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Re-evaluation of the enigmatic species complex *Saprolegnia diclina–Saprolegnia parasitica* based on morphological, physiological and molecular data

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Abstract

The phylogenetic relationships among isolates of the *Saprolegnia diclina–Saprolegnia parasitica* complex were investigated based on ITS rDNA sequences, and correlated with morphological and physiological characters. The isolates studied belong to five phylogenetically separate clades. The majority of presumed parasitic isolates, mostly isolated from fish lesions, fell within a clade that comprises isolates which has been variously named as *S. diclina* Type 1, S. parasitica, Saprolegnia salmonis or just as unnamed *Saprolegnia* sp. Presence of bundles of long-hooked hairs on secondary cysts, high frequency of retracted germination, and oogonia production at 7 °C (when occurring) were characteristic of this clade. A single isolate identified as *S. diclina* Type 2 clustered in a clade along with *Saprolegnia ferax* isolates. The isolates identified as S. diclina s. str. (*S. diclina* Type 3) distributed in two clades and appeared closely related to *Saprolegnia multispora* and to a number of Chilean isolates identified as *Saprolegnia australis.* The ITS sequences of clade I were almost identical even though the isolates were of diverse geographical origins and showed physiological and morphological differences and variations in their pathogenicity. This suggest these species reproduces clonally even in apparently sexually competent isolates. Adaptation to parasitism in Saprolegnia might have occurred at spore level by the development of long-hooked hairs to facilitate host attachment and selection of a retracting germination. The use of the name *S. parasitica* should be assigned to isolates of clade I that contained isolates forming cysts with bundles of long-hooked hairs.

Keywords: *Saprolegnia*; Oomycetes; Zoospore; Phylogeny; ITS rDNA; Freshwater; Salmonids; Pathogenicity; Fish pathology

1. Introduction

The Saprolegniaceae Kütz. ex Warm. are zoosporic water moulds belonging to the Oomycetes (Coker, 1923; Seymour, 1970). They include a number of economically important parasites on plant roots (Papavizas and Ayers, 1974), farmed freshwater animals and their eggs (Willoughby, 1978; Hatai et al., 1990; Fregeneda-Grandes et al., 2007) as well as wild populations of fish, crustaceans, and amphibians (Aller-Gancedo and Fernández-Díez, 1986; Cerenius and Söderhäll, 1992; Kiesecker et al., 2001; respectively). Species delineation in the genus *Saprolegnia* is largely based on the morphological traits of their sexual structures—oogonia, oospores, and antheridia (Coker, 1923; Seymour, 1970; Johnson et al., 2002). However, many species exhibit very similar or overlapping characters and isolates of *Saprolegnia* from animals often fail to produce sexual structures at all in vitro (see Table 1).

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Indeed the taxon, *Saprolegnia parasitica* was originally erected by Coker (1923) to accommodate all such asexual isolates of *Saprolegnia* that were parasitizing fish. The history and validity of this taxon has already been documented at length (Neish, 1976; Hughes, 1994) and the general consensus based on oogonium-based morphological criteria was that the taxon *S. parasitica* should be reduced to synonymy with the common saprobiotic species, *Saprolegnia diclina* (Seymour, 1970; Neish, 1976; Willoughby, 1978; Johnson et al., 2002).

Neish (1976) developed the concept of the *S. diclina–S. parasitica* species complex to encompass a number of genera with oogonia that have predominantly diclinous antheridial branches and thin-walled, unpitted or inconspicuously pitted oogonial walls (Fig. 4). This complex included *Saprolegnia australis* Elliott, *S. diclina* Humphrey, *S. parasitica* Coker, and *Saprolegnia shikotsuensis* Hatai, Egusa and Awakura (Hughes, 1994). Recently, several additional taxa fitting within this broad species concept have been described, including *Saprolegnia salmonis* (Hussein and Hatai, 1999) and *Saprolegnia multispora* (Paul and Steciow, 2004). Exactly which genera should be included or excluded from this complex has also been contentious (Johnson et al., 2002). The naming of fish-pathogenic *Saprolegnia* isolates was further complicated as a result of a detailed analysis of isolates taken from diseased fish (largely salmonids) and the background environment by Willoughby (1978). Based on oogonial morphology and the temperature required for oogonium formation, this author introduced the concept of three different subgroups of *S. diclina* which were designated Type 1, 2 and 3. *S. diclina* Type 1 isolates were all derived from lesions on salmonid fish and were broadly equivalent to *S. parasitica* sensu Coker (1923) and *S. diclina* Type 3 represented typical saprobiotic *S. diclina s. str.* isolates. Willoughby (1978) also recognized a third group, so-called *S. diclina* Type 2, which this author applied to a small group of isolates taken from coarse fish such as perch. As a result different authors have adopted different approaches to the taxonomic notation they attached to *Saprolegnia* isolates taken from fish (as illustrated in Table 1) which has led to an extremely confusing literature. Using traditional taxonomic criteria and keys, the species level identification of parasitic isolates of *Saprolegnia* has at best proven problematic and at worst, impossible.

However, it was clear from other morphological (cyst germination pattern, ornamentation of secondary cyst walls—reviewed by Beakes et al., 1994) and physiological characteristics (esterase isozymes, Beakes and Ford, 1983; repeated zoospore emergence [RZE], Cerenius and Söderhäll, 1985; Diéguez-Uribeondo et al., 1995) that isolates of *Saprolegnia* taken from a variety of amphibian, fish, and invertebrate lesions could be unambiguously distinguished from saprobiotic species, such as *S. diclina* and *Saprolegnia ferax* (Pickering et al., 1979; Hatai et al., 1990; Söderhäll et al., 1991; Fregeneda-Grandes et al., 2000). These findings were reinforced by subsequent molecular characterization of *Saprolegnia,* which also supported the separation of *S. parasitica* and *S. diclina s. str.* in two non-conspecific groups (Molina et al., 1995; Inaba and Tokumasu, 2002).

The most reliable morphological characteristic for the animal pathogenic group were the bundles of long-hooked hairs which decorated the secondary cyst coats, which can be seen under the electron-microscope (Pickering et al., 1979) or phase contrast microscopy (Willoughby, 1985). However, there is considerable variation both in the length and number of the long-hooked hairs associated with the secondary cysts (Pickering et al., 1979; Hatai et al., 1990; Fregeneda-Grandes et al., 2000). Curiously, those isolates with the longest spines (Type I, Fregeneda-Grandes et al., 2000, 2001) appeared to be less aggressive pathogens or even non-pathogenic, at least towards salmonid fish, than those with shorter spines (Type II, Fregeneda-Grandes et al., 2000, 2001) and these observations appear to be supported by other studies (Hatai et al., 1990; Hussein and Hatai, 1999; Stueland et al., 2005). This raises the question whether we are dealing with a single fish-pathogenic *Saprolegnia* species showing broad host range, wide morphological diversity and pathogenicity, or whether there are a number of species defined by their pathogenicity and cyst coat structure.

Currently, no molecular studies have been carried out in wide sample of isolates to clarify the phylogenetic relationships among isolates of *S. diclina–S. parasitica* complex and the taxonomic status of animal parasitic asexual isolates. Moreover, the taxonomic validity of certain morphological characters, e.g. bundles of long-hooked hairs in the cysts, retracted germination (also named indirect germination) or physiological characters that might represent an adaptation to parasitism, e.g. RZE (Cerenius and Söderhäll, 1985; Diéguez-Uribeondo et al., 1995), have not investigated in a wide sample of isolates. Therefore, the objective of this study was to investigate both phylogenetic and taxonomic aspects within the *S. diclina–S. parasitica* species complex. For this purpose, we have sequenced the internal transcribed spacer of nuclear ribosomal DNA and studied the cyst ornamentation, retracted germination, and ability to undergo RZE in a large and representative sample of isolates of *Saprolegnia* spp. and the *S. diclina–S. parasitica* complex obtained from different hosts and geographical origins.

2. Materials and methods

2.1. Isolates

A total of 128 isolates of worldwide distribution belonging to the *S. diclina–S. parasitica* complex (*S. australis*, *S. diclina* Type 1, *S. diclina* Type 2, *S. diclina* s. str., and *S. parasitica*), *Saprolegnia* sp., *S. ferax*, *Saprolegnia litoralis* and *Leptolegnia* sp. were included in this study. The origin of the isolates and their reference numbers are presented in Table 1. Vouchers of the cultures are kept in the fungal culture collections of the Departamento de Sanidad Animal