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Pathogenicity to Mekong Giant Catfish Eggs of Water Moulds Isolated in the Laboratory from Mekong Giant Catfish Eggs and Rearing Water

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ABSTRACT

Water moulds in the genera *Achlya* and *Saprolegnia* were isolated from eggs of the Mekong giant catfish (*Pangasianodon gigas*) and from the water in the hatching tank at the Inland Aquaculture Research Institute, Phra Nakhon Sriayutthaya province, from 2008 to 2010. The optimal temperature of almost all isolates was 30 °C. The *Achlya* spp. and *Saprolegnia* spp. could tolerate an NaCl medium at 10 and 25 parts per trillion (ppt), respectively. An exception was *Saprolegnia* sp. (E3/52-P2) which could tolerate NaCl up to 30 ppt. The isolates could grow in broth at pH 4–11, while the optimal pH for *Achlya* spp. and *Saprolegnia* spp. was pH 5 and pH6, respectively. The study on pathogenicity of the water moulds isolated in the laboratory showed that the isolates *Achlya* spp. (T.MCF1-02, E.MCF 2-001 and E4/52-10) and *Saprolegnia* sp. (E1/53-12) were pathogenic to the catfish eggs.

Keywords: eggs, Mekong giant catfish, *Pangasianodon gigas*, *Achlya* spp., *Saprolegnia* spp., pathogencity

INTRODUCTION

Fungal disease is a problem that occurs occasionally in fish farms and brood stock husbandry; it causes low productivity of fry and low production of fish cultures (Kwanprasert *et al*., 2007). Diseases caused by water fungi occur more often in the egg stage. The mortality rate of incubated eggs due to water fungal infection sometimes reaches 80–100% (Chukanhom and Hatai, 2004). Water moulds are classified in the order Saprolegniales which includes two families—the Saprolegniaceae (e.g. *Achlya*, *Brevilegnia*, *Dictyuchus*, *Saprolegnia* and *Thraustotheca*) and the Leptolegniaceae (*Aphanomyces Leptolegnia* and *Plectospira*)—totaling 132 species in about 20 genera (Dick, 2001). The Saprolegniales are the best-known group of water moulds. The most important water moulds in the order Saprolegniales are those water moulds in the genera *Achlya* and *Saprolegnia* that can be pathogens of many fish species and their eggs (Willoughby, 1994). Normally, fungal diseases are a secondary infection following an injury (Kitancharoen *et al.,* 1995). At present, the number of Mekong giant catfish in the Mekong River and its branches is being reduced to a critical level. Therefore, this species was put on the endangered species list under the Convention on International Trade in Endangered Species of

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Wild Fauna and Flora (CITES) (Poulsen *et al*., 2004). Control of fungal infection problems will result in improving the egg hatching rate and conserving the natural distribution of the Mekong giant catfish. Therefore, the purposes of this research were the isolation and identification of the genera of water moulds, the study of some of the morphological and biological characteristics and of the pathogenicity of the isolated water moulds on eggs in the laboratory.

MATERIALS AND METHODS

Isolation and identification

Eggs of the Mekong giant catfish and water samples were collected in the spawning seasons from 2008 to 2010 at the Inland Aquaculture Research Institute, Phra Nakhon Si Ayutthaya province Thailand. The samples were collected during July to September 2008 and May to August in both 2009 and 2010. The number and type of samples are shown in Table 1.

The samples were isolated by inoculation or spreading on to glucose-yeast extract agar (GY agar) according to Hatai and Egusa (1979) and incubated at 20 °C for 24–48 hr. For inhibition of bacterial growth, 500 $\mu g.mL^{-1}$ each of ampicillin and streptomycin sulphate was added to the medium. The purification process used a zoospore spread technique according to Choi *et al.* (1999) and was subcultured every month. For morphological observations, the fungi were inoculated into 50 mL of GY broth and incubated at 20 °C for 3–4 d. The young hyphae in the GY broth were washed five times in sterilized distilled water. Fungal isolates were identified to the genus level by the different structures of asexual stage development and the zoospores discharged in sterilized tap water, according to Munchan (2003).

Effect of temperature, NaCl and pH on fungal growth

The advancing edges of the fungal growth colonies were cut with a cork borer (8 mm in diameter) and placed onto the center of Petri dishes (90 mm in diameter) that contained 20 mL of GY agar; the plates were incubated at various temperatures—namely, 10, 20, 30 and 35 °C. Other sets of fungal samples were placed on GY agar containing NaCl (0, 5, 10, 15, 20 and 25 ppt) or GY medium with different pH levels (pH 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12) and incubated at 20 °C. The radial growth of each colony was measured with vernier calipers and recorded for 3 d. All experiments were performed in triplicate. Each experiment was compared with reference fungi—namely, *Saprolegnia diclina* NJM 0501 and *Achlya bisexualis* NJM 0611. All reference strains of freshwater fungi used in this study were supplied by Professor Dr. Kishio Hatai, Nippon Veterinary and Life Science University, Tokyo Japan. Statistical analysis was carried out using the NCSS 2007 program (Version 07.1.21, NCSS, Kaysville Utah) by a one-way ANOVA to compare the average diameter of the fungal

Table 1 Sampling data of eggs of Mekong giant catfish and water from hatching tank at the Inland Aquaculture Research Institute, Phra Nakhon Si Ayutthaya province, Thailand, from 2008 to 2010.

Sampling year	Type of sample	
	Number of eggs	Water from hatchery tank number
2008	85	16
2009	150	30
2010	150	30

colonies at the 95% confidence level. The optimum growth was defined as the examined fungal growth that produced an average diameter of the colony significantly ($P < 0.05$) bigger than the other groups.

Pathogenicity test of water moulds on eggs of Mekong giant catfish

The pathogenicity testing was conducted on the isolated fungi *Achlya* spp. T.MCF 1-02, E.MCF 2-001, E4/52-5, E4/52-8, E4/52-10, E4/52-11, E4/52-12, *Saprolegnia* spp. E1/53-12, and *A. bisexualis* NJM 0611. Zoospores were prepared by subculturing the isolates on GY agar for 2 d, before cutting them into agar blocks (1×1 cm^2); 2–3 pieces were put onto a plate with 20 mL GY broth and incubated at 20 °C for 2 d. Then, the hyphae were washed three times with sterilized tap water and immersed in 30 mL sterilized tap water on a plate for 18–24 hr and incubated at 20 °C. Zoospores that developed were released into the sterilized tap water on the plate. The zoospores were counted with a hemacytometer (Neubauer improved Bright-line; 0.0025 mm^2) and the concentration adjusted to 1×10^4 spores. mL^{-1}. Then, 30 eggs of the Mekong giant catfish that had been artificially fertilized were immersed for 1 hr in sterilized tap water on a plate containing zoospores at 1×10^4 spores.mL^{-1}. The control group was not immersed in the suspension of zoospores. All experimental groups were duplicated and the eggs were incubated at room temperature for 2 d. The amounts of fry hatched were recorded and analyzed. The presence of infection was determined by the appearance of fungal hyphae developing on the egg surface. The non hatched eggs were subcultured for fungal growth on GY agar. Some of the non hatched eggs were fixed with 10% neutral buffered formalin solution and then embedded into paraffin blocks so that histopathological slides could be prepared for examination of any histopathological characteristics.

RESULTS AND DISCUSSION

Isolation and identification

According to the morphological characteristics and zoospores released (Figure 1), as shown in Table 2, the isolated water fungi were identified as nine isolates of the genus *Achlya* and seven isolates of *Saprolegnia* (Munchan, 2003). Fungal isolates from the genus *Achlya* had a puffy and whitish colony appearance and aseptate hyphae. Furthermore, they took 3–4 d to become fully grown on GY agar and break down the GY agar into a circular shape. An asexual stage developed in 18–20 hr after immersion

Table 2 Genera of water moulds isolated from eggs of Mekong giant catfish and water samples of the hatching tank from 2008 to 2010.

Isolation year	Water mould isolated from eggs	Water mould isolated from hatching water
2008	*Achlya* spp. E.MCF1-02	*Achlya* spp. T.MCF2-001
2009	*Achlya* spp. (E4/52-2, E4/52-3, E4/52-5, E4/52-8, E4/52-10, E4/52-11 and E4/52-12)	–
	Saprolegnia spp.(E3/52-P1, E3/52-P2, E3/52-P3 and E3/52-P4)	–
2010	*Saprolegnia* spp. E1/53-12	*Saprolegnia* spp. (T3/53-3 and T3/53-5)

E = water moulds isolated from eggs of Mekong giant catfish; T = water moulds isolated from water of the hatching tank.

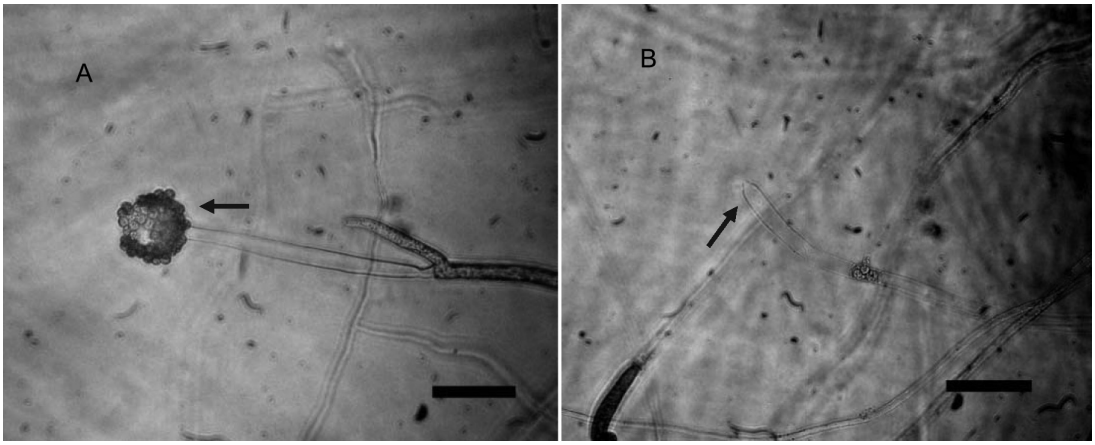

Figure 1 Arrow showing zoosporangium development after zoospore released (scale bar = 50 µm): (A) *Achlya* spp.; and (B) *Saprolegnia* spp.

in sterilized tap water on a plate at 20 °C. The zoospores released were of the achlyoid type. The zoospores occasionally discharged from a discharge pore and the spore clusters usually accumulated at the opening of a zoosporangium (Figure 1A). On the other hand, the fungal isolates from the genus *Saprolegnia* had a cotton-like, whitish colony appearance; their hyphae were aseptate and showed fast growth on a GY agar plate and fully occupied the plate in 2 d. An asexual stage developed in 18–20 hr after immersion in sterilized tap water on a plate at 20 °C. The zoospores released were of the saprolegnoid type. The zoospores were released and swam out from the opening of the zoosporangium (Figure 1B).

Effect of temperature, NaCl and pH on fungal growth

Water mould isolates of *Achlya* spp. (T.MCF 1-02, E.MCF 2-001, E4/52-2, E4/52-3, E4/52-5, E4/52-8, E4/52-10, E4/52-11 and E4/52-12) and *A. bisexualis* NJM 0611 could tolerate an NaCl medium at 10 ppt but could not grow in a higher NaCl medium at 15 ppt. Water mould isolates of the genus *Saprolegnia* spp. (E3/52-P1, E3/53-P3, E3/52-P4, E1/53-12, T3/53-3 and T3/53-5) could tolerate an NaCl medium at 25 ppt but could not grow in a higher NaCl medium at 30 ppt, with the exception of *Saprolegnia* sp. E3/52-P2 and *S. diclina* NJM 0501 that could tolerate a NaCl medium at 30 ppt but could not grow in a higher NaCl medium at 35 ppt (Table 3).

The optimal temperature for growth of the isolated *Achlya* spp. and *Saprolegnia* spp. was 30 °C (Table 3) which was similar to the study of Chukanhom and Hatai (2004) that reported the optimal temperature range for the growth of *S. diclina* NJM 0208 and *A. klebsiana* NJM 2010 was 25–30 and 30–35 °C, respectively. Chukanhom and Hatai (2004) also reported that *S. diclina* NJM 0208 could tolerate a high NaCl medium at 30 ppt but could not grow in a higher NaCl medium at 40 ppt and *A. klebsiana* NJM 2010 could tolerate an NaCl medium at 10 ppt but could not grow in an NaCl medium at 20 ppt, giving the same results as the present study. Almost all isolates could grow in GY broth at pH 4–11, while the optimal pH for *Achlya* spp. and *Saprolegnia* spp. was pH 5 and 6, respectively. The two reference stains, *A. bisexualis* NJM 0611 and *S. diclina* NJM 0501, could grow in GY broth at pH 4–11, while the optimal pH value was pH 5 and 6, respectively, (Table 3). However, the results of the present study with the GY medium at various pH levels

Table 3 Optimal temperature, salinity and pH effects on growth of isolated water moulds compared with *Saprolegnia diclina* NJM 9219 and *Achlya bisexualis* NJM 0611.

Isolation year	Water fungus	Temperature (°C)	Salinity (ppt)	pH
2008	*Achlya* spp. T.MCF 1-02	30	10	5
	Achlya spp. E.MCF 2-001	30	10	5
2009	*Achlya* spp. E4/52-2	30	10	5
	Achlya spp. E4/52-3	30	10	5
	Achlya spp. E4/52-5	30	10	5
	Achlya spp. E4/52-8	30	10	5
	Achlya spp. E4/52-10	30	10	5
	Achlya spp. E4/52-11	30	10	5
	Achlya spp. E4/52-12	30	10	5
	Saprolegnia spp. E3/52-P1	30	25	6
	Saprolegnia spp. E3/52-P2	30	30	6
	Saprolegnia spp. E3/52-P3	30	25	6
	Saprolegnia spp. E3/52-P4	30	25	6
2010	*Saprolegnia* spp. E1/53-12	30	25	6
	Saprolegnia spp. T3/53-3	30	25	6
	Saprolegnia spp. T3/53-5	30	25	6
Reference	*Achlya bisexualis* NJM 0611	30	10	5
	Saprolegnia diclina NJM 0501	30	30	6

ppt = Parts per thousand.

contrasted with Chukanhom and Hatai (2004) who reported that *A. klebsiana* NJM 0210 and *S. diclina* NJM 0208 could grow in GY medium at pH 4–10, with the optimal pH level being 6 and 7, respectively, while in the present study, the fungal isolates of *Achlya* spp. and *Saprolegnia* spp. could grow in the GY medium at pH 4–11, with an optimal pH level of 5 and 6, respectively. Therefore, the results suggested that increasing the water temperature to more than 30 °C with 30 ppt NaCl and pH 6 could prevent fungal infection during the hatching process of the Mekong giant catfish eggs. However, further study involving a safety test for the eggs must be undertaken.

Pathogenicity test of water moulds on eggs of Mekong giant catfish

After 2 d, the infection and percentage rates of non hatched Mekong giant catfish eggs were recorded. The results in Table 4 show that *Achlya* spp. T.MCF 1-02, E.MCF 2-001 and E4/52-10 and *Saprolegnia* sp. E1/53-12 could be pathogenic to eggs of the Mekong giant catfish because no hatched eggs were found. Therefore, the control group and *Achlya* spp. (E4/52-5, E4/52-8 and E4/52-11) could not be pathogens as no fungal hyphae were observed on eggs in these samples. The non hatched eggs were infected and entangled with hyphae on the second day after challenge. The same infected fungi used in the experiment were re-isolates from the non hatched eggs. The study on the histopathology of eggs with fungal infection showed the invasion of water mould mycelia from the cell membrane into the egg yolk of the Mekong giant catfish which induced a gap in the egg (Figure 2). These results clearly demonstrated that experimental fungi had a high pathogenicity to Mekong giant catfish eggs.

Table 4 Percentage average hatch rate of eggs of Mekong giant catfish after 2 d infection with water fungus zoospores.

Water fungus isolate	Percentage average hatch rate
Control group	8.3[a]
Achlya spp.T.MCF 1-02	0[b]
Achlya spp.E.MCF 2-001	0[b]
Achlya spp.E4/52-5	8.3[a]
Achlya spp.E4/52-8	8.3[a]
Achlya spp.E4/52-10	0[b]
Achlya spp.E4/52-11	8.3[a]
Achlya spp.E4/52-12	5.0[a]
Saprolegnia spp. E1/53-12	0[b]
A.bisexualis NJM 0611	0[b]

[a,b] = Different superscripts indicate a significant difference at $P < 0.05$.

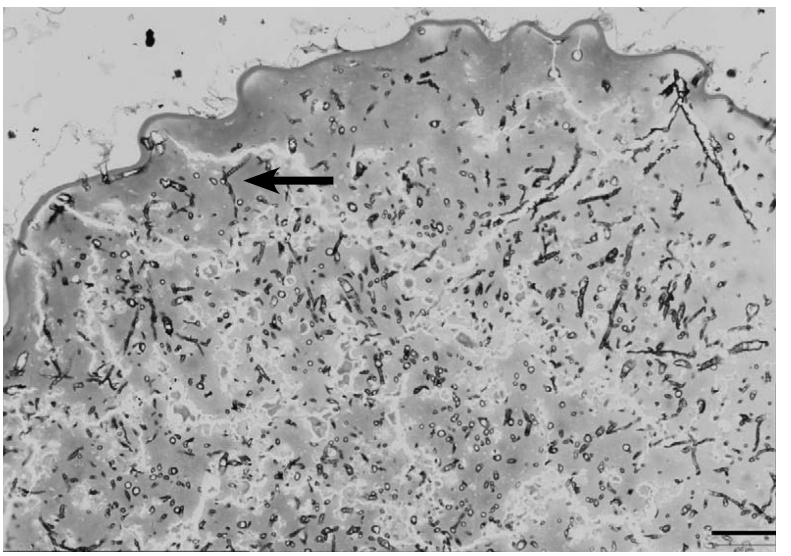

Figure 2 Arrow showing the invasion by water mould mycelia (dark lines) in eggs of the Mekong giant catfish, from cell membrane into egg yolk (light gray color) resulting in gap formation. (Staining with Grocott-Gomori methenamine-silver stain; power at 400×; scale bar = 50 µm.)

Almost all of the hyphae in the eggs had died, because protoplasm in the hyphae generally could not be observed.

CONCLUSION

Solving the fungal infection problem will improve the egg hatching rate and help to conserve the natural distribution of the Mekong giant catfish. Therefore, the purposes of this research were the isolation and identification of the genera of water moulds, study of some morphological and biological characteristics and a pathogenicity test of the isolated fungi on eggs in the laboratory.

The results showed that the optimal temperature of almost all isolates of *Achlya* spp. and *Saprolegnia* spp. was 30 °C. The *Achlya* spp. and *Saprolegnia* spp. could tolerate a NaCl medium at 10 ppt and 25 ppt, respectively. An exception was *Saprolegnia* spp. (E3/52-P2) which could tolerate up to 30 ppt. The isolates could grow in broth at pH 4–11, while the optimal pH for *Achlya* spp. and *Saprolegnia* spp. was pH 5 and 6, respectively. The study on pathogenicity testing of the water moulds isolated in the laboratory showed that the pathogens were *Achlya* spp. T.MCF1-02, E.MCF 2-001 and E4/52-10 and *Saprolegnia* spp. E1/53-12. The non hatched eggs were re-isolated in the same infected fungi used in the experiment. The histopathological detection on infected eggs clearly demonstrated that experimental fungi had a high pathogenicity to Mekong giant catfish eggs.

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