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Original research article

Sequence analysis and typing of *Saprolegnia* strains isolated from freshwater fish from Southern Chinese regions

Siya Liu [a], Pengpeng Song [a], Renjian Ou [a], Wenhong Fang [b], Mao Lin [c], Jiming Ruan [a], Xianle Yang [a], Kun Hu [a, *]

[a] National Pathogen Collection Center for Aquatic Animals, Shanghai Ocean University, Shanghai 201306, China
[b] East China Sea Fisheries Research Institute Chinese Academy of Fishery Sciences, Shanghai 200090, China
[c] Jimei University, Fujian 361021, China

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ABSTRACT

Saprolegniasis, caused by *Saprolegnia* infection, is one of the most common diseases in freshwater fish. Our study aimed to determine the epidemiological characteristics of saprolegniasis in Chinese regions of high incidence. *Saprolegnia* were isolated and identified by morphological and molecular methods targeting the internal transcribed spacer (ITS) ribosomal DNA (rDNA) and building neighbor-joining (NJ) and maximum parsimony (MP) phylogenetic trees. The ITS sequences of eight isolated strains were compared with GenBank sequences and all strains fell into three clades: CLADE1 (02, LP, 04 and 14), CLADE2 (S1), and CLADE3 (CP, S2, L5 and the reference ATCC200013). Isolates 02 and LP shared 80% sequence similarity with *S. diclina*, *S. longicaulis*, *S. ferax*, *S. mixta*, and *S. anomalies*. Further, isolates 04 and 14 shared 80% similarity with *S. bulbosa* and *S. oliviae*. Finally, extremely high ITS sequence similarities were identified between isolates S1 and *S. australis* (100%); CP and *S. hypogyna* (96%); and S2, L5, ATCC200013 and *S. salmonis* (98%). This research provides insights into the identification, prevention and control of saprolegniasis pathogens and the potential development of effective drugs.

1. Introduction

Saprolegnia is one of the main aquatic diseases caused by filamentous fungi infections, which can cause severe losses of freshwater fish in the wild and in commercial fish farms (Hatai & Hoshiai, 1992). These infections are usually termed saprolegniasis (Ke, Wang, Gu, Li, & Gong, 2009) and the main pathogens of *Saprolegnia* are Saproleniaceae *Saprolegnia* and Saproleniaceae *Ambisexualis*. These two pathogens are responsible for fungal infections of freshwater fish such as grass carp (*Ctenopharyngodon idellus*), crucian carp (*Carassius aumtus*), silver carp (*Hypophthalmichehys molitrix*), bighead carp (*Aristichthys nobilis*), Bluntnose black bream (*Megalobrama amblycephala*), Nile tilapia (*Oreochromis niloticus*), Yellow-headed catfish (*Pelteobagrus fulvidraco*), ChannelCatfish (*Ictalurus punctatus*), and their eggs. There is a large range of adaptive temperatures for *Saprolegnia* and cases of saprolegniasis occurring in the main regions where freshwater fish are aquacultured in China are reported all the year round. *Saprolegnia* infection spreads rapidly without a strict choice of hosts. It is hypothesized that dozens of fish species in different life stages including their eggs can be infected by *Saprolegnia*.

Currently, identification of *Saprolegnia* is mainly based on traditional morphological characteristics of the sexual reproductive structures such as oogonia, oospores and antheridia (Dick, 1969; Leclerc, Guillot, & Deville, 2000). However, many species exhibit very similar or overlapping characters and their morphology is not stable and constant (Dieguez-Uribeondo, Cerenius, & Söderhäll, 1996). Moreover, *Saprolegnia* from animals often fail to produce sexual structures *in vitro* (Dieguez-Uribeondo et al., 2007). Therefore, it is difficult and sometimes even impossible to identify, classify and name *Saprolegnia* using traditional morphological criteria (Seymour, 1970; Dieguez-Uribeondo et al., 2007).

Alternative approaches include polymerase chain reaction (PCR) amplification and sequencing of the internal transcribed spacer (ITS) region of ribosomal RNA (rRNA) genes. PCR amplification of rDNA is easy even from small quantities of DNA due to the high copy number, which makes it easy to analyze (Dieguez-Uribeondo et al., 2007; Paul & Steciow, 2004). Additionally, ITS has a high

* Corresponding author. National Pathogen Collection Center for Aquatic Animals, Shanghai Ocean University, 999 Hucheng huan Road, Shanghai 201306, China.
E-mail address: khu@shou.edu.cn (K. Hu).

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degree of variation even between closely related species, making PCR an ideal technique for identification and classification of *Saprolegnia*. In fact, molecular identification techniques based on ITS sequence analysis remedy the defects of traditional methods by morphology and has become a useful tool for species separation, identification and determination (Huang, Cerenius, & Söderhäll, 1994; Paul, 2001; Paul & Steciow, 2004; Paul, Galland, & Masih, 1999).

Previous works on *Saprolegnia* has been carried out, but mostly based on morphological observations (Bangyeekhun, Quiniou, Bly, & Cerenius, 2001; Fregeneda-Grandes, Rodríguez-Cadenas, & Aller-Gancedo, 2007). The Eastern, Southern and Central regions of China, the most important freshwater fish farming areas, have a high incidence of *Saprolegnia* infections. Saprolegniasis brings significant economic losses to freshwater fisheries every year resulting from transportation, injuries, low temperatures, and other factors (Howe, Gingerich, Dawson, & Olson, 1999; Sano, 1998). However, it has become increasingly difficult to prevent *saprolegniasis* in China's huge freshwater fish farming areas due to the unclear biological information about the nature of the pathogens. Variables that must also be taken into account when analyzing saprolegniasis include environmental conditions such as temperature, salinity, and pH, which differ from areas, climates, and seasons. The exact way in which these environmental conditions affect *Saprolegnia* strains and the unclear taxonomic classifications of the strains, hinder the development of epidemiological investigations and *Saprolegnia* research.

The objective of this study was to investigate both phylogenetic and taxonomic aspects of eight representative isolates of pathogenic *Saprolegnia* obtained from different hosts and geographical origins including Guangzhou, Hubei and Shanghai. For this purpose, we studied the morphological traits and sequenced the ITS to clarify their taxonomic positions. Moreover, the resistance of strains to temperature, pH and salinity was evaluated and the phylogenetic background was analyzed. This work was designed to provide basic support to epidemiological investigations and the control of *Saprolegnia* infection and saprolegniasis in freshwater fish.

2. Material and methods

2.1. Saprolegnia strains

The eight *Saprolegnia* strains were obtained from Hubei, Guangzhou, and Shanghai and their background information is presented in Table 1. The strains 02, 04 and 14 were isolated from Eggs of crucian carp. Eggs of *Megalobrama amblycephala* and water cultured crucian carp respectively at Shahu Town, Xiantao City, Hubei and L5 were isolated from water cultured crucian carp, Eggs of crucian carp and Methylene blue treated eggs of crucian carp respectively at FoshanBairong hatchery, Guangdong. The strains LP was isolated from the caudal fin of weever at Xiceng Town, Qingpu District, Shanghai. The strains of LP isolated from the skin of grass carp at Lingang New City River, Shanghai. The standard strain ATCC 200013 was purchased from American Type Culture Collection (ATCC).

2.2. Separation and purification

Separation and purification was performed as described by Oláh and Farkas with some modifications (Oláh & Farkas, 1978). The scrapings of isolates from the local lesions of diseased fish or their eggs were rinsed in 75% ethanol solution (chemically pure, Shanghai Anpel Scientific Instrument Co., Ltd., China) for 2–3 s and then dipped in sterile distilled water for 1–2 s before being placed on potato dextrose agar (PDA, Qingdao Hope Biotechnology Co., Ltd., Shanghai, China) plates. After incubation at 20 °C (Mir-253, Sanyo, Japan) for 24 h, the fungi colony grew to the edge of the plate, and heat-sterilized rapeseeds were scattered on the plates and incubated at 20 °C. The rapeseeds embedded in PDA were placed in sterilized and filtered river water and incubated at 20 °C until the zoospores ripened and released. Then 100 μL of *Saprolegnia* spore suspension was evenly coated on PDA plate and incubated at 20 °C until the fungi grow to cover the plate. A single colony was cut and put into another new PDA plate, in this way the strain was further purified by inoculations (Técher et al., 2010). If necessary, purification was repeated.

2.3. Saprolegnia morphological characteristics

Optical microscopy was used to assess the morphological characteristics of all the isolated strains.

2.4. Saprolegnia growth in different environmental factors

To measure temperature dependency of growth, PDA plates with *Saprolegnia* strains were punched into disks (diameter of 6 mm), placed in the centers of new PDA plates and incubated at 4 °C, 20 °C and 30 °C, respectively. To measure the pH dependency of growth, disks punched from *Saprolegnia* PDA plates were placed in the center of new PDA plates that were previously adjusted to pH 7, pH 8, pH 9 and pH 10 using 1 M HCl and 1 M NaOH (Shanghai Anpel Scientific Instrument Co., Ltd., Shanghai, China), then incubated at 20 °C. To measure the effect of NaCl concentration differences on *Saprolegnia* growth, PDA disks again were placed in the center of new PDA plates that included 0.5%, 1.0%, 1.5% and 2.0% NaCl, and incubated at 20 °C (Ali, 2005). All the PDA plates described above were incubated for 48 h and the radius (mm) of the developed fungus colonies were measured in triplicate at 12, 24, 36 and 48 h, respectively.

Table 1
Sources and background of the strains used in the present study.

Isolate	Host	Origin	Acquisition time
02	Eggs of crucian	Shahu Town, Xiantao City, Hubei	May 10, 2010
04	Eggs of megalobramaamblycephala	Shahu Town, Xiantao City, Hubei	May 10, 2010
14	Water cultured crucian	Shahu Town, Xiantao City, Hubei	May 10, 2010
S1	Water cultured crucian	FoshanBairong hatchery, Guangdong	March 10, 2011
S2	Eggs of crucian	FoshanBairong hatchery, Guangdong	March 10, 2011
L5	Methylene blue treated eggs of crucian	FoshanBairong hatchery, Guangdong	March 10, 2011
LP	Caudal fin of weever	Xiceng Town, Qingpu District, Shanghai	April 13, 2012
CP	Skin of grass carp	Lingang New City River, Shanghai	August 20, 2012
ATCC200013	Rainbow trout	ATCC (Kanagawa, Japan)	March 23, 1990

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