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ISOLATION AND IDENTIFICATION OF *Aphanomyces* SPECIES FROM NATURAL WATER BODIES AND FISH FARMS IN SELANGOR, MALAYSIA

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ABSTRACT

This study was conducted to isolate and identify fresh water fungi species from the Malaysian natural water bodies and fish farms and to examine the pathogenicity of the isolates as a confirmative identification tool for epizootic ulcerative syndrome (EUS) outbreak in Selangor state, Malaysia. For this aim, 165 water samples and 62 infected fish collected from 12 stations were tested in which 35 and 24 samples were found to be positive for fungi contamination and/or infection, respectively. The isolates were morphologically characterized; from 59 isolates, 32 were identified as *Saprolegina,* 21 as *Achlya* and 6 as *Aphanomyces* species. Experimental infection was carried out by intramuscularly injection of the *Aphanomyces* spp. isolates to the Malaysian moonlight gourami (*Trichogaster Microlepis*), where no mortality and no signs of EUS were observed in the fish groups. Histopathology test also revealed no signs of damage in the skin, muscles and other tissues following infection with the isolates indicating that all the *Aphanomyces* isolates were non-pathogenic.

Key words: *Aphanomyces*, *Saprolegnia*, *Achlya*, Epizootic Ulcerative Syndrome (EUS), histopathology

INTRODUCTION

Freshwater fish are an important source of food and protein for the traditional and cultural livelihood of many riparian communities in Malaysia. Assessments show that Peninsular Malaysia is among top ten countries in the world in the terms of numbers freshwater fish species recorded; it has been ranked fourth among the Asian countries by their freshwater fish species-to-area ration (Othman *et al.,* 2002; Chong *et al.,* 2010).

Oomycete (water mould) is an economically important group of mycotic agents causing epizootics ulcer among freshwater fish around the world (Dieguez-Uribenodo *et al.,* 1996). This group of fungi can infect host organisms when they are exposed to stress or when the environmental conditions and water quality change (Kiziewicz & Nalepa, 2008). Water mould belongs to the order Saprolegniales and family Saprolegniaceae containing 19 genera and about 150 species of which *Achlya*, *Aphanomyces* and *Saprolegnia* are significant as fungi infectious agents in aquaculture (Hatai & Hoshiai, 1993). *Saprolegnia* and *Aphanomyces* species have been shown to be responsible for serious infections in fish. Among 30 species of *Aphanomyces* only a few have been determined as disease-causing agents in freshwater animals (Kitancharoen & Hatai, 1997; Johnson *et al.,* 2002; Royo, 2004; Royo *et al.,* 2004; Takuma *et al.,* 2010). *Aphanomyces invadans* (also called *A. piscicida* and *A. invaderis*), for example, has been identied as a causative agent of epizootic ulcerative syndrome (EUS) in some fish like ayu, atlantic menhaden (Dykstra *et al.,* 1986), sea mullet and sandwhiting (Fraser *et al.,* 1992).

EUS is the most economically destructive diseases of fresh and brackish water farmed and wild fish in the Asia-Pacific region (Baldock *et al.,* 2005). The main EUS clinical signs are mycotic granulomas, red spots and dermal ulceration; that is why it is also named Mycotic Granulomatosis (MG)

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in Japan, Red Spot Disease in Australia (RSD) (Callinan & Keep, 1989) and Ulcerative Mycosis (UM) in the United States of America (Dykstra *et al.,* 1986). Identication of oomycetes is depend on morphology and sporulation characteristics like sexual (oogonium, antheridium, oospore) and asexual (sporangium, primary, secondary zoospors) structures (Sparrow, 1960). *Apahnomyces* species identication is basically encountered some taxonomic problems that make it difficult, one of them is lack of reference isolates for some species and another one is inability of large number of sterile animal parasitic species to produce reproductive structure that make them hard to identify morphologically and need to be identified using infection studies to determine their ability to parasitize their host or could be identified by using a number of physiological properties (Ballesteros *et al.,* 2006). Producing sexual reproductive characters are essential for zoosporic fungi identification but such structures have not been observed in cultures of *A. invadans*. Therefore, it is possible to be distinguished from saprophytic *Aphanomyces* species by pathogenic and growth characteristics (Lilley *et al.,* 2003).

The first EUS outbreak has been reported in farmed freshwater ayu (*Plecoglossus altivelis*) in Japan in 1971, and later in 24 countries within four continents, Northern America, Southern Africa, Asia and Australia (Oidtmann, 2011). EUS reported for the first time in southern peninsular Malaysia in 1979 and later, in December 1980, in rice-field fishes in northern Malaysia. The major affected species were snake skin gourami (*Trichogaster pectoralis)*, striped snakehead *(channa striata*), climbing perch (*Anabas testudineus*) and walking catfish (Lilley *et al.,* 2001). Regarding to the importance of EUS, OIE (World Organization for Animal Health) member countries are obliged to make an official notification in the case of any occurrence or outbreak of the disease (Oidtmann, 2011). So far, there are no studies on the aquatic pathogenic oomycetes and no scientific reports of EUS outbreak in Malaysia as a member of OIE. Furthermore, there are no studies on the aquatic pathogenic oomycetes. Hence, the present study was organized by the Faculty of Veterinary Medicine, University Putra Malaysia (UPM) to isolate and identify *Aphanomyces* spp., as causative agent of EUS outbreak, from water and infected fish in Selangor state, Malaysia.

MATERIALS AND METHODS

Sampling

One hundred sixty-five water samples and sixty-two infected fish were collected from 12 different stations (farms and natural water bodies) in Selangor state, Malaysia from May 2011 to May 2012. The stations were Kuala Kubu Bharu-North of Selangor, Sabak Bernam-North West of Selangor, Kuala Selangor-West of Selangor, Klang-West of Selangor, Hulu Langat-East of Selangor, Kuala Lumpur - Middle of Selangor, Rawang-Middle of Selangor, Serikembangan-Middle of Selangor, Puchong-Middle of Selangor, Putrajaya-South East of Selangor, Kualu Langat-South of Selangor, Sepang-South of Selangor. Twenty-two samples were collected from rivers, 18 from estuaries, 23 from lakes, 32 from ponds, 20 from streams and 50 from fish farms. Water samples were transferred to the Aquatic Animal Health laboratory at the Universiti Putra Malaysia in sterile 500 ml bottles. The fish were checked for any EUS characteristic clinical signs during the sampling program.

Fungi Isolation and Identification

Fungi species were isolated from the water samples by baiting method and using sterilized maize, green peas, fish meat, insect's wings and hemp seed as previously described by Stevens (1974). Fungus sporogenesis and typical asexual characteristics were checked under light microscope for genus identification. Infected baits with visible fungal colonies were transferred to autoclaved ex-mine pond water (APW) supplemented with penicillin-streptomycin (10 mg/l). After adding new baits, they were incubated again for another 14 days at room temperature (RT) to obtain pure cultures. To observe sexual stage sporogenesis, hyphae were baited with hemp seed in sterile tap water; after 5 days incubation at 20ºC (Chukanhom & Hatai, 2004), reproductive structures of the fungi were examined under a light microscope.

During sampling program, natural and farmed fish were examined for lesions. Wet mounts were prepared from the scraped skin of 45 infected fish to confirm the presence of fungus hyphae as a preliminary diagnostic method (Ferguson, 1989). Fungi were isolated from the fish samples according to the methods described by Hussein *et al.* (2001) and Stueland *et al.* (2005) with some modifications. Briefly, fungal infected fish were sacrificed with an overdose of MS-222 and affected muscles were excised carefully. To limit bacterial contamination, the tissue samples were washed with sterile distilled water before transferring into sterile glucose-yeast (GY) broth containing penicillin G (100 units/ml) and streptomycin (100 µg/ml). Pure cultures were obtained by repeatedly transferring the hyphae on GY agar supplemented with antibiotics. Subsequently, the hyphae were incubated in APW at 20ºC using hemp seed and examined under a light microscope for morphological identification (Sparrow, 1960; wolf, 1944).

To assess pathological changes in the infected fish tissues, immediately after anesthesia, a small portion of the skin and muscle from the infected area were collected and fixed in 10% buffered formalin for further histopathological test.

Pathogenicity Test

Aphanomyces spp. isolates were assessed for their ability to show the EUS clinical signs. The pathogenicity test was carried out using gourami (*Trichogaster microlepis)*, a fish species that is known to be susceptible to the EUS infection (AF Zali *et al.* unpublished data). Native and healthy fish with an average weight of 12 g and 70 mm in length were purchased from a pet shop. They were kept at 21ºC in 35x75cm glass tanks with de-chlorinated tap water and aerated filter. All the fish were acclimatized for a week and fed once daily with the commercially available feed pellets. Each group was contained 2 fish injected with the zoospores of the *Aphanomyces* isolate. As controls, another two fish were injected with APW.

Fungal sporulation was stimulated according to the method described by Vandersea *et al.* (2006) with some modifications. Briefly, 5 mm of the 4-day old hyphae on GY agar were inoculated into GY broth. After 4 days incubation at 25ºC, the resulted hyphae were collected and washed thrice with sterile deionized water followed by 24 hr incubation in 1 ml sterile deionized water at 20ºC. A 0.3–0.4 ml suspension of the zoospores (1000 zoospores/ml) were prepared; after anesthetizing the fish with MS-222 at 150 ppm, they were injected with the zoospore suspension via the intramuscular rout, at the left lateral body below dorsal fin. Fish in the both groups were checked for the clinical signs daily for 30 days. At the end of the experiments, fish were sacrificed with an overdose of MS-222, skin muscles at the injection area was taken and fixed in 10% buffered formalin for histopathological study.

Histopathology test

Histopathology test was done following the method described by Luna (1968). Fixed issues were decalcied, dehydrated, and then embedded in paraffin wax. Paraffin blocks were sectioned at 3 to 4 um with a rotary microtome. The slides were then stained with haematoxylin and eosin (H & E) and examined under a light microscope for ulcerative and granulomas reactions.

RESULTS

Among 165 water samples taken from water bodies, 35 samples were found to be positive for fungi growth on the maize and hemp seed baits of which, based on the morphological characteristics, 19 isolates were identified as *Saprolegnia* spp., 10 isolates as *Achlya* spp., and 6 isolates as *Aphanomyces* spp. (Table 1). Among 62 infected fish, 24 samples were positive for the fungal infection, *Saprolegnia* (13 samples) and *Achlya* (11 samples). Based on the light microscopic observations, the fungi isolates were characterized as follow:

***Saprolegnia* spp. isolates**

The *Saprolegnia* spp. isolates were observed as branching filamentous cells with non-septate hyphae, irregularly arranged spore in the sporangium, and zoospores escaping separately from the sporangium saprolegnoid type with no lateral branches from below (Fig. 1). The isolates produced cotton-like whitish colony on GY agar covering the plate after 2 days at 25ºC (Fig. 4a). Histopathology experiments from *Saprolegnia* infected fish tissues showed some necrosis in the skin and muscle, the common signs of *Saprolegina* infections (Fig. 5).

***Achlya* spp. isolates**

The *Achlya* spp. isolates were observed as stout aseptate branched hyphae with irregularly arranged spore in the sporangium. All of the spores encysting at the tip of the sporangium and sporangia were renewed by lateral branches from below. Zoospores were discharged achlyoid type from the end of the sporangium and accumulated at the tip of the tube (Fig. 2). Puffy and whitish colony observed on the GY agar which reached full plate after 5 day at 25°C (Fig. 4b). Histopathology experiments from infected fish tissues showed some necrosis in the skin and muscle of the infected fish with *Achlya* which are common in this kind of infection (Fig. 6).

***Aphanomyces* spp. isolates**

The *Aphanomyces* spp. isolates exhibited vegetative mycelium about 5–10 lm in diameter, aseptate, smooth, slightly wavy, moderately branched. The isolate produced sporangia with a single row of primary spores and the primary spores were eventually released and encysted at the hyphal tip forming spore-balls, characteristic for the genus *Aphanomyces* (Fig. 3). No oogonia or antheridia were observed, thus the strains appeared to be sterile and lacked sexual reproduction. Zoosporangia were slender with the same diameter as hyphae. The isolates produced star like colonies in GY broth at RT (Fig. 4c).

Pathogenicity

Aphanomyces spp. developed no sexually structure and could not be identied to species level; thus, they were injected to the EUS susceptible fish to observe any clinical sign like those causes by *Aphanomyces invadans* during EUS outbreak. All

the fish injected with the *Aphanomyces* spp. zoospores and/or APW (controls) only showed some reddening in the injection area; it disappeared at day three post injection (Fig. 7). Neither *Aphanomyces*-injected nor APW-injected fish showed any EUS characteristic clinical signs. No mortality and no change in swimming behavior were observed among the groups during the examination.

Histopathology

The pathological symptoms associated with the *Aphanomyces* isolated were found to be different from those resulting from *A. invadans* infection. The most striking characteristic of the EUS disease is mycelial growth into the host tissue, whereas no hyphae penetration or cell damage was detected in the skin and muscles tissues following injection with the *Aphanomyces* spp. isolated in this study.

Table 1. Selangor state water bodies sampling results

Row	Water Bodies	Temp. (°C)	pH	Sampling Month	Fungi Communication
1	Lake	31	8.35	May	*Saprolegnia* sp.
2	Stream	30.1	6.21	May	*Saprolegnia* sp.
3	Fish farm	29.2	7.3	May	*Achlya* sp.
4	Fish farm	29	7.68	May	*Saprolegnia* sp.
5	Fish farm	30	7.5	May	*Saprolegnia* sp.
6	Estuary	30.5	8.00	June	*Saprolegnia* sp.
7	Pond	31	6.8	June	*Achlya* sp.
8	Stream	31.8	6.9	June	*Aphanomyces* sp.
9	River	31.2	6.7	June	*Saprolegnia* sp.
10	Fish farm	31	6.5	July	*Achlya* sp.
11	Pond	30	6.6	July	*Saprolegnia* sp.
12	Fish farm	29	6.9	September	*Saprolegnia* sp.
13	Pond	31	7.9	September	*Saprolegnia* sp.
14	Fish farm	29.5	6.9	October	*Achlya* sp.
15	Fish tank	29	6.7	October	*Aphanomyces* sp.
16	Fish farm	30	7.8	October	*Achlya* sp.
17	River	29.5	6.75	November	*Saprolegnia* sp.
18	Estuary	30	7.1	December	*Aphanomyces* sp.
19	Pond	29	8.1	December	*Achlya* sp.
20	Estuary	29.4	7.7	December	*Saprolegnia* sp.
21	Pond	31	7.15	January	*Aphanomyces* sp.
22	Lake			January	*Achlya* sp.
23	Lake	29.5	6.99	January	*Saprolegnia* sp.
24	Stream	29.2	6.45	January	*Saprolegnia* sp.
25	Fish tank	28.8	6.3	January	*Aphanomyces* sp.
26	Lake	29	7.4	February	*Saprolegnia* sp.
27	Pond	28.3	6.6	February	*Aphanomyces* sp.
28	Fish tank	28	7.2	February	*Achlya* sp.
29	Fish tank	29.5	7	March	*Saprolegnia* sp.
30	Pond	29	7.5	March	*Achlya* sp.
31	Fish tank	29.6	6.9	March	*Saprolegnia* sp.
32	Pond	30	7.7	April	*Saprolegnia* sp.
33	Pond	29.7	6.5	April	*Achlya* sp.
34	Pond	30	6.8	April	*Saprolegnia* sp.
35	Stream	30.2	7.3	April	*Saprolegnia* sp.

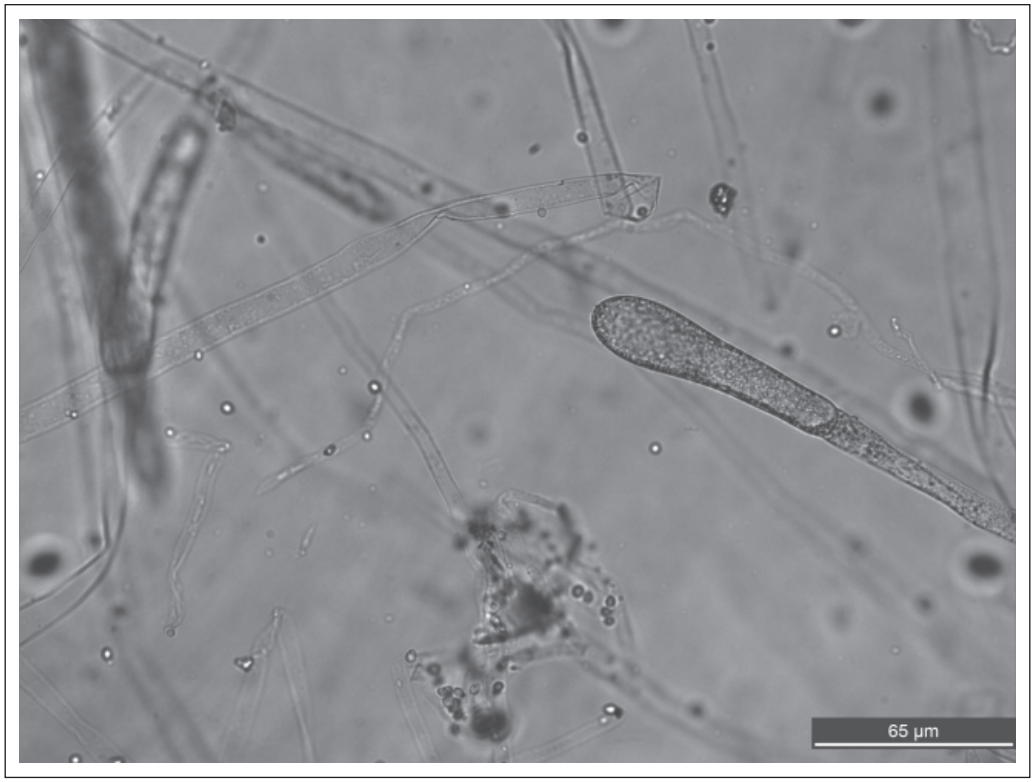

Fig. 1. Identical asexual reproduction *Saprolegnia* sp. isolated from water. Wet mount preparation of *Saprolegnia* is showing aseptate hyphae and mature sporangium (arrow) with zoospores inside.

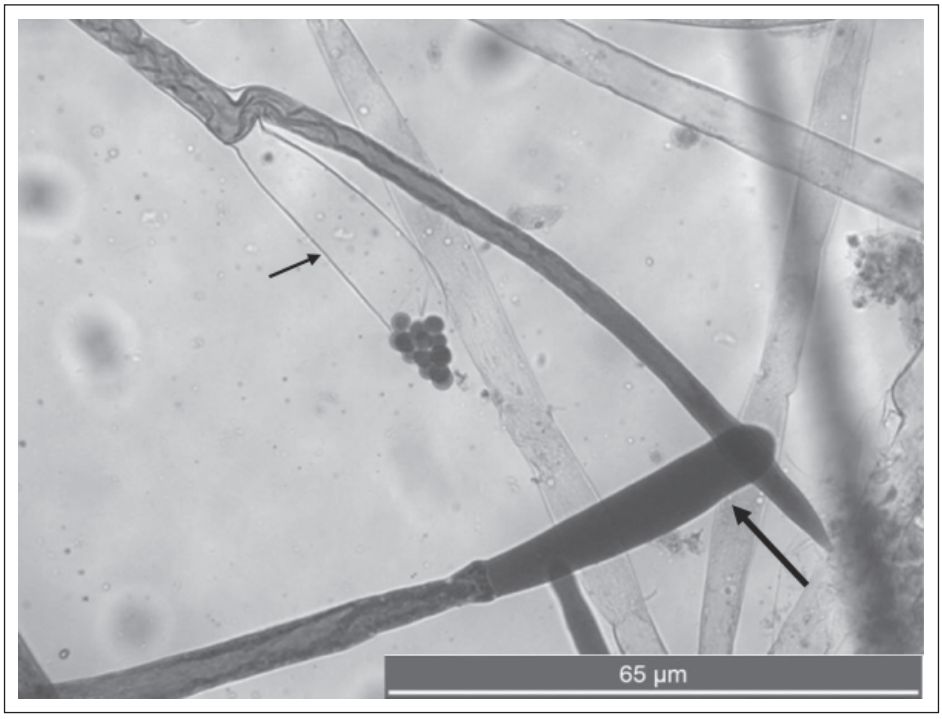

Fig. 2. Morphological characteristics of *Achlya* sp. isolated from cat fish. Wet mount preparation of *Achlya* is showing mature sporangium (large arrow) and sporangial renewal by external proliferation (small arrow).

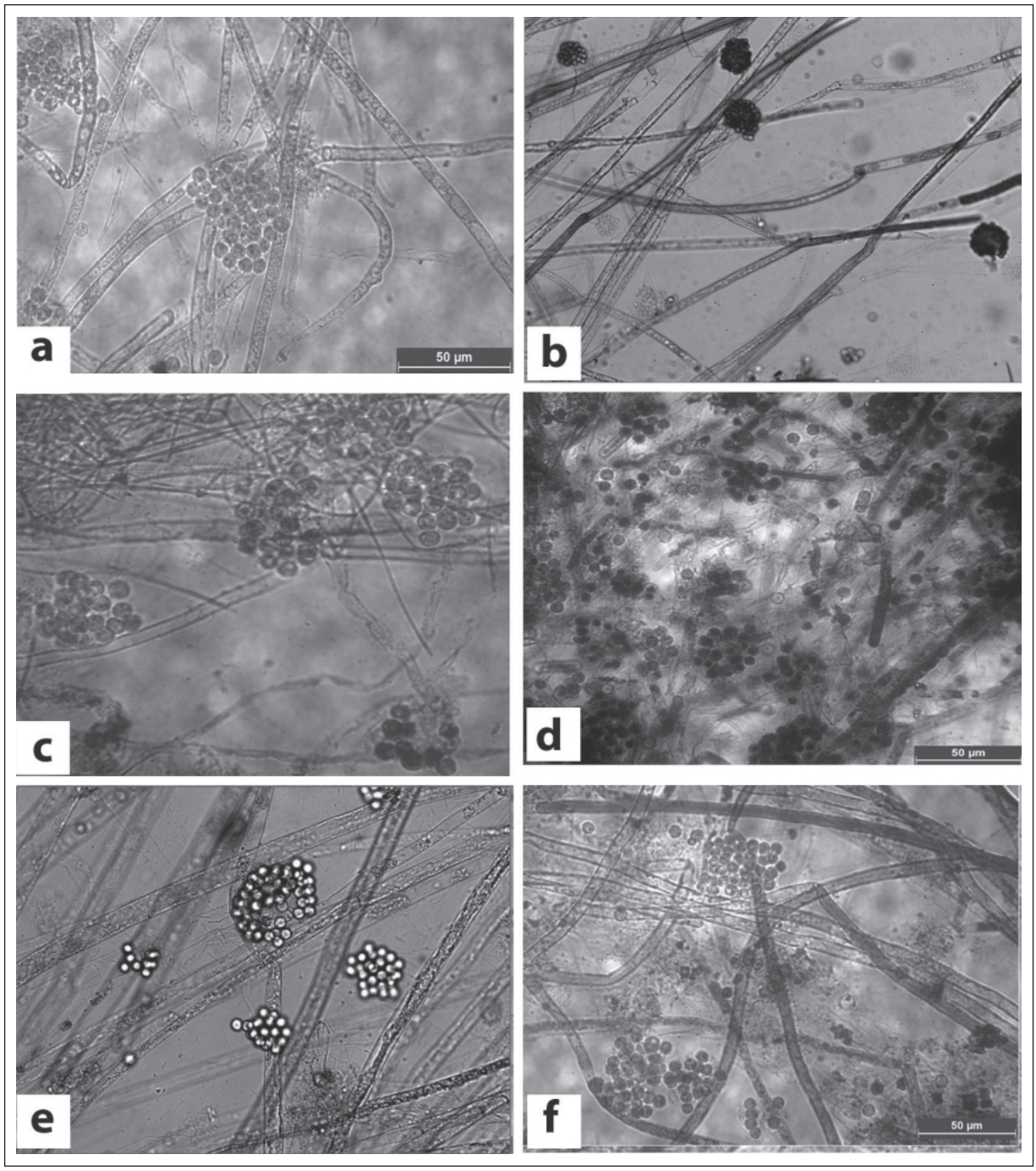

Fig. 3. Wet mount preparation of *Aphanomyces* spp. aseptate hyphae. (a) ASS1 isolated from stream (50 µm). (b) ASFT2 isolated from fish tank (50 µm). (c) ASR3 isolated from river (50 µm). (d) ASE4 isolated from estuary (50 µm). (e) ASP5 isolated from pool (50 µm). (f) ASFT6 isolated from fish tank (50 µm).

DISCUSSION

In the present study, aquatic fungi were isolated from water and also infected fish to investigate the outbreak of EUS in the Selangor state in Malaysia from May 2011 to May 2012. Fungi isolates were morphologically characterized; from 59 isolates, 32 were identified as *Saprolegina,* 21 as *Achlya* and 6 as *Aphanomyces* species. All the *Aphanomyces* species were isolated from the water samples and no evidence of infection with *Aphanomyces* was found in the fish samples. Many studies have attempted to isolate *Aphanomyces*, *Saprolegnia*, *Achlya* and other aquatic fungi from water or freshwater fish (Dykstra *et al.,* 1986; Czeczuga & Mazalska, 2000; Cail, 2002; Czeczuga & Muszynska, 2004; Chukanhom & Hatai, 2004; Czeczuga *et al.,* 2004; Prabhuji, 2005; Ramaiah, 2006; Fregenedaâ Grandes

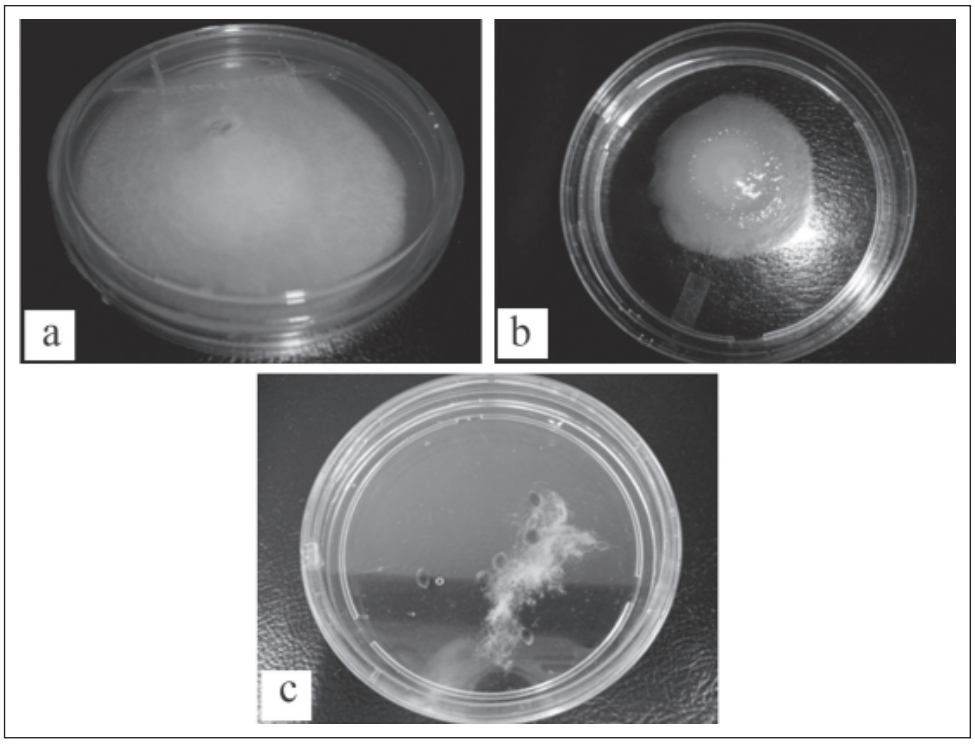

Fig. 4. Cultural characteristics of isolated fungi cultured on glucose-yeast (GY) media. (a) Cotton like and whitish colony of *Saprolegna* sp. (b) Puffy and whitish colony of *Achlya* sp. (c) A colony of the *Aphanomyces* sp. isolate ASFT6 growing on hempseed.

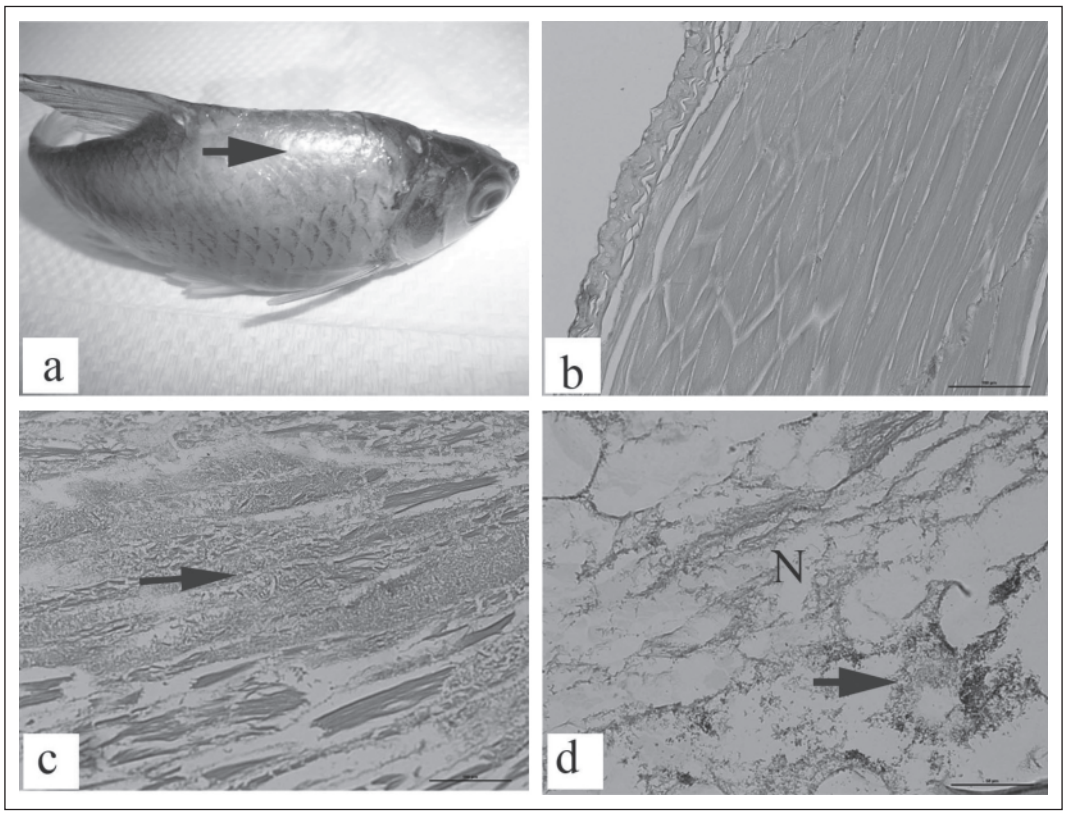

Fig. 5. Histopathological characteristics of diseased fish of skin of *Saprolegnia dicilina* infected carp. (a) Grey white mark on *Saprolegnia diclina* affected Carp. (b) Normal skin and muscle of uninfected carp. (c) Degeneration and severe necrotizing (arrow). (D) Severe necrosis (N) and distribution of melanin pigments (arrow) in skin. (H&E, X200).

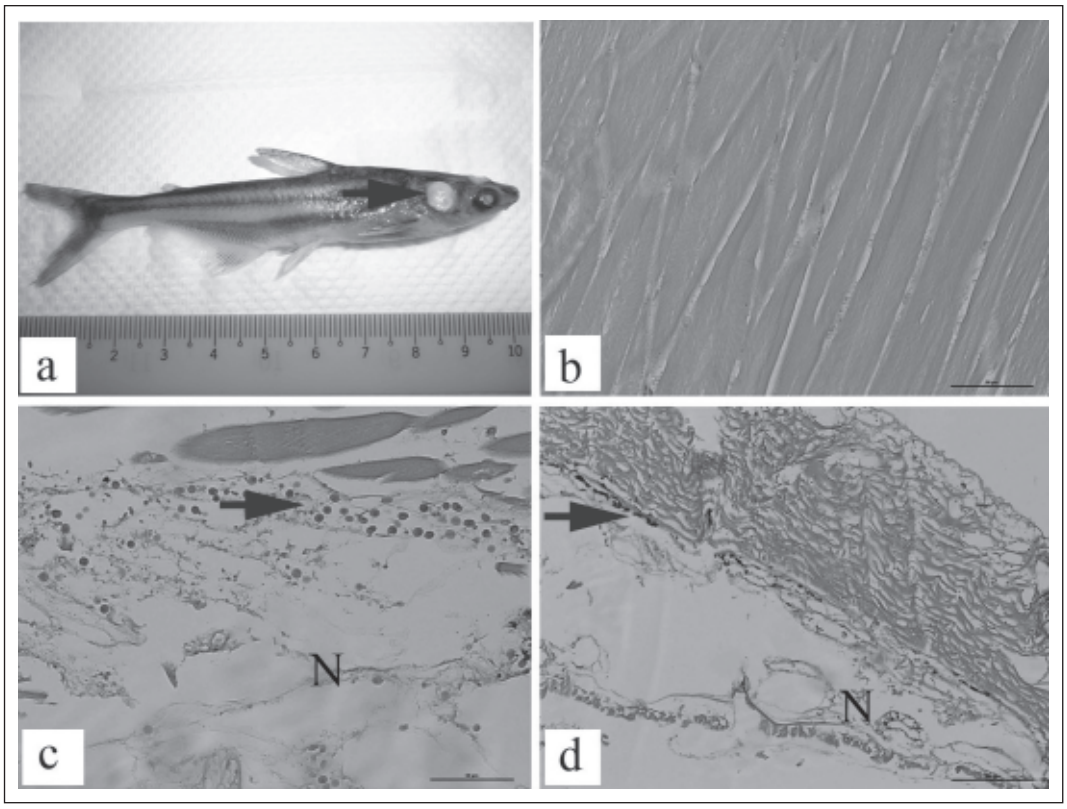

Fig. 6. Histopathological characteristics of diseased fish with *Achlya* of skin. (a) *Achlya* sp. affected Catfish (arrow). (b) Normal muscle of uninfected catfish. (c) Muscle necrosis (N) macrophages are engulfing muscle debris (arrow). (d) Muscle necrosis (N) with melanin pigments (arrow). (H&E, X200).

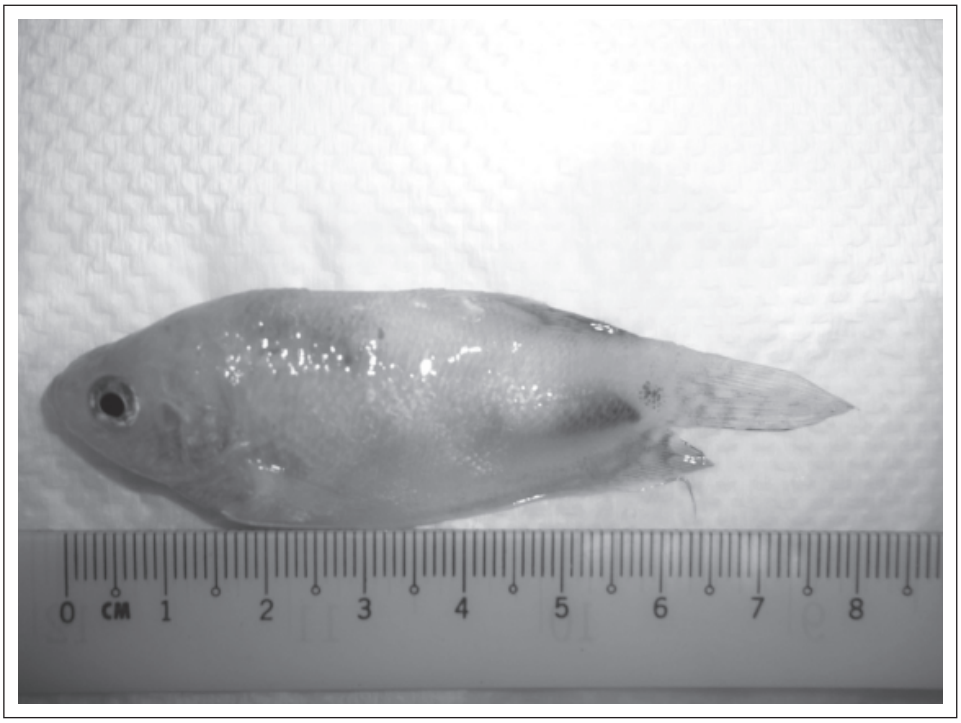

Fig. 7. Moonlight Gourami artificially injected with saprophytic *Aphanomyces* isolate ASFT6. Some reddening observed in injection area which was healed after 2 days.

et al., 2007; Kiziewicz & Nalepa, 2008; Czeczuga *et al.,* 2010; Takuma *et al.,* 2010), however there is no report of successful isolation of *A. inavadns* from water even in EUS outbreak areas. Several studies have attempted to isolate *A. invadans* from natural water bodies using bait and culture methods, although they have not been able to isolate it because of contamination of culture media by opportunistic fungi or bacteria (Panyawachira *et al.,* 2000).

Pathogenicity test for *Aphanomyces* spp. infection in the tropical moonlight gourami (*Trichogaster microlepis)*, a highly EUS -susceptible fish, revealed no sign of maycotic granuloma in the inoculated fish. A LD50 of 9.7 zoospores per fish has been estimated for *A. invadans*. It has been shown that a single zoospore is capable of initiating ulcer leading to fish mortality (Kiryu *et al.,* 2003); however, our finding showed no sign of disease even after injection with a high concentration of *Aphanomyces* zoospore indicating that all the *Aphanomyces* isolates were non-pathogenic.

These results are broadly agreed with those given by Roberts *et al.* (2006) which indicated a local host response followed by healing of the induced lesion of saprophytic strains of *Aphanomyces* and destruction of the mycelium in the injected healthy fish. The Results of Roberts' study showed that *Aphanomyces* isolated from EUS-affected fish in Thailand was slow-growing and unables to grow at 37°C and above. In comparison with local saprophytic *Aphanomyces*, this isolate also succeed in migrating into the tissues causing severe myonecrosis with chronic epithelial reaction when injected to highly EUS-susceptible fish (Roberts *et al.,* 2006). These findings are strongly agreed with the results of present study which showed that all the fish artificially injected with the saprophytic *Aphanomyces* isolates were recovered after 2 days post injections, without any histological damages.

EUS prevalence has been observed in susceptible wild estuarine fish populations in affected countries such as Australia (Callinan, 1997) and Zambia (Songe *et al.,* 2011). A significantly higher relative risk of EUS has been also reported in farmed fish when wild fish are presented in the pond (Khan *et al.,* 2002). Previous studies have shown that more than 100 fish species have been affected by EUS worldwide of which the most susceptible are belong to *Channa* spp., *Puntius* spp. (among the wild species) and Indian major carps, among the farmed fish (Naik *et al.,* 2012). There are several geographical regions which remain unaffected by the disease, however because of the epizootic nature and its broad range of susceptible fish species, the potential of onset of the disease would emerge in unaffected areas (Oidtmann, 2011).

In a word, the results of the present work showed that *Aphanomyces* spp. are common freshwater fungi in Malaysia water bodies. Although, EUS-causing *Aphanomyces* seem not to be endemic in Malaysia, favorable conditions such as climate, water quality and/or availability of susceptible species can made it susceptible for EUS outbreaks. Therefore, to avoid unexpected onset of the disease, continuous supervision for monitoring EUS outbreak in Malaysia and also development of specific molecular diagnostic methods for fast and easy detection of the pathogenic *A. invadans* are recommended.

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