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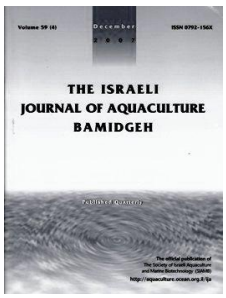


# *Saprolegnia* Pathogen from Pengze Crucian Carp (*Carassius auratus* var. Pengze) Eggs and its Control with Traditional Chinese Herb

**Haipeng Cao[1], Wenwei Xia[1], Shiqi Zhang[1], Shan He[2], Ruopeng Wei[3], Liqun Lu[1], Xianle Yang[1]***

[1] *Key Laboratory of Exploration and Utilization of Aquatic Genetic Resources, National Aquatic Pathogen Collection Center, Shanghai Ocean University, Shanghai 201306, China*

[2] *Shanghai Normal University, Shanghai 200235, China*

[3] *Shanxi Veterinary Drug and Feed Engineering Technology Research Center, Yuncheng 044000, China*

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Key words: *Saprolegnia* sp., phylogenetic analysis, *Pseudolarix kaempferi* Gord. (Pinaceae), saprolegniasis

**Abstract**

In the present study, a pathogenic strain, JL, was isolated from Pengze crucian carp (*Carassius auratus*) eggs suffering from saprolegniosis. It was initially determined as Saprolegnia sp. strain JL. *Saprolegnia* species have been implicated for significant fungal contamination, involving both living and dead fish and their eggs. A phylogenetic tree was constructed using the maximum parsimony method. The tree shows that the JL strain was closely related to *Saprolegnia parasitica* isolate SAP171, isolated from *Salmo trutta* suffering from saprolegniasis in Laukaa, Finland. The minimum inhibitory concentration (MIC) of 20 Chinese herbs was screened. *Pseudolarix kaempferi* Gord. (Pinaceae) was the most effective in inhibiting growth of the bacteria and was chosen for further trial. Significant protective efficacy of 52.63% and 73.68% was obtained against the JL strain in Pengze crucian carp eggs at *P. kaempferi* concentrations of 12.5 mg/ml and 25.0 mg/ml, respectively.

* Corresponding author. Tel.: +86-216-1900453, fax: +86-216-1900452, e-mail: hpcao@shou.edu.cn

## Introduction

The crucian carp, *Carassius auratus*, is a popular freshwater fish species with wide distribution in Asia, Europe, Africa, and North America. Crucian carp farming is an important industry, especially in China, with an annual production of 2 million tons. One of the most valuable crucian carps in China is the Pengze crucian carp, *C. auratus* var. Pengze. It is China's second largest export freshwater fish and 50,000 tons of its products are exported annually to Korea, Japan, Russia, and southeast Asia (Wang, 2009). However, one of the most serious problems in Pengze crucian carp hatcheries is oomycete infections caused by zoosporic fungi. During egg incubation, oomycete produce mycelia which grow and spread from dead to healthy eggs causing major financial losses.

Fungal infections of freshwater fish often affect wild and farmed fish in freshwater environments (Pickering and Wiloughby, 1982). One of the most destructive is *Saprolegnia* sp., which is widespread in freshwater habitats around the world and responsible for significant contaminations involving living and dead fish as well as incubating fish eggs (Noga, 1993). Losses of millions of pounds in the salmon aquaculture business in Scotland, Chile, Japan, Canada, and the USA were attributed primarily to saprolegniosis (Hussein and Hatai, 2002). Control of saprolegniasis is a problem since the effective malachite green treatment has been banned worldwide.

*Saprolegnia* species have been studied in rainbow trout eggs and zebra fish (Ke et al., 2009; Mousavi et al., 2009). In the present study, morphological characteristics of a pathogenic *Saprolegnia* isolated from Pengze crucian carp eggs suffering from saprolegniasis is described, and its taxonomic position is determined by a nucleotide BLAST search in the NCBI website and phylogenetic analysis based on ITS rDNA sequence. *Pseudolarix kaempferi* Gord. (Pinaceae) was one of 20 Chinese herbs screened as a potential drug for controlling *Saprolegnia* infection.

## Materials and Methods

*Egg samples.* Fifty Pengze crucian carp eggs suffering from saprolegniosis were obtained as samples from the Sand Lake Aquatic Technique Popularizing Station in Hubei, China, in May 2010, where 6 million Pengze crucian carp eggs are hatched annually.

*Isolation and purification of fungal strains.* The sampled eggs were disinfected for 2-3 seconds with 75% alcohol, then washed several times in sterile filtered water, placed on potato dextrose agar (PDA) plates (Sinopharm Chemical Reagent Co., Ltd) containing 100 ppm streptomycin and penicillin (Sinopharm Chemical Reagent Co., Ltd) to facilitate isolation of the fungus, and incubated at 25°C for 24 h. Autoclaved rape seeds were placed at the edges of colonies that grew on the PDA plates, and were incubated until they were covered with hyphae. These were then transferred to sterile filtered river water and incubated until zoospores were discharged. 100 µl Zoospores were spread on PDA plates and incubated at 4°C for 48-72 h until used.

*Artificial challenge test*. Approximately seven days prior to the test onset, isolates were subcultured in sterile filtered river water containing several autoclaved rape seeds at 25°C for 72 h. Zoospore suspensions were then collected. The test was carried out in nine glass petri dishes supplied with sterile filtered river water at 25°C. Each petri dish was randomly stocked with 40 healthy Pengze crucian carp eggs. These were challenged with the zoospore suspension at a concentration of $1 \times 10^6$/ml. Eggs in control dishes were held in sterile filtered river water only. The eggs were observed under a light microscope daily for five days. Eggs with hyphae were immediately removed for fungal isolation according to Ghiasi et al. (2010), and mortalities were recorded.

*Morphological observation.* Pathogenic isolates were grown on PDA plates with several autoclaved rape seeds at 25°C until the rape seeds were covered with hyphae. The seeds with hyphae were transferred to six-well cell culture plates containing sterile filtered river water and incubated at 25°C for 14 days. Observations under an inverted microscope were carried out every day to check the emergence of primary cysts, zoospore discharges, oogonia, antheridia, etc.

*DNA extract, PCR, and sequencing*. Genomic DNA was extracted from pure cultures of pathogenic isolates using the Universal Genomic DNA Extraction Kit Ver 3.0 (Takara

Biotechnology (Dalian) Co., Ltd.) following the manufacturer's instructions. The 750 bp of the internal transcribed spacer (ITS) gene was amplified by PCR using two ITS gene primers: 5'-TCCGTAGGTGAACCTGCGG-3' (ITS1) and 5'-TCCTCCGCTTATTGATATGC-3' (ITS4), and carried out according to the instructions of the Fungi Identification PCR Kit (Takara Biotechnology (Dalian) Co., Ltd.). 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 by the fluorescent labeled dideoxynucleotides termination method (with a BigDye terminator) on an ABI 3730 automated DNA Sequencer.

*Phylogenetic analysis*. The partial ITS rDNA sequence was assembled using MegAlign, Editseq, and Seqman software with a power Macintosh computer. Searches were done against the National Centere 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 maximum parsimony method as recommended by Chen and Liu (2007).

*Assay for susceptibility to Chinese herb extracts*. Prior to the susceptibility assay, twenty Chinese herbs were obtained from Shanghai Fosun Industrial Co., Ltd. and 100 g of each herb was extracted according to Qiu et al. (2010). The minimum inhibitory concentration (MIC) of each herb extract was determined by the dilution plate method described by Benger et al. (2004). The MIC was the lowest concentration of each herb extract that prevented any visible fungal colony growth on the PDA plates.

*Protective efficacy assay.* Healthy Pengze crucian carp eggs were obtained from Sand Lake Aquatic Technique Popularizing Station, Hubei, China, and maintained in three glass petri dishes supplied with sterile filtered river water at 25°C. Each dish was randomly stocked with 40 healthy eggs. Herb extracts were added to river water in the treatment dishes to final concentrations of 12.5 and 25.0 mg/ml. No extract was added to river water in the control dish. Eggs in the control and treatment dishes were then challenged with the zoospore suspension at a concentration of $1 \times 10^6$/ml. The tested eggs were observed under a light microscope daily for five days. Eggs with hyphae were immediately removed for fungal isolation, and dead eggs were recorded.

## Results

*Morphological characterization of the pathogenic Saprolegnia isolate*. Symptoms of Pengze crucian carp eggs suffering from saprolegniosis, covered with fungal hyphae, are shown in Fig. 1. Eight different fungal isolates were obtained from the infected eggs, but only one strain, named JL, was pathogenic, resulting in 45% mortality. The JL strain showed identical morphological characteristics of asexual and sexual reproduction as *Saprolegnia* sp., such as aseptate and sparingly branched hyphae (not shown), clavate and straight or slightly bent zoosporangia (Fig. 2a), sporangial renewal by internal proliferation (Fig. 2b), sporangial discharges of zoospores (Fig. 2c-f), encysted zoospores (Fig. 2g), reniform secondary zoospores (Fig. 2h), germinating spores (Fig. 2i), terminal and intercalary oogonia with centric oospores (Fig. 3a), oogonia with monoclinous, androgynous, and diclinous antheridia (Fig. 3b-d). Thus, the JL strain was initially determined as *Saprolegnia* sp. strain.

Fig. 1. Pengze crucian carp eggs suffering from saprolegniosis, arrows show contaminated areas.

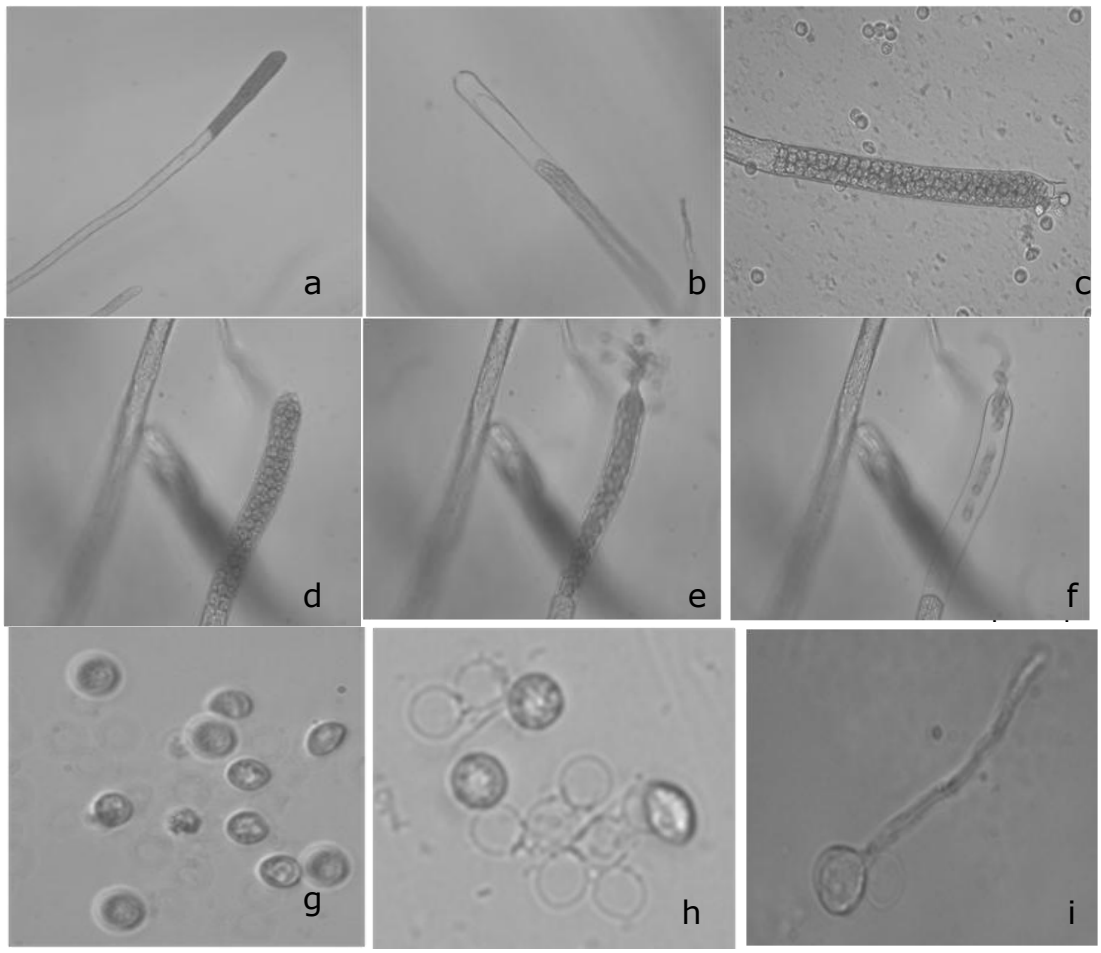


Fig. 2. Asexual reproduction of the JL strain of the *Saprolegnia* isolate: (a) immature zoosporangium, (b) sporangial renewal by internal proliferation, (c) saprolegnoid discharge of zoospores, (d-f) orderly release of primary zoospores from the apex of the zoosporangium, (g) primary and encysted zoospores, (h) empty cysts that underwent repeated zoospore emergence, and (i) germinating zoospores.

*Molecular identification and phylogenetic analysis.* The 750 bp ITS rDNA sequence of the JL strain was submitted to the GenBank database with the accession no. HM637287. Similarities between the ITS rDNA sequence of the JL strain and those of *Saprolegnia* strains in the GenBank database were 99.0%, confirming the initial identification. The phylogenetic tree, constructed using the maximum parsimony method, further demonstrated that the JL strain was closely related to the *Saprolegnia parasitica* isolate SAP171 (GenBank accession no. AM228804; Fig. 4) that was isolated from *Salmo trutta* suffering from saprolegniasis in Laukaa, Finland (Diéguez-Uribeondo et al., 2007). The molecular identification result of the phylogenetic analysis was consistent with that found through morphological identification.

*Susceptibility to Chinese herb extracts.* Seven of the twenty herb extracts showed good inhibition effects on *Saprolegnia* growth, i.e., MIC was below 5.0 mg/ml (Table 1).

*Protective efficacy*. Since as little as 1.25-2.50 mg/ml of *P. kaempferi* was effective in inhibiting hyphae growth, this herb was further screened for its potential to inhibit *Saprolegnia*. Results suggest good protective effects of *P. kaempferi* for controlling *Saprolegnia* infection on crucian carp eggs (Fig. 5). Significant protective efficacy against the JL strain in eggs (52.63% and 73.68%) was obtained at concentrations of 12.5 mg/ml and 25.0 mg/ml, respectively. The death of dead test eggs was caused by *Saprolegnia* sp., as determined by fungal isolation and molecular identification (data not shown). Thus, *P. kaempferi* is a potential drug for the successful treatment of saprolegniasis of carp eggs.

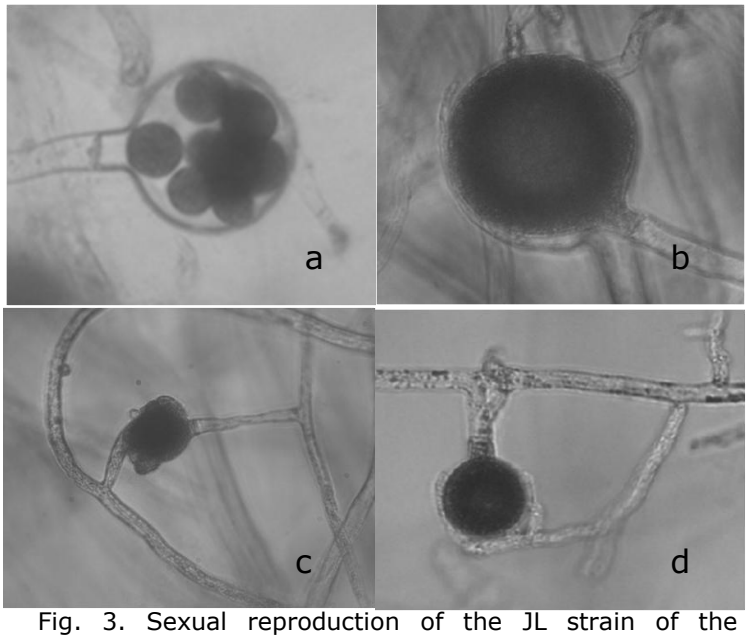


Fig. 3. Sexual reproduction of the JL strain of the *Saprolegnia* isolate: (a) mature oogonium with centric oospores, (b) oogonium with monoclinous antheridia, (c) oogonium with diclinous antheridia, and (d) oogonium with androgynous antheridia.

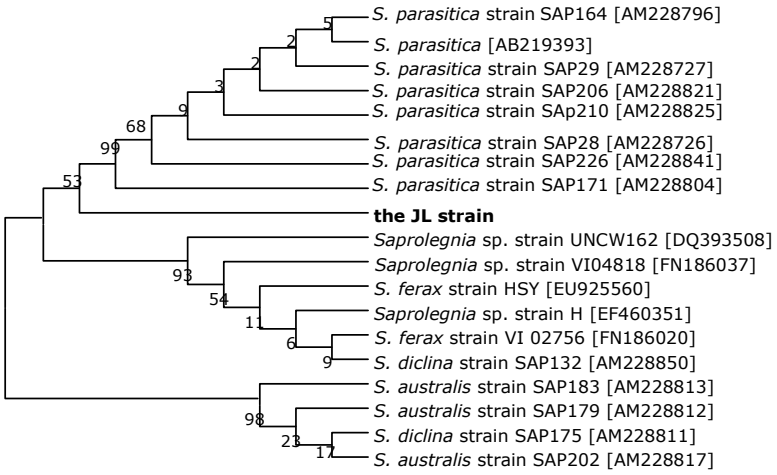


Fig. 4. Phylogenetic tree constructed using maximum parsimony method.

Table 1. Minimum inhibitory concentration (MIC) of the JL strain of *Saprolegnia* to 20 Chinese herb extracts ($7 \times 10^7$ spores/ml).

| *Herb* | *MIC (mg/ml)* |
|---|---|
| *Syzygium aromaticum* | 5.0-10.0 |
| *Fructus cnidii* | >10.0 |
| *Thallus laminariae* | >10.0 |
| *Fructus anisi stellati* | 2.5-5.0 |
| *Perilla frutescens* | >10.0 |
| *Sophora flavescens* | 2.5-5.0 |
| *Pseudolarix kaempferi* Gord. (Pinaceae) | 1.25-2.50 |
| *Polygonatum canaliculatum* | 2.5-5.0 |
| *Lithospermum erythrorhizon* | >10.0 |
| *Curcuma longa* | >10.0 |
| *Borneolum syntheticum* | >10.0 |
| *Cortex phellodendri* | 2.5-5.0 |
| *Fructus kochiae* | >10.0 |
| *Herba artemisiae scopariae* | >10.0 |
| *Foeniculum vulgare* | >10.0 |
| *Melaphis chinensis* | 2.5-5.0 |
| *Melia azedarach* L. | >10.0 |
| *Pericarpium citri reticulatae* | 5.0-10.0 |
| *Cortex dictamni* | >10.0 |
| *Fragrant litsea* | 2.5-5.0 |

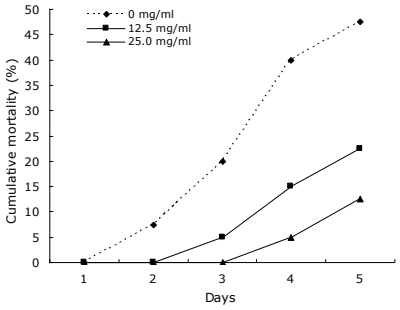


Fig. 5. Control by *Pseudolarix kaempferi* Gord. (Pinaceae) extract of artificial infection of the JL strain of *Saprolegnia* on Pengze crucian carp eggs.

## Discussion

In the present study, the naturally occurring pathogen was identified as *Saprolegnia* sp. based on morphological characteristics and phylogenetic analysis. Our findings confirm that *Saprolegnia* species are the major cause of saprolegniasis in aquaculture production (Bangyeekhun, 2003). Determination of *Saprolegnia* species is complex and sometimes confusing. However, several typical morphological features involving asexual and sexual reproductive organs serve for classic *Saprolegnia* identification (Stueland et al., 2005a). The JL strain in the present study was initially identified as *Saprolegnia* sp. based on typical morphological characteristics which comform with precise descriptions of *Saprolegnia* sp. by Van West (2009). However, *Saprolegnia* species are usually difficult or even impossible to identify by traditional morphological criteria alone. Therefore, we compared ITS regions to further identify the JL strain, as done to *Saprolegnia* isolates from salmonid fish (Whisler, 1996). The phylogenetic analysis based on the ITS rDNA

region further clarified the taxonomic position of the JL strain and confirmed its initial identification as *Saprolegnia* sp.

Zoospores of some moderately or highly pathogenic *Saprolegnia* strains have long hook cilia that are believed to increase *Saprolegnia* attachment efficiency (Beakes, 1982). However, no such cilia were observed on the zoospores of pathogenic strain JL or on other *Saprolegnia* pathogens such as *Saprolegnia* sp. strain SAP211 (GenBank accession no. AM228826) (Diéguez-Uribeondo et al., 2007). Other factors such as chymotrypsin-like activity could also contribute to their pathogenicity (Peduzzi and Bizzozero, 1977).

No significant differences were found between the susceptibility of *Saprolegnia* pathogens to antifungal chemicals (Stueland et al., 2005b). Thus, only the JL strain was chosen for the susceptibility and protective efficacy assay. To date, the few chemicals used to control saprolegniasis include hydrogen peroxide, salt, ozone, formaldehyde, and formalin formulations (Forneris et al., 2003; Rach et al., 2004; Gieseker et al., 2006), but these treatments do not totally arrest the growth of *Saprolegnia* species (Van West, 2006). In our study, *P. kaempferi* completely inhibited the growth of the JL strain and, at concentrations of 12.5 mg/ml and 25.0 mg/ml, exhibited significant protective efficacy of 52.63% and 73.68%, respectively, against experimental *Saprolegnia* infections of eggs. This could be due to its ability to produce pseudolaric acid A and B, which possess antifungal activities (Yang and Yue, 2001). In addition, field trials showed that when *P. kaempferi* was applied for five days, the incidence rates of saprolegniosis were reduced by up to 80% in crucian carp eggs and *Megalobrama amlycephala* eggs at Sand Lake, Hubei, China (data not shown). Thus, *P. kaempferi* is promising as an anti-*Saprolegnia* drug for controlling saprolegniasis.

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