RESEARCH LETTER

Saprolegnia diclina: another species responsible for the emergent disease '*Saprolegnia* infections' in amphibians

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Abstract

Many amphibians are known to suffer embryonic die-offs as a consequence of an emergent disease known as '*Saprolegnia* infections'. Thus far, the only species of *Saprolegnia* shown to be involved in natural infections is *Saprolegnia ferax*. In this study, we have isolated and characterized another *Saprolegnia* species responsible for '*Saprolegnia* infections' on embryos of *Bufo calamita* in mountainous areas of central Spain. The strain was identified as belonging to *Saprolegnia diclina* based on morphological, physiological and molecular characters (sequencing of the internal transcribed region of ribosomal DNA). Zoospores of the new strain were able to infect embryos of *Bufo calamita*, and the symptoms observed were the same as those observed in natural infections. The results presented emphasize the need to carry out isolations and characterizations of the species and/or strains involved in this emergent disease. This will be important in order to design strategies to prevent the impact and spread of species (or strains) pathogenic to amphibians.

Introduction

In recent years, a decline in amphibian populations has been noted worldwide (Blaustein & Wake, 1990; Wake, 1991; Houlahan *et al*., 2000). Around one-third of the amphibian species known are currently classified as threatened according to the Global Amphibian Assessment (2006). Several factors appear to be involved in this abrupt decline and their importance is now the subject of extensive studies. Both abiotic factors, e.g. habitat loss or fragmentation, water pollution and increased UV-B radiation (Beebee & Griffiths, 2005), and biotic factors, e.g. introduction of exotic species (Kats & Ferrer, 2003) and the proliferation of emergent amphibian diseases (Daszak *et al*., 2003), seem to be involved in this decline. For example, the fungal disease chytridiomycosis, caused by *Batrachochytrium dendrobatidis*, is affecting more than 100 species of amphibian world wide (Rachowicz *et al*., 2005). It was first noted in pristine areas of Australia and Costa Rica (Daszak, 1998), and further investigations have associated the occurrence of this fungus with mass mortalities in several regions around the world (Ouellet *et al*., 2005; Rachowicz *et al*., 2005). Another commonly cited disease related to amphibian mortality is the so-called '*Saprolegnia* infections' affecting aquatic stages of several species (Blaustein *et al*., 1994; Kiesecker & Blaustein, 1997). This disease has been associated with the extinction of populations of *Rana pipiens* and *Bufo terrestris* (Bragg, 1958, 1962). Increased mortality in the salamander *Ambystoma maculatum* (Walls & Jaeger, 1987), and massive deaths of *Bufo calamita* and *Rana temporaria* eggs have also been related to this type of infection (Banks & Beebee, 1988; Beattie *et al*., 1991). The lethal effects of '*Saprolegnia* infections' have been shown on embryos of *Bufo boreas* and *Rana cascadae* (Kiesecker & Blaustein, 1995).

The genus *Saprolegnia* belongs to the *Oomycetes*, which are heterokont organisms (phylogenetically not true fungi). Some *Oomycetes* have evolved to a parasitic lifestyle by developing adaptations in their zoosporic phase (Cerenius & Söderhäll, 1985; Judelson & Blanco, 2005). Parasitic species are some of the most devastating organisms known on both farmed and wildlife populations of plants and aquatic animals (The World Conservation Union, 2007). In spite of the knowledge and experience gained from extensive research on *Oomycetes*, few studies have been carried out on

the so-called '*Saprolegnia* infections' of amphibians. Species of the genus *Saprolegnia* are generally considered to be saprobes that live on decayed plant or animal debris. However, this genus also includes parasites on freshwater animals and their eggs, and some of these are responsible for economically important diseases affecting farmed and wild-life populations of aquatic animals (Willoughby, 1978; Söderhäll *et al.*, 1991; Kiesecker *et al.*, 2001; van West, 2006.). In amphibians, the term '*Saprolegnia* infections' is usually used to refer to eggs harbouring colonies that resulted in a cotton-like appearance. However, in most cases, no species or even a genus has been characterized or identified as the causative organism. Although several species of *Saprolegnia* might be involved in embryonic die-offs, thus far, only *Saprolegnia ferax* has been identified as being associated with embryonic mortality of amphibian populations in the field (Blaustein *et al.*, 1994; Kiesecker *et al.*, 2001).

The identification of *Saprolegnia* species using traditional taxonomic criteria and keys on parasitic isolates has, at best, proven problematic. A recent study based on morphological, physiological and molecular data of *Saprolegnia* isolates has shown a number of species misassignments evident for *Saprolegnia diclina*, *Saprolegnia australis*, *Saprolegnia litoralis* or *S. ferax* due to similarity of oogonial morphology (Diéguez-Uribeondo *et al.*, 2007). The aim of the present study was to make the first complete characterization of a strain of *Saprolegnia* involved in '*Saprolegnia* infections' by using morphological, physiological and molecular features.

Materials and methods

Isolation of the fungus

Eggs of *Bufo calamita* showing symptoms of '*Saprolegnia* infections' were collected from several mountain ponds in Sierra de Gredos (Ávila, Spain) where a high infection rate has been observed. Isolations were carried out from colonized pieces of infected eggs. These were washed in distilled water with 100 mg L^{-1} penicillin C and placed onto potato dextrose agar (PDA). To prevent bacterial growth, penicillin C was added to the agar to a final concentration of 100 mg L^{-1}. A piece of infected egg was placed on top of the agar and into a previously placed glass ring 3 cm in diameter to protect the growing fungus from bacteria. The isolates were maintained on PDA and stored under the strain name SAP007 in the culture collection of the Real Jardín Botánico (Madrid, Spain).

Strain characterization

Production of sexual structures was studied by growing the isolate on hemp seeds and corn meal agar for 10 days at 20 °C. The cultures were observed under an inverted microscope to check the production of sexual structures. Sexual structures and coat ornamentation of the secondary cysts, and germination were examined under an Olympus BX51 compound microscope (Olympus Optical, Tokyo, Japan). Light micrographs were captured using a Qimaging Micropublisher digital camera (Qimaging, Burnaby, BC, Canada) as described in Diéguez-Uribeondo *et al.* (2003). To test the strain growth rates, the isolates were cultured in PDA Petri dishes and maintained at 15 and 25 °C. The diameter of the colonies was measured every 12 h.

Secondary cysts were obtained as described in Diéguez-Uribeondo *et al.* (1994) and were observed using phase contrast microscopy at 400 × and 1000 × as described in Diéguez-Uribeondo *et al.* (2007). Images of cyst coat ornamentation were improved by using the software Syncroscopy-Automontage (Microbiology International Inc., Frederick, MD, USA) as described in Diéguez-Uribeondo *et al.* (2003). The percentage of cysts undergoing retracted germination was measured after incubation of a cyst suspension in 25% glucose yeast broth at 20 °C for 3 h as described in Yuasa *et al.* (1997). Three separate replicates were made for each isolate and at least 50 cysts were observed on each ocassion.

Zoospore production was performed as described by Diéguez-Uribeondo *et al.* (1994). The zoospores were encysted by agitation with a vortex mixer. The cysts were incubated at 13 °C for 10 h, and the number of swimming zoospores, undeveloped cysts and germinated cysts were counted. The swimming zoospores were collected and the production of a new generation was studied as above. This procedure was repeated three times.

DNA extraction, PCR amplification and sequencing

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) 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 EMBL database under accession number 281399.

Virulence assay

Freshly oviposited *Bufo calamita* eggs (<24 h, stage <10 according to Gosner, 1960) from four different clutches with no signs of *Saprolegnia* infection were also collected in Sierra

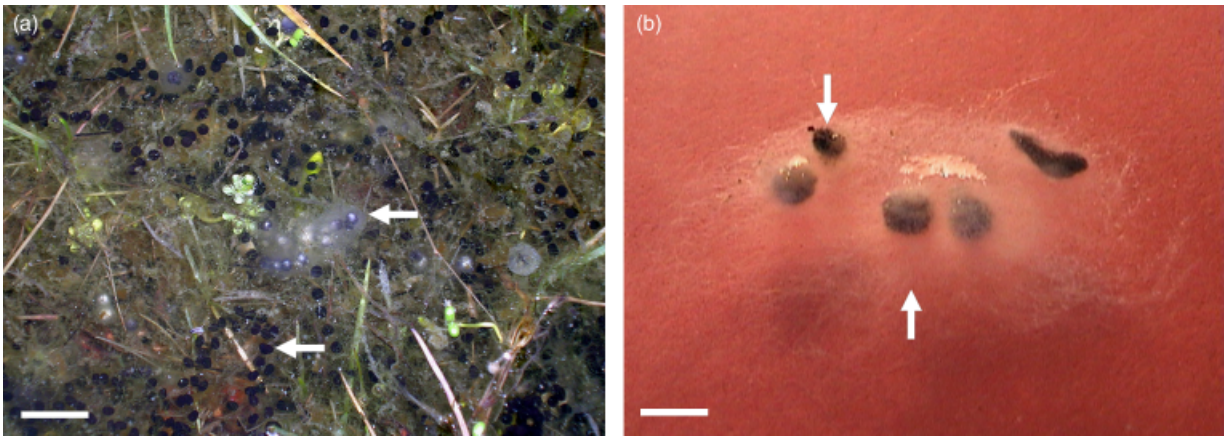

Fig. 1. (a, b) *Saprolegnia* infections in *Bufo calamita* embryos. (a) Strings of eggs, of *B. calamita* naturally infected with *Saprolegnia diclina* sp.; (b) infected embryos, after challenging them with zoospores of *S. diclina*. The pathogen grows as cotton-like masses, c. Scale bars: (a) 15 mm; (b) 3 mm.

de Gredos, in a location close to where the *Saprolegnia* strain was obtained. Zoospore production was performed as described by Diéguez-Uribeondo *et al.* (1994) and zoospore yield was estimated by using a Neubauer counting chamber.

The virulence experiment was carried out in 100-mL plastic containers containing 80 mL mineral water and 20 eggs (5 eggs $\times$ 4 clutches). Each container was randomly assigned to one of the three treatments: control (no zoospores added), low zoospore concentration (10^3 zoospores mL^{-1}), and high zoospore concentration (10^4 zoospores mL^{-1}). Each treatment was replicated six times.

Containers were kept in an aquarium containing 30 L water maintained at 14 °C with a Selecta 285 W refrigerator (J.P. Selecta SA, Abrera, Spain). Temperature inside the containers was checked periodically and did not vary more than 1 °C from the temperature in the aquarium. The experiment was finished after 11 days of exposure, when hatchlings were able to swim. We checked containers daily and recorded the number of dead and infected embryos to calculate mortality rates.

Statistical analysis

To analyse the effect of zoospore concentration on embryonic survival, we used a repeated-measures ANOVA with the increase in mortality rate (arcsin-square root transformed) over time as the dependent variable. To determine the incubation time (days after exposure) required for symptoms of '*Saprolegnia* infections' to develop, we used one-way ANOVAs with daily mortality rates as dependent variables. To estimate mean time of survival in each of the treatments, we used a survival analysis. Differences in time of survival caused by zoospore concentrations were checked with a log rank comparison.

The zoospore concentration that caused embryonic mortality was determined via a *posthoc* test. SPSS 11.5 for Windows® (SPSS Inc., Chicago, IL) was used for statistical analyses.

Results

Strain characterization

Eggs collected from all the ponds showed characteristic '*Saprolegnia* infections' (Fig. 1a and b). The isolated strains exhibited typical morphology of *Saprolegnia* (Fig. 2a). Secondary cysts of the isolates did not show the presence of bundles of long hairs that is characteristic of *Saprolegnia parasitica* (Fig. 2b). All isolates cultured in hemp seeds and corn meal agar produced sexual structures with similar morphological characters (Fig. 2c–f). These features are characteristic of *S. diclina*, e.g. diclinous antheridial branches (Fig. 2c and d) and spherical unpitted oogonia (Fig. 2e) with centric oospores (Fig. 2f). Radial growth rates of isolates were similar, and the elongation rate was 26 mm day^{-1} with an optimum temperature at 25 °C. All five isolates produced secondary cysts that were able to produce at least three successive generations of zoospores. Retracted germination occurred in less than 3% of germinating cysts.

The ITS sequences of the isolates were identical. A BLAST search of the sequence of the isolates showed 100% similarity to Genbank sequence AM228848 corresponding to isolate SAP243 of *S. diclina* (Diéguez-Uribeondo *et al.*, 2007) and 99% similarity (the sequence differed in one base for ITS1 and four bases in ITS2 out of the 673 bases compared) to Genbank sequence AM228844 corresponding to isolate FBA810/APCC no.203a (ATCC56851) of *S. diclina*

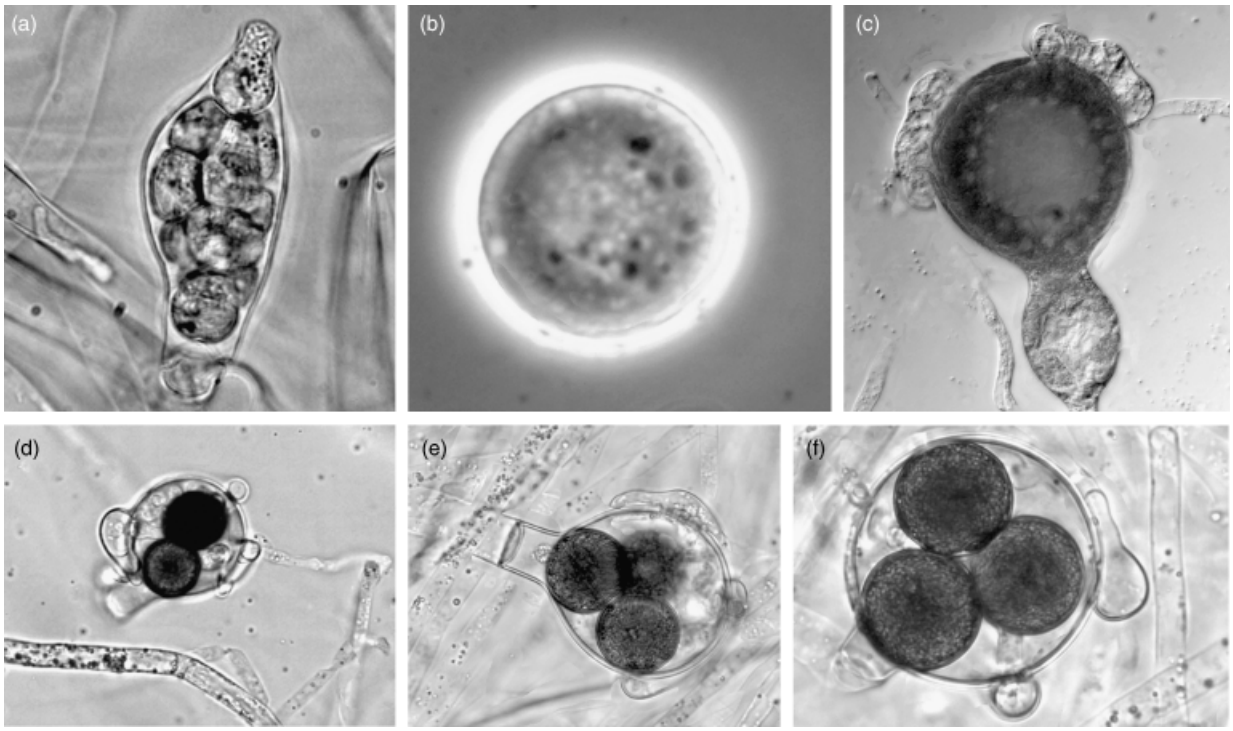

Fig. 2. (a–f) Characteristic spore morphology of *Saprolegnia diclina*. (a) Light micrograph of an asexual sporangium; (b) phase contrast light micrograph of a secondary cyst with no visible hairs on its surface; (c) oogonium, being fecundated by the antheridia; (d) fecundation by tubular diclinous antheridium (antheridium and oogonium in different hyphae); (e) unpitted wall of the oogonium; (f) spherical centric oospores.

(Willoughby, 1978). As all morphological, physiological and genetic features studied were identical, the isolates were considered to be isolates of the same strain.

Virulence assay

For virulence experiments we used only one of the isolates. Mortality in controls was less than 6%, no '*Saprolegnia* infections' were observed and no isolates were obtained. Repeated-measures ANOVA showed that mortality of embryos exposed to *S. diclina* zoospores significantly increased over time ($F_{2,15} = 8.093$, $P = 0.004$). Cumulative survival over time in each treatment is shown in Fig. 3. Significant differences among treatments were found for this variable (Log Rank = 150.43, 2 *df*, $P < 0.001$).

Daily ANOVAs revealed that significant mortality occurred after 4 days of exposure ($F_{2,15} = 6.652$, $P = 0.009$), and *posthoc* tests showed that both zoospore concentrations tested were lethal at this period, with mortality rates of 16% at low zoospore concentration and 36% at high zoospore concentration. At the end of the experiment, 47% of embryos exposed to the low zoospore concentration and 84% of those exposed to the high zoospore concentration died (Fig. 4; $F_{2,15} = 8.640$, $P = 0.003$). All dead embryos exposed to zoospore treatments exhibited '*Saprolegnia* infections' (Fig. 1b). All the isolates obtained from the colonizing organism were morphologically and genetically identical to the strain used in the experiment.

Discussion

Emergent diseases have acquired great importance for amphibian conservation due to widespread declines and extinctions in this group of animals. '*Saprolegnia* infections' have been shown adversely to affect several species of amphibians at their embryonic or larval stages. However, with the exception of *S. ferax* (Blaustein *et al*., 1994; Kiesecker *et al*., 2001), no specific identification of the pathogens producing these infections has been made. In this study, we demonstrate that *S. diclina* is also responsible for amphibian embryonic mortalities in the environment; the strain producing this mortality has been characterized using a combination of morphological, physiological and molecular features. The results indicate that this strain is pathogenic to *Bufo calamita* embryos as it meets Koch postulates: (1) it was constantly associated with the disease; (2) it was isolated from infected embryos and grown in pure culture; (3) the original disease and symptoms occurred in healthy embryos when they were inoculated with the pathogen from pure culture; and (4) the same strain was reisolated from

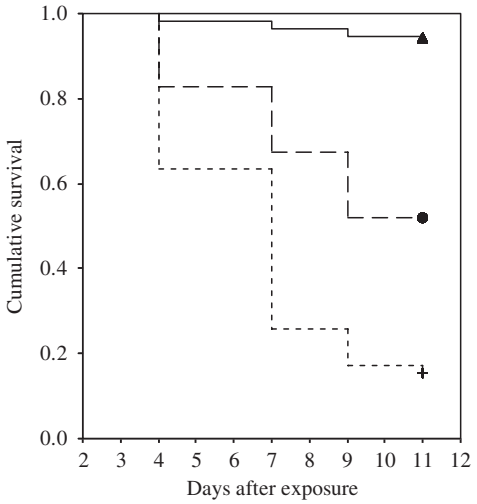

Fig. 3. Results of the survival analysis to test the cumulative survival (percentage of individuals that remained alive at each particular time after challenging with spores) over time in each of the three zoospore treatments. Continuous line represents control embryos, dashed line represents embryos exposed to 10^3 zoospores mL^{-1}, and dotted line represents embryos exposed to 10^4 zoospores mL^{-1}. Triangles, circles and crosses represent combined data for each treatment.

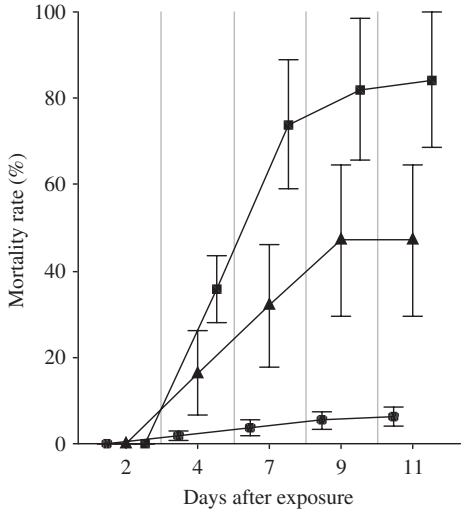

Fig. 4. Mean (± SE) mortality rates of *Bufo calamita* embryos exposed to three zoospore concentrations of pathogenic *Saprolegnia diclina*. Black circles represent control animals; black triangles represent animals exposed to 10^3 zoospores mL^{-1}; black squares represent animals exposed to 10^4 zoospores mL^{-1}.

challenged eggs under experimental conditions. This strain is currently affecting spawns of *Bufo calamita* and threatening their populations in mountainous areas of central Spain (M. J. Fernández-Benéitez, personal observations).

To our knowledge, this is the first time that the lethal effect of '*Saprolegnia* infections' on amphibians has been reported and demonstrated in Spain. *Saprolegnia diclina* has never previously been reported anywhere as naturally occurring in amphibian egg die-offs. Robinson *et al.* (2003) have previously shown that a strain belonging to the *S. diclina–parasitica* complex and that an asexual isolate of *Saprolegnia* (obtained from waters in which hyphal infection of amphibian eggs had been observed in the previous breeding season) were lethal to common frog (*Rana temporaria*) and common toad (*Bufo bufo*) eggs under laboratory conditions. Other species of *Saprolegnia*, and also *Achlya* (*Oomycetes*), have been observed growing on the spawn of nine amphibian species (Czeczuga *et al.*, 1998). However, the relationship of this species to embryonic die-offs is doubtful given that the authors did not study their pathogenicity. The species observed might reflect either that there is a different prevalence of *Saprolegnia* species in distinct freshwater ecosystems, or that eggs studied were colonized by opportunistic *Saprolegnia* rather than by primary invaders.

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 colonized eggs of salmonids (Fregeneda-Grandes *et al.*, 2007). *Saprolegnia diclina* is also known to be frequently observed and isolated from fish eggs (Fregeneda-Grandes *et al.*, 2007). Interestingly, the strain isolated in this study caused high embryonic mortality even at the lowest zoospore concentration, suggesting an adaptation to infect amphibian eggs as a substrate. The virulence of the strain can be related to ecophysiological properties such as the ability to undergo repeated zoospore emergence, although this remains speculative. This physiological pathway has been proposed as a possible adaptation to parasitism in the closely related genus *Aphanomyces* (Cerenius & Söderhäll, 1985). Species exhibiting this physiological property can form several generations of their infective unit, e.g. zoospore, instead of germinating after being triggered by unspecific stimuli. In *S. diclina* and also the fish pathogenic *S. parasitica*, some strains exhibit this physiological pathway whereas in others only a small percentage of the cysts can undergo repeated zoospore emergence (Bangyeekhun *et al.*, 2003; Diéguez-Uribeondo *et al.*, 2007). This ability may increase the likelihood that the pathogenic strain will find an appropriate substratum to germinate, e.g. amphibian eggs, thus ensuring their survival, as proposed for *Saprolegnia* strains isolated from salmonids in Finland (Bangyeekhun *et al.*, 2003). The presence of this character should be investigated in other strains involved in embryonic die-offs of amphibians to determine whether it may constitute a virulence factor, and whether or not other highly pathogenic strains or species of *Saprolegnia* may be present in the environment.

Future studies need to be done regarding identification and characterization of species of *Saprolegnia* causing amphibian embryo die-offs and to differentiate opportunistic *Saprolegnia* growing in dead eggs from species or strains of

Saprolegnia able to grow as primary invaders in amphibian eggs. This is a crucial aspect for designing strategies in order to prevent the impact and spread of species (or strains) pathogenic to amphibians. These strategies might include, for example, the management of species that can act as vectors for pathogenic *Saprolegnia* species, such as in fish and crustacean restocking practices (Wood & Willoughby, 1986; Söderhäll *et al*., 1991; Kiesecker *et al*., 2001).

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