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FULL PAPER

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# Freshwater fungi isolated from eggs of the common carp (*Cyprinus carpio*) in Thailand

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**Abstract** Fungal infection in the eggs of freshwater fish is well known as a problematic disease. We had a chance to examine fungal infection in common carp (*Cyprinus carpio*) eggs at fish farms in Khon Kaen province, northeast Thailand, in February 2002, attempting to isolate fungi from eggs with fungal infection at three fish farms (A, B, and C). Nineteen stocks of fungi from farm A, 2 fungi from farm B, and 2 fungi from farm C were isolated and 3 of them were identified as *Saprolegnia diclina*, *Achlya (A.) klebsiana*, and *Allomyces (Al.) arbuscula. S. diclina*, *A. klebsiana*, and *Al. arbuscula* grew well at 25°–30°C and pH 6–7, at 30°–35°C and pH 6–7, and at 30°–40°C and pH 6–8, respectively. *Saprolegnia diclina* grew in a medium containing NaCl up to a concentration of 3.0%, whereas *A. klebsiana* and *Al. arbuscula* grew poorly in 1.0% NaCl. Artificial infection to platyfish (*Xiphophorus maculatus*) was also made using the 3 fungi selected, in which injured fish were exposed to $10^4$ spores/ml of *S. diclina* and *A. klebsiana* and showed 100% of mortality, but none in the other experiments. This article includes the first description of *Allomyces arbuscula* from fish eggs in Thailand.

**Key words** *Achlya klebsiana* · *Allomyces arbuscula* · Common carp eggs · Pathogenicity · *Saprolegnia diclina*

## Introduction

Freshwater fish are an important protein source for the people of Thailand. Recently, however, the fish population in natural rivers has been decreasing year by year. As a result, fish culture now is becoming an economically important industry. Percentages of seed production for cultured freshwater fishes in Thailand are as follows: tilapia 30.3%, walking catfish 28.6%, common silver barb 16.3%, snake skin gourami 8.7%, striped catfish 4.5%, common carp 2.3%, and other fishes 9.3% (Fisheries Economics Division 2002). Common carp is one of the most economically important fish cultured in Thailand. In Khon Kaen province, in northeast Thailand, there are about 200 fish hatcheries at present. However, diseases caused by lower fungi occur very often at the egg stage. The mortality rate due to fungal infection reaches sometimes 80%–100% of incubated eggs. Fungal infection in eggs of common carp, *Cyprinus carpio* Linnaeus, has been known as a problematic infection at several hatcheries in Thailand. However, there are no reports on fungal infection in Thailand so far, and we had a chance to survey fungal infection in eggs of the common carp hatched in spawning tanks at three farms in Khon Kaen province in February 2002.

Fungal infection in fish eggs is usually caused by the fungi of the family Saprolegniaceae, including the genera *Saprolegnia* and *Achlya* (Wesley and Wolf 1937; Srivastava and Srivastava 1977; Srivastava 1980; Sati 1985; Padmakumar et al. 1985; Czeczuga and Woronowicz 1993; Czeczuga et al. 1995; Kitancharoen and Hatai 1998). Willoughby and Lilley (1992) reported the first records of *Allomyces* as a saprophyte on the dead fish in water in Thailand. Khulbe et al. (1995) succeeded in isolating *Allomyces anomalus* Emerson from the Gomati River in India. This species has also been reported from common carp eggs and showed poor pathogenicity to common carp eggs (Sati 1983) and to beta eggs (William et al. 1962).

In this article, we describe three species of fungi isolated from common carp eggs with fungal infection, especially *Allomyces arbuscula* Butler, isolated from Thailand for the first time.

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## Materials and methods

### Isolation and identification

Eggs of the common carp *Cyprinus carpio* infected by fungi were collected from spawning tanks at three fish farms (A, B, and C) in Khon Kaen province, northeast of Thailand, in February 2002. The eggs with fungi were inoculated on GY agar (Hatai and Egusa 1979) at 25°C and 500 μg/ml each of ampicillin and streptomycin sulfate were added to the medium to reduce bacterial contamination. A pure culture was made by the single spore culture method. For morphological observations, the isolated fungi were inoculated into GY broth and incubated at 25°C for 3–4 days. The young hyphae in GY broth were then washed 3–4 times in sterile tap water and incubated at 25°C. The fungi were identified according to Cocker and Matthews (1937), Sparrow (1960), Scott (1961), Seymour (1970), Johnson (1956), and Karling (1977) from the morphological characteristics on hemp seeds cultures in sterile tap water.

### Effect of various temperatures on fungal growth

Temperature range and optimum temperature for growth were determined using the mycelia of the fungi. The advancing edge of growing colonies were cut with a no. 2 cork borer (5.5 mm in diameter) and placed onto center of Petri dishes that contained 25 ml GY agar, instead of potato dextrose agar medium (PDA) (Nissui Seiyaku, Tokyo, Japan) in the case of *Allomyces arbuscula* NJM 0212. Plates were incubated at various temperatures: 5°, 10°, 15°, 20°, 25°, 30°, 35°, and 40°C. The radial growth of the colony was measured with vernier calipers every day. All experiments were performed in duplicate.

### Effect of sodium chloride (NaCl) on fungal growth

An agar block of the isolate was inoculated on GY agar plates containing various concentration of NaCl (0%, 1.0%, 2.0%, 3.0%, and 4.0%) and incubated at 25°C. The radial growth of the colony was measured as already mentioned.

### Effect of pH on fungal growth

The GY broth containing 15 mM GTA buffer solution [3,3-dimethylglutaric acid, 0.80 g; Tris (hydroxymethyl) aminomethane, 0.61 g; 2-amino-2-methyl-1,3-propanediol, 0.53 g; DW 1000 ml] was adjusted to pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, or 12.0 by adding 1N HCl or 1N NaOH. The GY agar block with young mycelia was cut off with a no. 2 cork borer; the block was then put into a test tube containing 5.0 ml GY broth with GTA buffer. Each test medium was incubated at 25°C, and growth of hyphae was observed by naked eye every day for 1 week. After observation for 1 week, the negative test agar blocks were transferred onto GY agar to reconfirm hyphal survival. All experiments were done in duplicate.

### Pathogenicity test

The test was done using the platyfish *Xiphophorus maculatus* Günther in a 2.0 l plastic cabinet tank with 1.0 l dechlorinated tap water. Healthy fish were purchased from a pet shop in Tokyo, Japan. The fish averaged 1.13 ± 0.17 g in weight and 32.25 ± 1.78 mm in length. Each fish was artificially injured by removing 2–3 scales at left lateral body and mild scalping. Two fish were placed in each plastic cabinet tank and challenged with the fungal zoospores. Zoospores at $1.0 \times 10^4$, $1.0 \times 10^3$, and $1.0 \times 10^2$ spores/ml were used as the treatment in each group; in contrast, as the control group, fish were contacted by sterile tap water only. Dead or moribund fish were removed from the tank and immediately examined on a routine basis. Confirmation of the causative agent was achieved by reisolation of the fungus on GY agar from the infected fish. The experiments were continued for 10 days at 15°C.

## Results

### Isolation and identification

Brood stocks of common carp *C. carpio*, 300–500 g in body weight, were kept in a concrete tank at 25°–27°C. An artificial net made from plastic rope was added to the tank for attachment of eggs; after 24 h the plastic net was transferred to the hatchery tank, and fungal infection appeared 24 h later. Infection rates reached 40%, 50%, and 60% in farms A, B, and C, respectively. The infected eggs changed from the normal transparency to a whitish color, and numerous hyphae were observed with the naked eye. Nineteen fungi from farm A, 2 fungi from farm B, and 2 fungi from farm C were isolated from the infected eggs. Twenty-two fungi belonging to Saprolegniales and 2 fungi belonging to Blastocladiales were placed into pure isolation (Table 1). Isolates NJM 0208, NJM 0210, and NJM 0212 of the 23 isolates were selected for more detailed identification. The isolated NJM 0208, NJM 0210, and NJM 0212 were identified as *Saprolegnia diclina* Humphrey, *Achlya klebsiana* Pieters, and *Allomyces arbuscula*, respectively. The morphological characteristics of the fungi are as follows.

### *Saprolegnia diclina* NJM 0208

Fungus characteristics was as follows: cotton-like whitish colony on GY agar, reached full plate after 2 days, at 25°C. Hyphae in GY broth were stout, aseptate, branched with numerous shiny spherical granules, 7–17 μm in diameter. Zoospore formation was observed about 12–18 h after mycelia were transferred into sterile tap water at 20°C. Zoospores were discharged from the end of the discharge tube and swam away from the tip of the tube in the manner of the "saprolegnoid type," in which discharge time was 1–5 min. Zoospores were elongate. Encysted zoospores were globose, (6–)8(–12) μm in diameter. The antheridial

**Table 1.** Summary of fungi isolated from common carp eggs

| Farms | No. of examined eggs | No. of isolated fungi | Identification of isolated fungi | | Remarks[a] |
|---|---|---|---|---|---|
| A | 15 | 19 | *Saprolegnia diclina* | 15 | NJM 0208 |
| | | | *Achlya klebsiana* | 2 | |
| | | | *Allomyces arbuscula* | 2 | NJM 0212 |
| B | 2 | 2 | *Achlya klebsiana* | 2 | NJM 0210 |
| C | 2 | 2 | *Saprolegnia diclina* | 2 | |

[a] Fungi examined in more detail in this study

branches were single or the sparingly branched diclinous type. In the sexual stage, oogonia were found on top of the principal vegetative thalli after incubation on hemp seed for 4–5 days at 20°C; the oogonia were mainly spherical in shape, (35–)68–78(–102) μm in diameter. The oogonial wall was smooth, pitted only under point of attachment of antheridial brances. Oospores were mainly spherical in shape, (4–)7–10(–13) oospores/oogonium, (18–)24(–29) μm in diameter; inside mature oospore mainly centric, filled oospores.

From these morphological characteristics and mode of zoospore release, the fungus was identified as *Saprolegnia diclina* Humphrey.

Specimen examined: The strain NJM 0208 was isolated from common carp eggs, *C. carpio* with fungal infection, obtained from hatchery fish farm in Khon Kaen province, Thailand, in February 2002.

*Achlya klebsiana* NJM 0210

The fungus had morphological characteristics as follows: puffy and whitish colony on GY agar, separable agar, and reached full plate after 5 days, at 25°C. Hyphae in GY broth were stout, aseptate, and branched with numerous shiny spherical granules. The hyphae were 15–30 μm in diameter. Zoospore formation was observed about 8–12 h after mycelia were transferred into sterile tap water, at 25°C. Zoospores were discharged from the end of the discharge tube and accumulated at the tip of the tube in the manner of the "achlyoid type," in which the discharge of the zoospore continued for 1–4 min. Zoospores were elongated in shape. Encysted zoospores were globose, (6–)10(–14) μm in diameter. In the sexual stage, oogonia were found on top of the principal vegetative thalli after incubation on hemp seeds for 3–4 days at 20°C. The antheridial branches were single or of the sparingly branched diclinous type. The oogonia were mainly spherical in shape, (37–)56(–76) μm in diameter. The oogonial wall was smooth and unpitted. Oospores were mainly spherical in shape, (1–)3–4(–8) oospores/oogonium, (18–)22(–29) μm in diameter. Mature oospores were mainly eccentric and not filled.

From these morphological characteristics and mode of zoospore release, the fungus was identified as *Achlya klebsiana* Pieters.

Specimen examined: Strain NJM 0210 was isolated from common carp, *C. carpio*, eggs with fungal infection, obtained from hatchery fish farm in Khon Kaen province, Thailand, in February 2002.

*Allomyces arbuscula* NJM 0212 (Fig. 1)

Hyphae in GY broth small, slender, rhizoid present, branches usually dichotomous, separated from the nodes by distinct and complete septa, (7–)12(–14) μm in diameter. Zoospores escaping singly, discharging through usually one or two apical holes, the discharging of the zoospores continued for 2–10 min, usually having one flagellum, oval when swimming, amoeboid before encysting. Encysting zoospores, round, (8–)12(–15) μm in diameter. Zoosporangia oval, 12–39 × 46–61 μm in size, formed at terminal and sympodially arranged, often clustered on the short branches, rarely in chains. Resistant sporangia borne in the same way as the zoosporangia, changing later the zoosporangia, the same size and shape with zoosporangia, at maturity enclosed in a thin sheath, and the wall brown with finely pitted wall. Zoospores finally fall through an apical pore of the zoosporangia. Sexual stage of the fungus was not observed.

From these morphological characteristics the fungus was identified as *Allomyces arbuscula* Butler.

Specimen examined: The strain NJM 0212 isolated from common carp eggs, *C. carpio*, with fungal infection, obtained from a hatchery fish farm in Khon Kaen province, Thailand, in February 2002.

Effect of various temperatures on fungal growth

*Saprolegnia diclina* NJM 0208 grew at a range of 5°–35°C with optimal temperature at 25°–30°C. *Achlya (A.) klebsiana* NJM 0210 grew at a range of 5°–35°C with optimal temperature at 30°–35°C. *Allomyces (Al.) arbuscula* NJM 0212 grew at a range of 20°–40°C with optimal temperature at 30°–40°C (Table 2).

Effect of sodium chloride (NaCl) on fungal growth

*Saprolegnia diclina* NJM 0208 was able tolerate up to 3.0% NaCl. No growth was observed on GY agar containing 4.0%NaCl, whereas *A. klebsiana* NJM 0210 and *Al. arbuscula* NJM 0212 were able to tolerate up to 1.0% NaCl, but no growth was observed on GY agar containing 2.0% NaCl (Table 3).

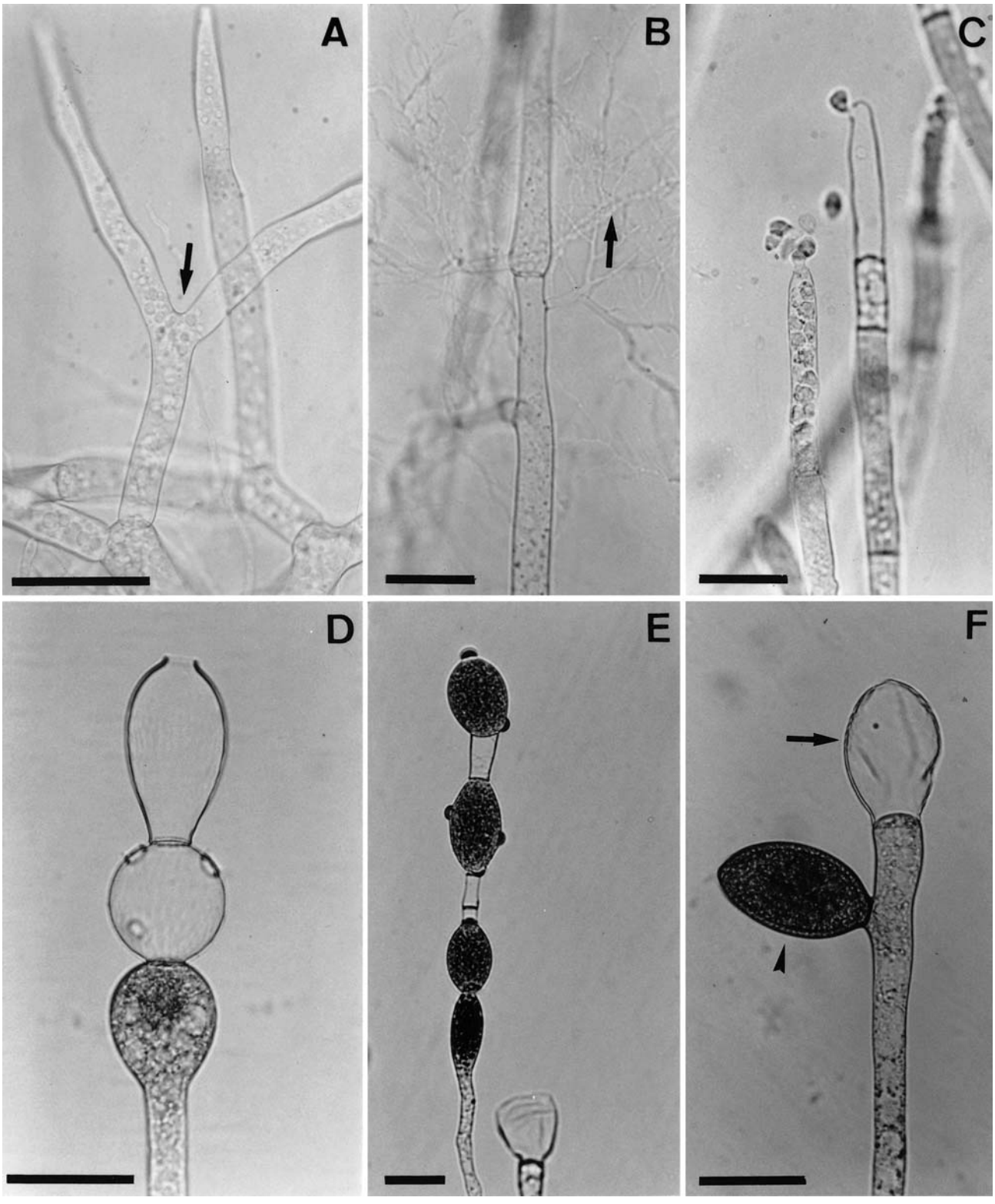


**Fig. 1.** Morphological characteristics of *Allomyces arbuscula* NJM 0212. **A** Vacuolate vegetative hyphae showing dichotomous branch (*arrow*), grown in GY broth. **B** Septate hypha with rhizoid structure (*arrow*). **C** Zoosporangia showing "swimming away from zoosporangia" type of zoospore discharge. **D** Empty zoosporangia in chains and various sites of exit pores. **E** Young vegetative hyphae showing young thin-walled zoosporangia. **F** Resistant sporangium (*arrowhead*) and empty sporangium (*arrow*). *Bar* 50 μm

**Table 2.** Effect of various temperatures on the growth of fungi isolated from common carp eggs, *Saprolegnia* (*S.*) *diclina* NJM 0208 and *Achlya* (*A.*) *klebsiana* NJM 0210 on GY agar (PDA for *Allomyces* (*Al.*) *arbuscula* NJM 0212)

| Isolates | Days after incubation | Temperature (°C) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 |
| *S. diclina* | 2 | 2.0[a] | 6.8 | 19.0 | 32.5 | 37.5 | 37.5 | 28.8 | 0 |
| NJM 0208 | 4 | 8.0 | 19.8 | 37.5 | 37.5 | 37.5 | 37.5 | 37.5 | 0 |
| | 6 | 14.8 | 30.5 | 37.5 | 37.5 | 37.5 | 37.5 | 37.5 | 0 |
| *A. klebsiana* | 2 | 0 | 0.5 | 7.8 | 14.5 | 20.8 | 27.0 | 25.3 | 0 |
| NJM 0210 | 4 | 0.5 | 2.5 | 18.3 | 29.5 | 36.8 | 37.5 | 37.5 | 0 |
| | 6 | 1.0 | 4.3 | 28.5 | 37.5 | 37.5 | 37.5 | 37.5 | 0 |
| *Al. arbuscula* | 2 | 0 | 0 | 0 | 0.8 | 2.4 | 12.4 | 11.2 | 14.8 |
| NJM 0212 | 4 | 0 | 0 | 0 | 4.3 | 10.0 | 28.1 | 28.5 | 32.4 |
| | 6 | 0 | 0 | 0 | 9.5 | 20.2 | 37.5 | 37.5 | 37.5 |

PDA, potato dextrose agar
[a] Mean radius of colony (mm); maximum radius of the fungi growth in this experiment was 37.5 mm

**Table 3.** Effect of salinity on the growth of fungi isolated from common carp eggs on GY agar at 25°C

| Isolates | Days after incubation | Sodium chloride (%) | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 |
| *S. diclina* | 2 | 37.5[a] | 22.8 | 14.8 | 2.0 | 0 |
| NJM 0208 | 4 | 37.5 | 37.5 | 30.8 | 4.5 | 0 |
| | 6 | 37.5 | 37.5 | 37.5 | 11.0 | 0 |
| *A. klebsiana* | 2 | 18.0 | 1.0 | 0 | 0 | 0 |
| NJM 0210 | 4 | 37.5 | 8.3 | 0 | 0 | 0 |
| | 6 | 37.5 | 17.3 | 0 | 0 | 0 |
| *Al. arbuscula* | 2 | 2.3 | 0 | 0 | 0 | 0 |
| NJM 0212 | 4 | 2.8 | 0 | 0 | 0 | 0 |
| | 6 | 3.0 | 0.5 | 0 | 0 | 0 |

[a] Mean radius of colony (mm); maximum radius of the fungi growth in this experiment was 37.5 mm

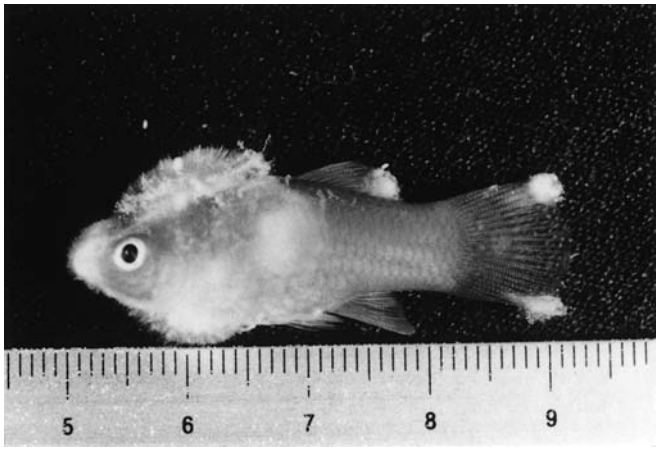


**Fig. 2.** Injured platyfish artificially infected with *Saprolegnia diclina* NJM 0208 at $1.0 \times 10^4$ spores/ml. Note fins and injured area were infected with the fungi. *Bar* in centimeters

### Effect of pH on fungal growth

*Saprolegnia diclina* NJM 0208 and *A. klebsiana* NJM 0210 grew in GY broth at a range of pH 4–10 with optimal pH at 6–7. *Allomyces arbuscula* NJM 0212 grew in GY broth at pH 5–9 with optimal pH at 6–8 (Table 4).

### Pathogenicity test

Artificial infection of platyfish (*Xiphophorus maculatus*) was done using *S. diclina* NJM 0208, *A. klebsiana* NJM 0210, and *Al. arbuscula* NJM 0212. The fungal lesions were cotton like, whitish in color, and appeared 24 h after challenging; the fish finally died within 3 days (Fig. 2). Mortality in injured fish challenged with $10^4$ spores/ml of *S. diclina* NJM 0208 and *A. klebsiana* NJM 0210 was 100%. The mortality, however, was zero in the other treatments (Table 5).

## Discussion

Fungi were collected from common carp eggs with fungal infection at fish farms in Khon Kaen province in the course of the survey in February 2002 and were identified as *Saprolegnia diclina*, *Achlya klebsiana*, and *Allomyces arbuscula*.

The range of optimal temperature for hyphal growth of *S. diclina* NJM 0208, *A. klebsiana* NJM0210, and *Al. arbuscula* NJM 0212 was 25°–30°C, 30°–35°C, and 30°–40°C, respectively. The results were different from those in freshwater fungi isolated from the temperate zone in that they tolerate temperatures up to 30°C (Hussein and Hatai 1999). All the isolates, especially *Al. arbuscula*, tolerated at high temperature up to 40°C. It was suggested that the isolates had adapted to the environment of the tropics.

The isolate of *S. diclina* NJM 0208 was able to tolerate in the environment up to 3.0% of NaCl, whereas the isolates of *A. klebsiana* NJM 0210 and *Al. arbuscula* NJM 0212 were able to tolerate up to 1.0% NaCl. It was suggested that all the fungi can survive in high salinity freshwater and also in brackish water. All the isolates grew at a wide range of pH 4.0–10.0 (5.0–9.0 for *Al. arbuscula*) and the optimum pH for growth was pH 6.0–8.0. These results suggest that fungal growth is not strongly affected by the pH of freshwater.

**Table 4.** Effect of various pH values on the growth of fungi isolated from common carp eggs in GY broth at 25°C

| Isolates | Days after incubation | pH[b] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| *S. diclina* | 2 | −[a] | ± | + | + | + | + | ± | − | − | − |
| NJM 0208 | 4 | − | + | ++ | +++ | +++ | ++ | + | ± | − | − |
| | 6 | − | + | +++ | +++ | +++ | ++ | + | + | − | − |
| *A. klebsiana* | 2 | − | ± | + | + | + | + | ± | − | − | − |
| NJM 0210 | 4 | − | + | ++ | +++ | +++ | ++ | + | ± | − | − |
| | 6 | − | + | +++ | +++ | +++ | ++ | + | + | − | − |
| *Al. arbuscula* | 2 | − | − | − | + | + | + | − | − | − | − |
| NJM 0212 | 4 | − | − | ± | + | + | + | ± | − | − | − |
| | 6 | − | − | + | ++ | ++ | ++ | + | − | − | − |

[a] Fungal growth: −, no growth; + to +++, slight to excellent growth
[b] Adding 15 mM GTA for pH control

**Table 5.** Pathogenicity of fungi isolated from common carp eggs to platyfish *Xiphophorus maculatus*

| No. of zoospores challenged (spores/ml)[a] | *S. diclina* NJM 0208 | | *A. klebsiana* NJM 0210 | | *Al. arbuscula* NJM 0212 | |
|---|---|---|---|---|---|---|
| | Injured | Intact | Injured | Intact | Injured | Intact |
| $1.0 \times 10^4$ | 100[b] | 0 | 100 | 0 | 0 | 0 |
| $1.0 \times 10^3$ | 0 | 0 | 0 | 0 | 0 | 0 |
| $1.0 \times 10^2$ | 0 | 0 | 0 | 0 | 0 | 0 |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 |

[a] Two platyfish were used in each experiment
[b] Seven-day cumulative mortality of platyfish artificially infected with the fungi

In the infection study, both *S. diclina* and *A. klebsiana* showed low pathogenicity to the platyfish *X. maculatus,* which was similar to the findings by Vishniac and Nigrelli (1957) and William et al. (1962), although *Al. arbuscula* was not pathogenic. These results suggest that *Al. arbuscula* was saprophytic in nature and the fungus might be infected secondarily on dead eggs, as previously reported by William et al. (1962) and Willoughby and Lilley (1992). Although the pathogenicity of the isolates to common carp eggs was not fully examined in this study, the fungi *S. diclina* and *A. klebsiana* might be pathogens of *C. carpio* eggs, because infection in the eggs of *C. carpio* has often been observed at the hatchery.

This is the first report of *Al. arbuscula* that was isolated from fish eggs in Thailand.

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