Saprolegnia australis R. F. Elliott 1968 infection in Prussian carp *Carassius gibelio* (Bloch, 1782) eggs and its control with herb extracts

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Summary

In order to control saprolegniosis in Prussian carp (*Carassius gibelio* (Bloch, 1782) eggs, it is important to screen herb extracts as potential anti-*Saprolegnia* drugs in Prussian carp hatcheries. For this purpose, an oomycete water mould (strain SC) isolated from Prussian carp [*Carassius gibelio* (Bloch, 1782)] eggs suffering from saprolegniosis was characterised morphologically as well as from ITS rDNA sequence data. Initially identified as a *Saprolegnia* sp. based on its morphological features, the constructed phylogenetic tree using the neighbour joining method further indicated that the SC strain was closely related to *Saprolegnia australis* R. F. Elliott 1968 strain VI05733 (GenBank accession no. HE798564), and which could form biofilm communities as virulence factors. In addition, aqueous extracts from forty Chinese herbs were screened as possible anti-*Saprolegnia* agents. Among them, a 1 g ml⁻¹ extract from *Radix sanguisorbae* was the most efficacious anti-*Saprolegnia* agent, indicated by the minimum inhibitory concentration that was as low as 256 mg L⁻¹. Relative survival of 73 and 88% was obtained against the SC strain in fish eggs at concentrations of 256 and 1280 mg L⁻¹, respectively. This is the first known report of *Saprolegnia australis* R. F. Elliott 1968 infection in *C. gibelio* (Bloch, 1782) eggs involving the screening of *R. sanguisorbae* extracts as potential anti-*Saprolegnia* agents.

Introduction

Prussian carp, *Carassius gibelio* (Bloch, 1782), is a popular freshwater fish species in China, Japan, Korea, East Europe, Siberia, the Russian Far East, Sakhalin and the basins of the largest Central Asian rivers Syr Darya and Amu Darya (Apalikova et al., 2011). Especially in China, Prussian carp farming has become an emerging industry in the province of Hubei, Jiangsu, Hunan, Guangdong, Jiangxi, Anhui, Shandong, Sichuan, Guangxi, Liaoning, etc., and has brought

great profits in recent years. Annual production was nearly 2.2 million tonnes in 2010 (Ge and Miao, 2011). However, under intensive culture conditions, serious problems in Prussian carp hatcheries occur due to saprolegniosis caused by zoosporic oomycete fungi. During egg incubation, the oomycetes produce mycelia, which grow and spread from dead to healthy eggs thereby causing major economic losses (Noga, 1993; Cao et al., 2012a). More attention needs to be paid to control oomycete infections during incubation of the Prussian carp eggs.

Major pathogens of many fish species (Pickering and Willoughby, 1982) are species of the saprolegniaceous water mould *Saprolegnia*, which can be found in most freshwater habitats and are responsible for significant infections of both living and dead fish as well as their eggs (Noga, 1993; Hussein and Hatai, 2002). A few studies have been conducted on *Saprolegnia* species of rainbow trout, zebra fish, silver crucian carp, Pengze crucian carp and yellow catfish eggs (Ke et al., 2009; Mousavi et al., 2009; Cao et al., 2012a,b). However, no specific report is available for *S. australis* R. F. Elliott 1968 infection in Prussian carp and its eggs. Additionally, the control of saprolegniosis remains a worldwide problem after the effective malachite green treatment was banned. Relevant studies need to be extensively conducted on the *Saprolegnia* pathogens and potential anti-*Saprolegnia* drugs.

In the present study, the pathogenic *S. australis* R. F. Elliott 1968 of Prussian carp eggs suffering from saprolegniosis was isolated and its taxonomic position determined using phylogenetic analysis based on ITS rDNA sequence; its susceptibility to forty different Chinese herb extracts was further assayed to screen for potential anti-*Saprolegnia* drugs.

Materials and methods

Experimental fish eggs

A saprolegniosis outbreak occurred in June 2012 in a Prussian carp hatchery in Guangdong province, China. More than fifty infected Prussian carp eggs were examined externally and microscopically for the presence of oomycetes.

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Another fifty affected fish eggs (as described by Evelyn et al., 1986) were sampled and transported to the laboratory. Healthy Prussian carp eggs used in the study were obtained from another hatchery with no history of any unknown mortalities or abnormalities (Cao et al., 2012b).

Isolation of fungi

The infected fish eggs were first disinfected for 2–3 s with 75% alcohol, then washed several times in sterile filtered water and plated on potato dextrose agar (PDA) (Sinopharm Chemical Reagent Co., Ltd) containing 100 mg L⁻¹ streptomycin and penicillin (Sinopharm Chemical Reagent Co., Ltd) to reduce bacterial contamination and incubated at 20°C for 24 h. Autoclaved rape seeds were immediately placed at the edge of colonies grown on PDA plates and incubated until they were infested with hyphae. These were then transferred to sterile filtered river water and incubated until the zoospores were discharged. One hundred µl of zoospores were then spread on PDA plates and incubated at 4°C for 48–72 h.

Infectivity experiment

Approximately 7 days prior to starting the test, rape seeds infested with the isolate were placed in sterile filtered river water at 20°C for 72 h to induce zoospore formation; zoospore suspensions were then collected as described by Hussein and Hatai (2002). Then 720 healthy fish eggs were randomly distributed among six small glass aquaria (120 eggs per aquarium) containing aerated sterile filtered river water at 20°C. The fish eggs in the challenged aquarium were exposed to the isolate's zoospores at a concentration of 2×10^5 ml⁻¹; in the control aquarium the eggs were held in aerated sterile filtered river water without the addition of zoospores. Each treatment was conducted in triplicate. The fish eggs were observed daily for 5 days. Eggs with hyphae were immediately removed for fungal isolation and identification according to Ghiasi et al. (2010). Infection of eggs by the isolate was recorded only if the challenge strain was re-isolated and identified.

Morphological observation

The isolate was grown on PDA plates with several autoclaved rape seeds at 20°C until the rape seeds were infested with the isolate as previously described. Rape seeds with hyphae were then transferred to 6-well cell culture plates containing sterile filtered river water and incubated at 20°C for 14 days. Daily observations using an inverted microscope were carried out to check the emergence of zoosporangia, zoospore discharges, sporangial renewal and oogonia.

Molecular identification

Genomic DNA was extracted from pure cultures of the isolate using a Universal Genomic DNA Extraction Kit vers. 3 (Takara Biotechnology, Dalian). The internal transcribed spacer (ITS) gene was amplified by PCR using a pair of ITS

Table 1
MICs of extracts from Chinese herbs against the SC isolate and two control strains

Chinese herbs	MIC (mg L ⁻¹)		
	Strain SC	Strain JL	Strain HP
<i>Bupleurum chinense</i>	ND	ND	ND
<i>Catsia tora</i> Linn	ND	ND	ND
<i>Cortex albiziae</i>	ND	ND	ND
<i>C. cinnamomi</i>	ND	ND	ND
<i>C. phellodendri chinensis</i>	ND	ND	ND
<i>Dryopteris setosa</i>	ND	ND	ND
<i>Eucommia ulmoides</i>	ND	ND	ND
<i>Exocarpium benincasae</i>	ND	ND	ND
<i>Fibraurea tinctoria</i> Lour.	ND	ND	ND
<i>Flos carthami</i>	ND	ND	ND
<i>Folium artemisiae argyi</i>	ND	ND	ND
<i>F. nelumbinis</i>	ND	ND	ND
<i>F. isatidis</i>	4096 ± 0	5461 ± 2365	5461 ± 2365
<i>Fructus carpesii</i>	ND	ND	ND
<i>F. lyciibarbari</i>	ND	ND	ND
<i>F. mume</i>	5461 ± 2365	5461 ± 2365	6827 ± 2365
<i>Galla chinensis</i>	8192 ± 0	ND	8192 ± 0
<i>Herba menthae</i>	ND	ND	ND
<i>H. artemisiae annuae</i>	5461 ± 2365	6827 ± 2365	5461 ± 2365
<i>Jasminum sambac</i> (Linn.) Aiton	ND	ND	ND
<i>Melia azedarach</i> Linn.	ND	ND	ND
<i>Omphalia lapidescens</i> Schroet	ND	ND	ND
<i>Pericarpium citri reticulatae viride</i>	ND	ND	ND
<i>P. granati</i>	5461 ± 2365	4096 ± 0	5461 ± 2365
<i>Plantago asiatica</i> L.	ND	ND	ND
<i>Radix astragali</i>	ND	ND	ND
<i>R. aucklandiae</i>	ND	ND	ND
<i>R. et rhizoma rhei</i>	ND	ND	8192 ± 0
<i>R. glycyrrhizae</i>	ND	ND	ND
<i>R. ophiopogonis</i>	ND	ND	ND
<i>R. paeoniae rubra</i>	ND	ND	ND
<i>R. rehmanniae preparata</i>	427 ± 149	427 ± 149	512 ± 0
<i>R. sanguisorbae</i>	256 ± 0	256 ± 0	256 ± 0
<i>R. sophorae flavescentis</i>	ND	ND	ND
<i>Rhizoma atracylodis macrocephalae</i>	ND	ND	ND
<i>R. phragmitis</i>	ND	ND	ND
<i>R. polygonati odorati</i>	ND	8192 ± 0	ND
<i>Salvia miltiorrhiza</i>	ND	ND	ND
<i>Sterculia lychnophora</i>	ND	ND	ND
<i>Trachycarpus fortunei</i>	ND	ND	ND

ND = herb extract MIC > 8192 mg L⁻¹.

gene primers (ITS1): 5'-TCCGTAGGTGAACCTGCGG-3' as well as (ITS4): 5'-TCCTCCGCTTATTGATATGC-3', and carried out according to the instructions of the fungi identification PCR kit (Takara Biotechnology, Dalian). Amplification was done after 35 cycles of denaturation at 94°C for 0.5 min, annealing at 58°C for 0.5 min and extension at 72°C for 1.0 min followed by a final extension at 72°C for 5 min using a PCR minicycler (Eppendorf Ltd., Germany). The PCR product was electrophoresed on 1% agarose gel and observed via ultraviolet trans-illumination. Sequencing

was performed using a fluorescent labeled dideoxynucleotides termination method (with BigDye terminator) on an ABI 3730 automated DNA Sequencer.

The partial ITS rDNA sequence was assembled using MegAlign and Seqman software with a Macintosh computer. Searches were done with the National Centre for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) program. The ITS rDNA gene sequence of the pathogenic isolate was constructed using the neighbour joining method (Chen and Liu, 2007).

Susceptibility assay to Chinese herb extracts

The susceptibility assay of the herb extracts against the isolate and the control *Saprolegnia* strains was conducted in triplicate. Forty Chinese herbs were obtained from Shanghai Fosun Industrial CO., LTD. (Table 1). A concentration of 1 g ml^{-1} of aqueous extract from each Chinese herb was prepared using the boiling method as described by Au et al. (2001). Herb extracts at concentrations of $0\text{--}8192 \text{ mg L}^{-1}$ were used to assay the minimum inhibitory concentration (MIC). MIC of each herb extract was defined as the lowest concentration that did not allow any visible fungal colony growth, determined through the dilution plate method as described by Stueland et al. (2005a). *S. parasitica* strain JL and *S. ferax* strain HP, previously isolated and identified by Cao et al. (2012a,b), were used as controls. Data were presented as the mean $\pm$ the standard deviation (SD). $P < 0.05$ was considered statistically significant using one-way analysis of variance.

Protective efficacy assay

The protective efficacy assay was conducted in triplicate. Healthy fish eggs were obtained from Sand Lake Aquatic Technique Popularizing Station, Hubei China and maintained in twelve 10-L glass aquaria supplied with single-pass aerated sterile filtered river water at 20°C . Each aquarium was randomly stocked with 100 healthy eggs. The 1 g ml^{-1} of herb extracts were added directly to aerated sterile filtered river water in the treatment aquaria at final concentrations of 256 and 1280 mg L^{-1} ; the control aquarium received no treatment. In the positive control treatment, aquaria malachite green was added at a final concentration of 0.2 mg L^{-1} , as recommended by Sudova et al. (2007). All test eggs were exposed to the isolate's zoospores at a concentration of $2 \times 10^5 \text{ ml}^{-1}$. The eggs were observed daily for 5 days; eggs with hyphae were immediately removed for fungal isolation and identification according to Ghiasi et al. (2010). Egg infections were recorded only if the challenge strain was re-isolated and identified. The cumulative infection rate and relative survival percentage were calculated using the following formulas: infected eggs/total eggs $\times 100$, cumulative infection rate in the control group-cumulative infection rate in the treatment group/cumulative infection rate in the control group $\times 100$ (Baulny et al., 1996; Khomvilai et al., 2006).

Results

Morphological characterization

The SC strain was demonstrated as the causative agent of the Prussian carp egg saprolegniosis, exhibiting a colonization

Fig. 1. Morphological characteristics of the SC strain. (a) cylindrical zoosporangium (arrow); (b) sporangial renewal by internal proliferation (arrow); (c) saprolegnoid discharge of zoospores (Arrow); (d) immature oogonium (arrow)

Fig. 2. Constructed ITS rDNA phylogenetic tree of 18 known *Saprolegnia* strains and the SC isolate using the neighbour-joining method. Bootstrap values (%) shown next to the clades, accession numbers indicated beside the strain names, and scale bars represent distance values

rate of 73% and with apparent external signs similar to the naturally infected fish eggs (data not shown). No mortality or visible changes were observed in the control group. The SC strain exhibited identical morphological characteristics in asexual and sexual reproductions with *Saprolegnia* sp. as described by Johnson et al. (2003), e.g. hyphae aseptate; zoosporangia cylindrical, renewed internally with the secondary ones nesting inside discharged primary ones; zoospores dimorphic, discharge and behaviour saprolegnoid; oogonia lateral and spherical (Fig. 1). This initially identified the SC strain as a *Saprolegnia* isolate.

Phylogenetic analysis

The 750 bp ITS rDNA sequence of the SC strain was submitted to the GenBank database with the accession no. JN662488. Similarities between the ITS rDNA sequence of the SC strain and those of *Saprolegnia* strains in the GenBank database were 99.0 to 100%, which confirmed the initial identification. The constructed phylogenetic tree using the neighbour joining method further demonstrated that the SC strain was closely related to *Saprolegnia australis* R. F. Elliott 1968 strain VI05733 (GenBank accession no. HE798564) (Fig. 2) that could form biofilm communities as the virulence factor (Ali et al., 2013). The molecular identification result from phylogenetic analysis was consistent with that found through morphological identification.

Susceptibility to the Chinese herb extracts

The susceptibility of the SC strain to 40 herb extracts is shown in Table 1. Extracts from *R. sanguisorbae* and *R. rehmanniae preparata* were demonstrated to show significant inhibition effects on the hyphae growth as indicated by their MICs ≤ 1024 mg L⁻¹ for the SC isolate and the two control

Fig. 3. Protective efficacy of *Radix sanguisorbae* extract on *Saprolegnia australis* infection in Prussian carp eggs. A, Cumulative infection rate in control group; B, Cumulative infection rate in treatment group with 256 mg L⁻¹ herb extract; C, Cumulative infection rate in treatment group with 1280 mg L⁻¹ herb extract; D, Cumulative infection rate in treatment group with 0.2 mg L⁻¹ malachite green. Data points = mean values of three replicates; vertical bars on data points = standard deviation of the mean

strains ($P < 0.05$). In addition, the *R. sanguisorbae* extract had the best potential to control the SC isolate and the two control strains, because concentrations as low as 256 mg L⁻¹ of *R. sanguisorbae* was effective at inhibiting hyphae growth.

Protective efficacy

The protective efficacy of the *R. sanguisorbae* extract on the *Saprolegnia* infection to Prussian carp eggs is shown in Fig. 3. Relative survival of 73 and 88% was obtained against

the SC strain on eggs at concentrations of 256 and 1280 mg L⁻¹, respectively, which was 22 and 37% higher than that achieved with a concentration of 0.2 mg L⁻¹ of malachite green. The death of all the test eggs was caused by *Saprolegnia australis* R. F. Elliott 1968, as determined by fungal isolation and molecular identification (data not shown).

Discussion

Saprolegnia australis R. F. Elliott 1968 has been implicated in significant oomycete infections of crayfish (Krugner-Higby et al., 2010). However, little information is available on its infection of Prussian carp eggs. In the present study, the naturally occurring pathogen of saprolegniosis-infected Prussian carp eggs was identified as a *Saprolegnia australis* R. F. Elliott 1968 strain. The findings further confirmed that *S. australis* R. F. Elliott 1968 was a major cause of saprolegniosis in aquaculture production (Krugner-Higby et al., 2010). In addition, the present results suggest that *R. sanguisorbae* extract has the potential to control saprolegniosis during fish egg incubation. This herbal extract can be applied directly to fish hatching pools, and its use will relieve some of the environmental pressures in a fish hatchery.

Identification of the *Saprolegnia* species is complex and sometimes confusing. However, several typical morphological features involving asexual and sexual reproductive organs serve as classic indicators of *Saprolegnia* (Stueland et al., 2005b). Thus, the SC strain in the present study was initially identified as a *Saprolegnia* sp. based on its morphological characteristics that conform to the descriptions of *Saprolegnia* sp. (Ke et al., 2009) (Fig. 1). However, *Saprolegnia* species are usually difficult or even impossible to identify by traditional morphological criteria alone. Therefore, the ITS region of the SC strain is compared to those of *Saprolegnia* isolates (Whisler, 1996). The phylogenetic analysis based on ITS rDNA region (Fig. 2) further clarified the taxonomic position of the SC strain and confirmed its initial identification as a *Saprolegnia* sp.

Herb extracts as alternative saprolegniosis therapies attract a great deal of attention due to their sufficient quantities, low toxicity and costs (Cooper et al., 1997; Cao et al., 2006, 2012a; Cai et al., 2010). *R. sanguisorbae*, an oriental herb medicine in China for hemostasis, has been used to treat hematemesis, hemoptysis, melena and hypermenorrhea (Chen et al., 2001). This herb is found to show high antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Streptococcus hemolyticus* (Xia et al., 2009), as well as against coronavirus through protein synthesis reduction (Kim et al., 2010). However, little is known of its antimicrobial activity against oomycete pathogens. In our study, the growth of the SC isolate and the control strains was completely inhibited at as little as 256 mg L⁻¹ extract from *R. sanguisorbae* (Table 1), providing evidence of its potent antifungal property. This herb extract at concentrations of 256 and 1280 mg L⁻¹ exhibited significant relative survival of 73 and 88%, respectively, against experimental *Saprolegnia australis* R. F. Elliott 1968 infection in fish eggs

(Fig. 3). The patterns of susceptibility of the SC strain to herbal extracts are similar to those found among other *Saprolegnia* species and antifungal chemicals (Stueland et al., 2005a).

In conclusion, based on morphological features and phylogenetic analysis, the causative agent of saprolegniosis in Prussian carp eggs was identified as *Saprolegnia australis* R. F. Elliott 1968. This study reported the first *Saprolegnia australis* R. F. Elliott 1968 infection in Prussian carp eggs and demonstrated for the first time *R. sanguisorbae* extract as a promising anti-*Saprolegnia* drug for the control of egg saprolegniosis.

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