow and enzyme secretion which lead to death of eggs (Fadaeifard et al., 2011). Other species of *Saprolegnia* have been observed growing on the spawn of amphibian and reptilian species as well (Czeczuga et al., 1998; Fernández-Benéitez et al., 2008; Petrisko et al., 2008). In contrast to some general assumptions of the opportunistic nature of the pathogenicity of *S. diclina*, recent studies appear to indicate that *S. diclina* is adapted to colonize as a saprothroph egg of salmonids. *S. diclina* is also frequently observed and isolated from different species of adult fish and their eggs (Czeczuga and Muszyńska, 2000; Fregeneda-Grandes et al., 2007). The fungus also observed growing on eggs of the sea trout *Salmo trutta* m. trutta in River Biała, Krasna and Supraśl near Białystok in Poland. 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 in fresh water in hatcheries at Miastko town (farmed form) (Czeczuga et al., 2005). The ubiquitous presence of one or more species of *Saprolegnia* indicates that these fungi might have a role in the biological recycling at farm fisheries. According to Willoughby (1994) FLO which belongs to genus *Saprolegnia* can be one of the fungal causes in freshwater fishes and their eggs. Our results showing the occurrence of *S. diclina* on eggs of *C. carassius* confirm this. All morphological, physiological and genetic features studied were identical, and then the isolates were considered to be from those of the same strain. This study represents the first isolation of *S. diclina* in fresh waters in Poland and also this study is the first one that describes *S. diclina* by using molecular features (ITS rDNA) in Poland.

Conclusion

In the present study, we successfully isolated fungus-like organisms (FLO) of *S. diclina* from spring water of Białystok and determined it by using eggs of crucian carp as bait. The results show that the sequence of our isolate correspond to the species *S. diclina* using the sequence comparisons of two ITS nuclear DNA for species identification.

Thus, this study represents the first isolation (based on molecular analysis) of *S. diclina* in Poland fresh waters.

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