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Molecular identification of a bronopol tolerant strain of *Saprolegnia australis* causing egg and fry mortality in farmed brown trout, *Salmo trutta*

*Svetlana REZINCIUC, Jose-Vladimir SANDOVAL-SIERRA, Javier DIÉGUEZ-URIBEONDO**

Department of Mycology, Real Jardín Botánico CSIC, Plaza Murillo 2, 28014 Madrid, Spain

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ABSTRACT

Some species of the genus *Saprolegnia*, such as *Saprolegnia diclina* and *Saprolegnia ferax* are responsible for devastating infections on salmonid eggs. Members of this group cause saprolegniasis, a disease resulting in considerable economic losses in aquaculture. Although both *S. diclina* and *S. ferax* have received much attention, the role of other *Saprolegnia* species in infecting fish eggs is less known. For this purpose, we have investigated the aetiology of chronic egg mortality events occurring in farmed brown trout, *Salmo trutta*. A total of 48 isolates were obtained from eggs with signs of infection as well as from water samples. A molecular analysis based on nrDNA internal transcribed spacer (ITS) operational taxonomic units indicated that the majority of the isolates correspond to *Saprolegnia australis*. All isolates of *S. australis* exhibited the same random amplified polymorphic DNA (RAPD) band patterns suggesting that a single strain is implicated in egg infections. The isolates followed Koch postulates using trout eggs and fry. Under standard concentrations of bronopol commonly used in farms, these isolates could grow, but not sporulate. However, both growth and sporulation were recovered when treatment was removed. This study shows that *S. australis* can infect and kill salmon eggs, and helps in defining oomycetes core pathogens.

Introduction

Oomycetes are responsible for devastating diseases on plants and animals (Diéguez-Uribeondo *et al.* 1996; Kamoun 2001; van West 2006; Margulis & Chapman 2009). Within the Oomycetes, the genus *Saprolegnia* contains some of the most important pathogens causing environmental damage and economically important loses in aquaculture (van West 2006; Walker & van West 2007). In aquaculture, infections of fish eggs are a major economic problem in many freshwater species (Neish & Hughes 1980; Willoughby 1994; Robertson *et al.* 2008; van den Berg *et al.* 2013). Over the last decades, an increasing prevalence and severity of saprolegniosis in fish and their eggs has been reported in the aquaculture industry (Bruno & Wood 1999; van West 2006; Phillips *et al.* 2008; Robertson *et al.* 2008). This disease was efficiently controlled by treating infections with malachite green (Alderman 2006). However, malachite green was banned in 2002 due to health concerns of its carcinogenic effects (van West 2006). Two commercial products that contain either formalin or bronopol are currently used for *Saprolegnia* control (Braidwood 2000), but none of them reaches the effectiveness of malachite green.

* *Corresponding author*. Departamento de Micología, Real Jardín Botánico CSIC, Plaza Murillo 2, 28014 Madrid, Spain. Tel.: +34 91 420 30 17; fax: +34 91 420 01 57.
E-mail address: dieguez@rjb.csic.es (J. Diéguez-Uribeondo).

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Moreover, the use of formalin is now under strict control in EU and according to the commission regulation (EC, No710/2009) may soon be banned. This has led to a continuous search of new treatments of this disease and several new alternatives are being studied.

Designing novel treatments often requires knowledge about the pathogen aetiology and its tolerance to treatment components (Srivastava 1987; Tamperi 1998; Zaror et al. 2004). In spite of the significant efforts expended to prevent egg losses due to *Saprolegnia*, few studies have been focused on identifying the species responsible for *Saprolegnia*-egg infections in fish aquaculture. To our knowledge, very few studies have been focused on identifying to species level the *Saprolegnia* isolates involved in the massive egg die-offs. *Saprolegnia* species are the main oomycete associated with mass mortalities of salmonid eggs (Willoughby 1994; Robertson et al. 2008; van den Berg et al. 2013). Some *Saprolegnia* spp. are consistently isolated from freshwater fish eggs (Pickering & Willoughby 1982; Roberts 1989; Rand & Munden 1993; Robertson et al. 2008; van den Berg et al. 2013), *Saprolegnia diclina* represents the most frequently isolated species (Rand & Munden 1993; Fregeneda-Grandes et al. 2007; Robertson et al. 2008; van den Berg et al. 2013). In addition, *Saprolegnia ferax* and *S. diclina* have also been found to be responsible for '*Saprolegnia* infections' in amphibian eggs, which is an emergent disease associated with climate change (Blaustein et al. 1994; Kiesecker et al. 2001; Fernández-Benéitez et al. 2008).

The characterization of *Saprolegnia* using traditional taxonomic criteria on parasitic isolates has proven problematic (Johnson et al. 2002; Diéguez-Uribeondo et al. 2009; Sandoval-Sierra et al. 2013). In *Saprolegniales*, morphological, physiological, and pathological characters seem to provide little information for species determination (Diéguez-Uribeondo et al. 1995; Sandoval-Sierra et al. 2013). Physiological characters, however, are sometimes used to differentiate individual degrees of pathogenicity and host specificity (Neish 1976; Willoughby 1978; Wilioughby & Copland 1984; Holub et al. 1991; Diéguez-Uribeondo et al. 1995, 1996). Identification and subspecific differentiation at population or clone level generally are carried out using physiological and molecular markers (Medlin et al. 2006; Alpermann 2009). DNA fingerprinting techniques appear to be useful to distinguish different clonal lineages within populations (Williams et al. 1990; Demeke et al. 1992). Random amplified polymorphic DNA (RAPD) has been used in the oomycetes as well to distinguish different strains, species, and clones. In the case of the crayfish pathogen *Aphanomyces astaci*, a closely related species, genetic diversity among several isolates was studied by using this technique (Huang et al. 1994). The RAPD–polymerase chain reaction (PCR) has also been applied successfully in some other species of oomycetes, *i.e. Phytophthora* spp. (Ersek et al. 1994) and *Saprolegnia* spp. (Diéguez-Uribeondo et al. 1996). Random DNA markers have a wide application in genetic diversity studies. However, they are rarely used for species identification. The problem when applying random markers on population studies is that it is needed to know that all samples belong to the same species. In *Saprolegnia*, a recent molecular taxonomic study has validated a number of taxa, and now allows the identification of isolates to species level using molecular techniques (Sandoval-Sierra et al. 2013).

Therefore, the aim of this study was to identify the aetiology of egg die-offs in a salmonid farm, and to determine whether this is caused by a particular strain or clone of *Saprolegnia* with specific properties. For this purpose, we have applied a combination of recently developed method, molecular taxonomy, and traditional PCR–RAPD.

Materials and methods

Collection of samples and isolations

Samples were taken during 2012 and 2013 from a fish farm located in Anotz, Navarra, Spain (lat. 42.86°, long. −1.82°, 561 m) that were having problems with *Saprolegnia* infections on salmonid eggs. Water temperature of this farm ranged from 8 °C to 10 °C. In 2012, *Saprolegnia* samples were collected from this farm from two sources, one set of samples consisted of trout eggs with visible signs of *Saprolegnia* infection. From each hatching case of eggs, up to five infected eggs were randomly selected and placed into 50 ml empty falcon tubes. The second set of samples was in the form of water samples collected into 50 ml falcon tubes that contained hemp seeds as bait for the *Saprolegnia* to grow on. The hemp seeds were incubated at 15 °C for 3 d in the water. In 2013, infected trout eggs were collected as described above. However, water samples were not collected.

Isolations were made by depositing a piece of infected egg or hemp seed on top of a peptone–glucose–agar plate (PGA) (Söderhäll et al. 1978). The *Saprolegnia* from the infected eggs grew within a glass ring previously placed on the medium following the method of Cerenius et al. (1987) to protect the growing isolates from bacteria the media was supplemented with 100 mg l^{-1} ampicillin (Sigma, Aldrich). Plates were incubated at 25 °C for 2 d after which isolates were transferred into new PGA media. For each isolate, a single spore culture was obtained as described in Cerenius et al. (1988) and stored at 4 °C in the culture collection of the Real Jardín Botánico (Madrid, Spain).

DNA extraction and PCR conditions

Total genomic DNA was extracted from mycelia that were grown in drop cultures on PGA for 2–3 d at 20 °C as described in Diéguez-Uribeondo et al. (2007). DNA extractions were carried out using DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA). The internal transcribed spacer region (ITS) was amplified using universal primers for eukaryotes ITS5 and

Table 1 – Nucleotide sequences of random primers used in PCR amplification.

Primer	Sequence	Primer	Sequence
A10	5′-GTGATCGCAG-3′	B01	5′-GTTTCGCTCC-3′
A12	5′-TCGGCGATAG-3′	B04	5′-GGACTGGAGT-3′
A16	5′-AGCCAGCGAA-3′	B05	5′-TGCGCCCTTC-3′
A20	5′-GTTGCGATCC-3′	B06	5′-TGCTCTGCCC-3′
OPT20	5′-GACCAATGCC-3′	B09	5′-TGGGGGACTC-3′
OPU13	5′-GGCTGGTTCC-3′	B19	5′-ACCCCCGAAG-3′

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