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Characterization of *Achlya* Infection on Giant Gourami (*Osphronemus goramy*) in Indonesia Based on Morphology and Molecular Method

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ABSTRACT

Saprolegniasis on Gourami in Indonesia was not reported based on the morphological and molecular characterization of the causative fungus. This study purposed to characterize the fungi that caused saprolegniasis on Gourami in Java, Indonesia based on morphology and molecular methods. The morphological method included the characterization of fungal structure and clinical signs due to fungal infections in Gourami. The molecular methods included analyzing the fungal internal transcribed spacer (ITS) rDNA sequences and their phylogeny. Fungal DNA was extracted, amplified by PCR, quantified with electrophoresis, and sequenced by Sanger's method. The sequence was compared with the GenBank nucleotide database by BLASTn, alignment with MUSCLE, and phylogeny with maximum likelihood and neighbor-joining by MEGA XI. In the results, isolates were identified as *Achlya* based on achlyoid spore release from sporangium with more than one row of spores. All isolates of *Achlya* spp. had monoecious thallus, multi-oospores and non-ornamented oogonia, mature or immature oospores. *Achlya* sp. isolates 1, 2, 3, 5, 6, 7, and 8 had subcentric oospore, diclinous antheridia, or diclinous and hypogynous antheridia. *Achlya* sp, isolate 4 had subcentric or centric oospore and diclinous antheridia. As clinical signs, the mycelium of *Achlya* spp. grew on the integument of fins, operculum, mouth, and the body of infected Gourami, with a reddish lesion and integument erosion around the mycelium. Molecularly *Achlya* spp. isolates 1, 2, 3, 5, 6, 7, and 8 were not identified with another *Achlya* that has species epithet name in the GenBank based on percent identity (<94%), and phylogenetically the isolates were separately independent clusters. *Achlya* sp. isolate 4 was identified as *Newbya oblongata* (synonym of *Achlya oblongata*) based on percent identity (99,84%) and phylogenetically clustered with *Newbya oblongata* LC149928.1

INTRODUCTION

Achlya is a genus of water molds or fungal-like organisms in the order Saprolegniales **(Leclerc *et al*., 2000; Jhonson Jr. *et al*., 2002; Beakes *et al*., 2014)**. Some genera from order Saprolegniales including *Achlya*, *Saprolegnia*, *Dictyuchus*, and *Aphanomyces* could be a pathogen in fish **(Robert, 2012)**. *Saprolegnia* and *Achlya* were more frequently identified in cases of saprolegniasis from various fish species **(Hussein *et al*., 2013; Shin, *et al*., 2017; Sarowar *et al*., 2019)**. The term saprolegniasis or saprolegniosis refers to fish diseases or other aquatic animal diseases that were caused by fungal-like organisms from the *Saprolegniales* order **(Johnson Jr *et al*., 2002; Robert, 2012)**.

Achlya had some species that were pathogenic to fish. The various species of *Achlya* identified from the saprolegniasis of freshwater fish in India, including *Achlya prolifera* **(Srivastava & Srivastava 1977)**, *A. orion* **(Srivastava 1978)**, *A. klebsiana* **(Sati, 1991)**, *A. flagellata* **(Khulbe *et al.*, 1995)**, and *A. ambisexualis* **(Dubey *et al*., 2018)**. **Panchai *et al.* (2014)** reported that *A. bisexualis*, *A. diffusa*, *A. klebsiana,* and *A. prolifera* were identified from the saprolegniasis of *Oreochromis niloticus* in Thailand. An *Achlya oblongata* was identified from seabass (*Lates calcarifer*) in Sabah, Malaysia **(Lau *et al*., 2018)**. Species *A. americana* and *A. bisexualis* were isolated from rice fish (*Oryzias sinensia*) in Korea **(Choi *et al.*, 2019)**. In Indonesia, *Achlya* was reported to be the cause of saprolegniasis in tilapia (*Oreochromis* sp.), pangasius (*Pangasius* sp.), betutu (*Oxyeleotris marmorata*), and common carp (*Cyprinus carpio*) **(Yuasa, 2003)**.

Gourami (*Osphronemu goramy*) or giant Gourami was one of the important commodities in Indonesian aquaculture whose production was often disrupted by the presence of disease, including saprolegniasis **(Rahmantya *et al*., 2018)**. The risk of disease transmission in Gourami increased due to poor intensive cultivation and exchange of live fish on the fish market, especially at the fry stage. The disease was detrimental to Gourami productivity because it could infect fish in the embryo to the adult stage and could be transmitted from the infected fish to healthy fish in the population **(Irianto, 2005; Roberts *et al*., 2012)**. The diagnosis of fish diseases, including Gourami saprolegniasis, was done by characterizing and identifying the infecting pathological agent **(Austin & Newaj-Fyzul, 2017)**.

Morphological characterization was the first step commonly used to characterize pathological agents, including in saprolegniasis. Initial morphological characterization by observing the presence and appearance of hyphae directly from infected fish specimens could be carried out to suspect the disease agent. Isolation and culture of hyphae from the infected fish specimen were needed to reveal other important characteristics for further characterization and identification **(Robert, 2012; Paladini *et al.*, 2017)**. Morphology of the sporangium including the arrangement of spores in the sporangium, the pattern of spore release from the sporangium, and the development of spores and spore cysts, were defining characteristics of the genus in order Saprolegniales **(Jhonson Jr. *et al*., 2002)**. Characteristics of oogonium, oospore, and antheridium were needed to determine species in order Saprolegniales although these characteristics were difficult to obtain in laboratory conditions **(Lone & Manohar, 2018)**. Taxonomy of fungi that caused saprolegniasis has been reported based on morphological and physiological characteristics **(Stueland *et al*., 2005)**, and also based on morphological and molecular characteristics **(Sandoval-Sierra & Dieguez-Uribeondo, 2015)**.

Molecular characteristics and phylogenetic analysis can strengthen the identification results more objectively. The molecular characteristics of fungi or fungal-like organisms could be determined from the sequence profiles in the internal transcribed spacer (ITS) rDNA region **(Steciow *et al*., 2014; Sandoval-Sierra *et al*., 2014a, b; Bastide *et al*., 2015)**. The phylogenetic analysis on various genera of Oomycota was prepared based on molecular data from the ITS rDNA region. Phylogenetic analysis was carried out on the Saprolegniaceae family involving 40 species from 10 different genera. Cladistic analysis of ITS rDNA sequences places 14 species of *Achly*a grouped and separated from other genera **(Leclerc *et al*., 2000)**.

Molecular characteristics and phylogenetic analysis based on ITS rDNA sequence were reported to characterize and identify *Achlya catenulata* sp. nov. collected from a mangrove swamp in Brazil. This new species was placed within the *Achlya* sensu stricto

clade **(Jesus *et al*., 2015)**. Characterization and phylogenetic analysis based on ITS rDNA sequence were reported to analyze and identify various species of *Achlya* from some fish specimens in Thailand **(Panchai *et al.* 2014)**, India **(Dubey *et al*., 2018)**, Malaysia **(Lau *et al*., 2018)**, and Korea **(Choi *et al.*, 2019)**. ITS rDNA sequences had never been reported to characterize, identify, and analyze the phylogenetic of *Achlya* for fish specimens in Indonesia.

This study aimed to characterize the fungi that caused saprolegniasis on Gourami in Indonesia based on morphology and molecular methods. The morphological method included observing the characteristics of the sexual and asexual structure of fungi and clinical signs on fungal-infected fish. The molecular methods included the analysis of the internal transcribed spacer (ITS) rDNA sequences of fungi and their phylogeny.

MATERIALS AND METHODS

1. Sample collection, fungal isolation, and morphological characterization

Samples were collected by purposive sampling method from saprolegniasis cases on Gourami in Central and West Java, Indonesia. Fish specimens were collected from ponds and fish markets in several districts in Java, including Banyumas, Magelang, Sleman, and Tangerang. The live fish specimen was collected based on the presence of mycelium on infected fish. The clinical signs were observed, and the mycelium was collected from the fish body. Mycelium was obtained by collecting it from the infected fish using tweezers. The mycelium was rinsed with sterile water and then transferred into a labeled sample bottle containing sterile water for isolation and cultured in the laboratory

The mycelium was isolated using glucose yeast extract agar (GY-agar) for 3 days at room temperature (± 25°C). The grown mycelium in the GY-agar was cut and reculture in a new GY-agar to get a pure isolate. 5mm pieces of pure isolated mycelium on 3-day-old agar were cultured in sterile water media for 24 hours (at ± 25°C) to obtain sporangium and spore/ zoospore structures **(Hussein *et al*., 2001)**. Mycelium was examined for thalus, hypha, sporangium, zoospore release pattern, gemmae types, and sexual structure. Sexual structures of fungi (oogonium, oospore, and antheridium) were prepared with cultured mycelium isolates in glucose yeast extract broth (GY-broth) media for 6 days, and then transferred in sterile cannabis seeds water media for 3- 4 weeks at room temperature (± 25°C) **(Hussein *et al*., 2001; Johnsons Jr. *et al*., 2002)**.

One pure isolate of the fungi (*Achlya* sp.5) was experimentally infected on Gourami to confirm the clinical signs. Infection was carried out on juvenile Gourami (8- 10cm in length) which were exposed to 2 x 10^5 zoospores/ l for 24 hours. The fish were then kept in clean water and observed for the development of infection for 12 days **(Hussein & Hatai, 2002; Hussein *et al*, 2013)**.

2. DNA extraction and amplification

The mycelium from a 6-day-old glucose yeast extract broth culture was harvested and washed with sterile distilled water. The mycelium was drained from the water and used for DNA extraction. Samples were extracted with DNeasy Plant Mini Kit (Qiagen), and amplified with Polymerase Chain Reaction (PCR) procedure (Bio-Rad Thermocycler) in ITS1, 5,8S, and ITS2 regions **(Sandoval-Sierra *et al*., 2014a, b)**. Forward primers were ITS5 (5'-*GGAAGTAAAAGTCGTAACAAGG-3')* and reverse primers were ITS4 (5'-TCCTCCGCTTATTGATATGC-3') **(White *et al*., 1990)**. Each PCR reaction was carried out in a 25µl final volume, containing 12,5µl MyTaq (Bioline), 0,5µl primer forward

(concentration 20µl), 0,5µl primer reverse (concentration 20 µM), 2µl DNA template (80-160 ng), and 9,5µl dH20 (nuclease-free water). The reaction began with initial denaturation (2 minutes at 95°C), followed by 35 cycles of denaturation (10 seconds at 95°C), annealing (15 seconds at 53°C), extension (10 seconds at 72°C), and 1 cycle of final extension (10 minutes 72°C). A total of 3µl of each PCR product was mixed with 2µl of loading dye, used for electrophoresis on a 0,8% agarose gel, and visualized under UV light **(Sandoval-Sierra *et al.*, 2014a)**, with modification.

3. Sequence of ITS rDNA, sequence comparison, and phylogenetic analysis

The amplification product was sequenced with Sanger's sequencing method in 1st BASE DNA Sequencing Services. The sequencing result was tested by the BLASTn (nucleotide Basic Local Alignment Search Tool) program to compare with the nucleotide sequence database (from GenBank) and calculate the statistical significance. The sequences examined its phylogeny with maximum likelihood and neighbor-joining in Kimura-2 methods and bootstrapping 1000 replicates using the MEGA XI application.

RESULTS

Samples collection, fungal isolation, and morphological characterization

A total of thirty infected Gourami were collected. Twenty-five infected Gourami specimens were collected from ponds and fish markets in Central Java, and five from fish markets in West Java. There were eight fungal isolates from all infected Gourami specimens. The seven isolates from Central Java were *Achlya* isolates 1, 2, 3, 4, 5, 6, and 7. One isolate from West Java was *Achlya* isolate 8 (Table 1).

Morphological characterization resulted in eight isolates included in the genus *Achlya*. The isolates were clustered into two groups, group 1 included *Achlya* sp. isolates 1, 2, 3, 5, 6, 7, and 8, and group 2 included *Achly*a sp. isolates 4. *Achlya* sp. group 1 was isolated from fry and adult Gourami from several locations in Central Java (isolate 1, 2, 3, 5, 6, 7) and West Java (isolate 8). *Achlya* sp. group 2 (isolate 4) was isolated from larvae of Gourami from Central Java (Table 1).

The eight isolates were included in the genus *Achlya* based on the pattern of spore release from the sporangium, the development of spores, and spore cysts. The sporangia were cylindrical, fusiform, or clavate in shape, with spores arranged in more than one row. Primary sporangium from all isolates showed an achlyoid pattern for spore release. The secondary sporangium was generally in an achlyoid pattern for spore release, but rarely in an aplanoid pattern (the spores germinating *in situ*). Spores were monomorphic, the primary spore was cysted as a spore ball on the sporangium orifice, and the secondary spore was released from the spore ball as a flagellated zoospore. Flagellated secondary zoospores were encysted and germinated as new hyphae (Table 2).

Achlya spp. group 1 was described as follows: thallus monoecious, sporangia renewal sympodially or cymosely; gemmae present; oogonium single or long catenulate, multi-oospored and non- ornamentated oogonia (oogonial wall smooth); Oogonial stalk single or sympodially branched, generally long-stalked (1- 6 times the diameter of oogonium); oospore rarely mature, if mature subcentric; antheridial branch diclinous, rarely hypogynous; antheridia with long or short projection attachment of oogonia (Fig. 1). *Achlya* sp. group 2 was described as follows: thallus monoecious; sporangia renewal sympodially; gemmae present; single or short catenulate oogonium, multi-oospored and non- ornamented oogonia (oogonial wall smooth); oogonial stalk single, generally short-stalked (⅓- 2 times the

diameter of oogonium); oospore generally mature, subcentric or centric; antheridial branch diclinous with long projection attachment of oogonia (Fig. 2). Comparison of *Achly*a sp., both group 1 and group 2, were described in Table (2).

Table 1. Infected Gourami specimens and *Achly*a isolates from some ponds and fish markets in Java, Indonesia

Sampling location		Fish specimens	Clinical signs	Isolate	Isolate remarks
Central Java	Fish market 1	5-fry, live	Mycelium on the dorsal fin, anal fin, and pectoral fins	*Achlya* sp.1	ASP01
	Fish pond 1	4-fry, live	Mycelium on the dorsal fin and part of the body, integument with a reddish color around the mycelium	*Achlya* sp.2	ASP02
	Fish market 2	6-fry, live	Mycelium on the dorsal fin and caudal fin	*Achlya* sp. 3	ASP03
	Fish pond 2	3-larva, die, moribund	Mycelium around the body	*Achlya* sp. 4	ASP04
	Fish market 3	6-fry, live	Mycelium on dorsal fins, anal fin, caudal fin, mouth, operculum, and body, integument with reddish color around mycelium	*Achlya* sp. 5	ASP05
	Fish market 4	2-adult, live	Mycelium on the dorsal fin, caudal fin, and part of the body, integument with reddish color around mycelium	*Achlya* sp. 6	ASP06
	Fish market 5	2-fry, live	Mycelium on dorsal fin and anal fin	*Achlya* sp. 7	ASP07
West Java	Fish market 6	5-fry, live	Mycelium on dorsal fin and anal fin	*Achlya* sp. 8	ASP08

Table 2. Comparison of *Achlya* spp. isolates characteristics in group 1 and group 2

Character	*Achlya* sp. group 1	*Achlya* sp. group 2
Thallus	• monoecious	• monoecious
Sporangium	▪ cylindrical, fusiform, or clavate ▪ sporangium renewal sympodially or cymosely ▪ spore release achlyoid (primary and secondary sporangium); rarely aplanoid (secondary sporangium) ▪ spores more than one row	▪ cylindrical, fusiform, or clavate ▪ sporangium renewal sympodially ▪ spore release achlyoid (primary and secondary sporangium); rarely aplanoid (secondary sporangium) ▪ spores more than one-row
Spore	▪ monomorphic ▪ primary spore non-motile, encysted on orifice after release ▪ secondary spore flagellated, motile, encysted, and germinated as new hyphae	▪ monomorphic ▪ primary spore non-motile, encysted on orifice after release ▪ secondary spore flagellated, motile, encysted, and germinated as new hyphae
Oogonium	▪ single or long catenulate oogonium, ▪ multi-oospores and non-ornamented oogonia ▪ oogonial stalk single or sympodial-ly branched ▪ long-stalked oogonium (1- 6 times	▪ single or short catenulate oogonium, ▪ multi-oospores and non-ornamented oogonia ▪ oogonial stalk unbranched ▪ short-stalked oogonium (⅓- 2 times the diameter of oogonium)

Character	*Achlya* sp. group 1	*Achlya* sp. group 2
	the diameter of oogonium)	
Oospore	▪oospores rarely mature ▪oospore subcentric	▪oospores generally mature ▪oospore sub centric or centric
Antheridium	▪antheridial branch diclinous rarely reduced as hypogynous ▪long or short projection attachment to oogonium	▪antheridial branch diclinous ▪long projection attachment to oogonium
Gemmae	Present, single, or catenulate	Present, single, or catenulate

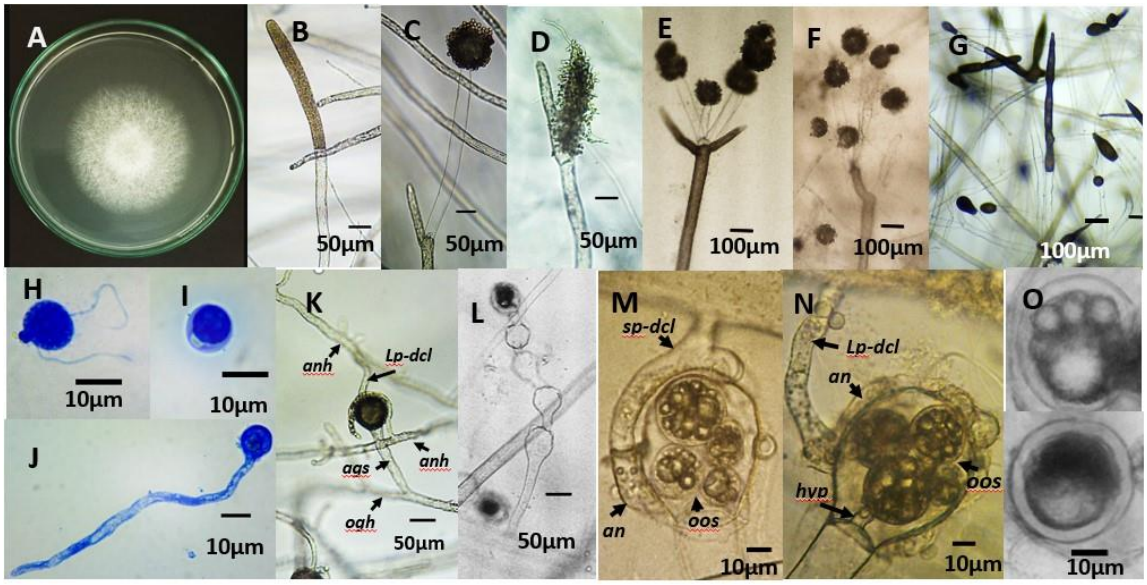

Fig. 1. Morphology of isolates *Achlya* spp. group 1 showing: **A.** Mycelium growth in GY-agar (3 days incubation), **B.** Cylindrical sporangium, **C.** Achlyoid pattern, **D.** Aplanoid pattern, **E.** Sporangium renewal cymosely, **F.** Sporangium renewal sympodially, **G.** Gemmae, single and catenulate, H, I, and J, spores and cyst were stained with lactophenol cotton blue (LCB), **H.** Biflagellate secondary zoospore, **I.** Zoospore encysment, **J.** Germinating cyst with new hyphae, **K.** Lateral and subspherical oogonium, antheridial hyphae (*anh*) with diclinous branch and long projection attachment to oogonia (*Lp-dcl*), oogonial hyphae (*ogh*) with long oogonial stalk, **L.** Long catenulate oogonia, oospore aborted, **M.** Antheridium branch diclinous with short-projection attachment to oogonia (*sp-dcl*), antheridial cell tubular (*an*), multi-oospores and non-ornamented oogonia, oospore (*oos*) immature, **N.** Antheridial branch diclinous with long projection (*Lp-dcl*) attachment, antheridial reduced to hypogynous (*hyp*), **O.** Sub centric oospore

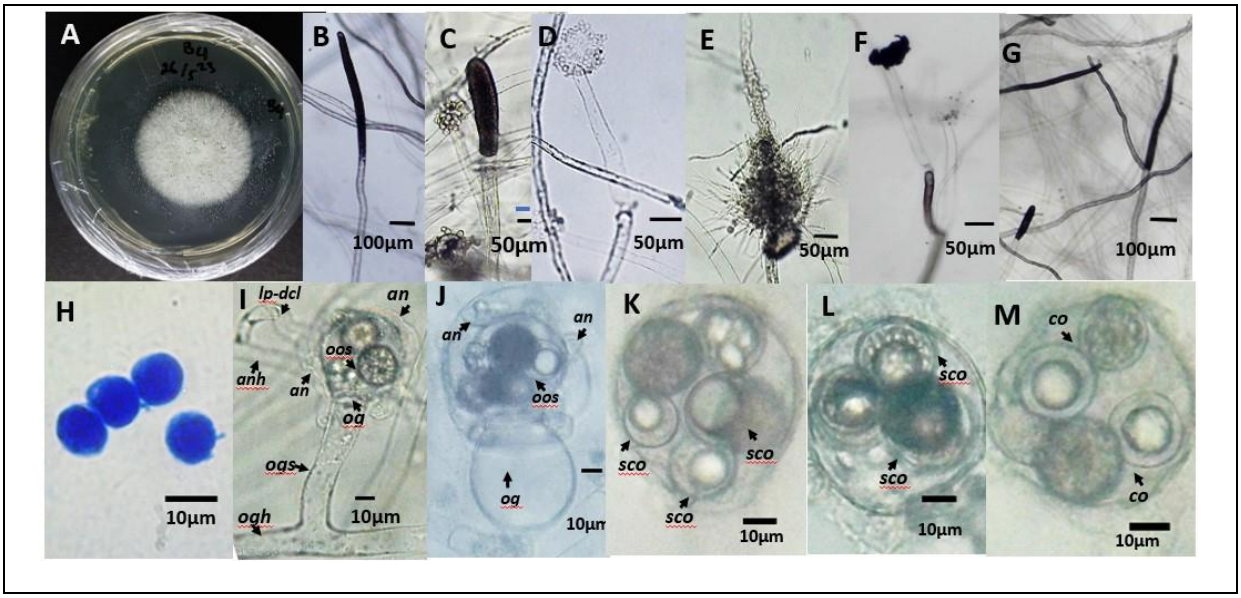

Fig. 2. Morphology of isolates *Achlya* spp. group 2 showing: **A.** Mycelium growth in GY-agar (3 days incubation), **B.** Cylindrical sporangium, **C.** Clavate sporangium, **D.** Achlyoid pattern, **E.** Aplanoid pattern, **F.** Sporangium renewal sympodially, **G.** Gemmae, single and catenulate, **H.** Zoospores and cyst were stained with lactophenol cotton blue (LCB), **I.** Lateral and subspherical oogonium (*og*) oogonial hyphae (*ogh*), oogonial stalk *(ogs*), and oospore (*oos*), antheridial cell (*an*), antheridial hyphae (*anh*) with diclinous branch and long projection attachment to oogonium(*lp-dcl*), **J.** Short catenulate oogonia *(og)*, **K.** Sub spherical oogonium with spherical oospore, multi-oospores and non-ornamented oogonia, oospore mature sub centric (*sco*), **L.** Spherical oogonium, sub centric oospore (sco), and **M.** Centric oospore (*co*)

Morphological characterization of the clinical sign from infected Gourami was observed from field specimens and experimental specimens. All specimens showed cottony mycelium in the infected integument, especially in infected fins. Various saprolegniasis conditions in infected Gourami were found in the field specimens (Table 1). The difference in mycelium thickness and its growth location in the integument of infected Gourami were found in the field specimens. The mycelium growth was found in the integument of fins, operculum, head, mouth, and part of the infected body on field specimens. Reddish color or erosion of the integument around the mycelium growth were the other signs of an advanced infection that was found in the field specimens (Fig. 3).

An isolate of *Achlya* spp. (*Achlya* sp. isolate 5) had infected experimentally on Gouramy, to confirm the clinical signs. Fish on the second day after 24 hours exposed to the zoospore, showed thin mycelium at the tip of the dorsal fin, ventral fin, or anal fin. The mycelium then grew thicker to the body at the base of the fins. Mycelium was transmitted to the operculum, mouth, and any part of the body, it grew wider and thicker on the sixth day of infection. There were reddish lesions and integument erosion around the mycelium on the infected integument (Fig. 3).

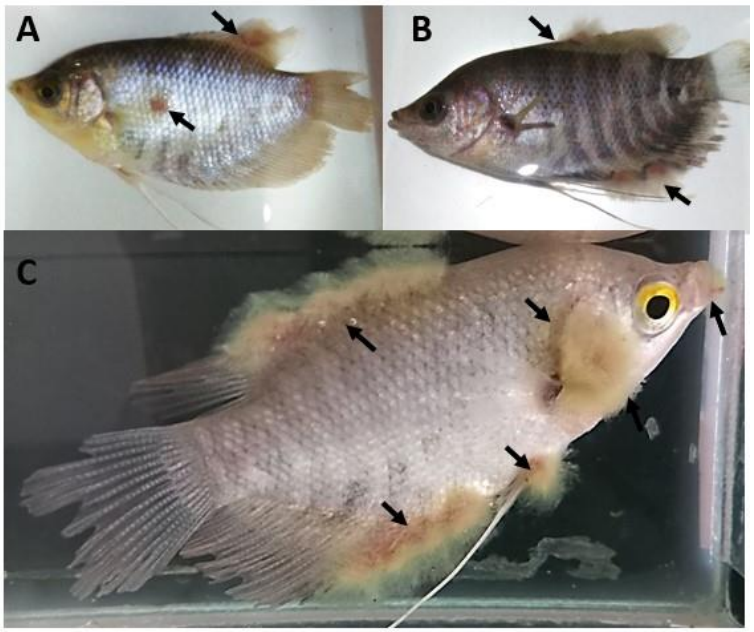

Fig. 3. Clinical signs of saprolegniasis infection on juvenile Gourami (8- 10cm in length) showing: **A.** & **B.** Field specimen, and **C.** Experimentally specimen (sixth days infected with *Achlya* sp. isolate 5). Mycelium and lesion on the integument around the mycelium growth indicated by the arrow

2. DNA extraction and amplification

The result of amplification in the ITS region showed sharp bands. Molecular characterization results of the ITS region of the DNA isolate visible in around 750bp as a single band on electrophoresis (Fig. 4).

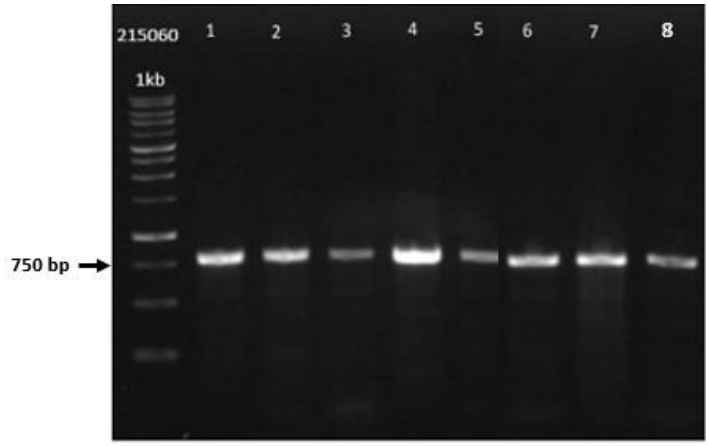

Fig. 4. DNA amplification result of *Achly*a spp. isolated from *Gourami* in the ITS region. The amplicon of each isolate was described in columns 1- 8 (electrophoresis on 0,8% agarose with 1kb DNA ladder)

3. Sequencing, comparing sequence, and phylogenetic analysis

The result of sequencing the DNA isolate sample is provided in Ab1. Format and FASTA format were fixed with BioEdit application and compared with sequence in GenBank data with BLASTn. The results of the compared isolates sequence showed that the *Achlya* isolates 1, 2, 3, 5, and 6 had the highest percent identity with *Achlya* sp. KJ511776.1 and KJ511774.1 (99,08% for *Achlya* isolate 1, 3, 5, 6, and 98,94% for isolate 2), *Achlya* sp.

isolates 7 and 8 with *Achlya* sp. KJ511775.1 (98,34% for *Achlya* sp. isolate 7 and 99,45% for isolate 8), however the percent identity of these isolates with the *Achlya* that has species epithet name on the GenBank was less than 94%. *Achlya* sp. isolates 4 had more than 99% of percent identity with *Achlya* sp. KJ511773.1, and KJ511777.1, *Achlya bisexualis* MT908192.1, and MT908917.1, and *Newbya oblongata* LC149928.1. *Achlya* sp. isolates 4 had the highest percent identity with *Achlya* sp. KJ511773.1 and KJ511777.1 (100%) and then *Newbya oblongata* LC 149928.1 (99, 85%). The two highest percent identities of *Achlya* spp. 1- 8 sequences that were analyzed with BLASTn are described in Table (3).

Table 3. The results compared the *Achlya* spp. sequence isolated from Gourami in Indonesia with *Achlya* species from GenBank (BLASTn)

Isolate	BLAST result accession number	Query cover	Identity %	Country	Host	Reference
***Achlya* sp. 1**	*Achlya* sp. KJ511776.1	95%	99,08	Thailand	*Oreochromis niloticus*	**Panchai *et al*., 2014**
	Achlya sp. KJ511774.1	95%	99,08	Thailand	*O. niloticus*	**Panchai *et al*., 2014**
***Achlya* sp. 2**	*Achlya* sp. KJ511776.1	93%	98,94	Thailand	*O. niloticus*	**Panchai *et al*., 2014**
	Achlya sp. KJ511774.1	93%	98,94	Thailand	*O. niloticus*	**Panchai *et al*., 2014**
***Achlya* sp. 3**	*Achlya* sp. KJ511776.1	96%	99,08	Thailand	*O. niloticus*	**Panchai *et al*., 2014**
	Achlya sp. KJ511774.1	96%	99,08	Thailand	*O. niloticus*	**Panchai *et al*., 2014**
***Achlya* sp. 4**	*Achlya* sp. KJ511773.1	96%	100	Thailand	*O. niloticus*	**Panchai *et al*., 2014**
	Newbya oblongata LC149928.1	82%	99.85	Malaysia	*Lates calcarifer*	**Lau *et al*., 2018**
***Achlya* sp. 5**	*Achlya* sp. KJ511776.1	88%	99,08	Thailand	*O. niloticus*	**Panchai *et al*., 2014**
	Achlya sp. KJ511774.1	88%	99,08	Thailand	*O. niloticus*	**Panchai *et al*., 2014**
***Achlya* sp. 6**	*Achlya* sp. KJ511776.1	96%	99,08	Thailand	*O. niloticus*	**Panchai *et al*., 2014**
	Achlya sp. KJ511774.1	96%	99,08	Thailand	*O. niloticus*	**Panchai *et al*., 2014**
***Achlya* sp. 7**	*Achlya* sp. KJ511776.1	94%	97,76	Thailand	*O. niloticus*	**Panchai *et al*., 2014**
	Achlya sp. KJ511775.1	90%	98,34	Thailand	*O. niloticus*	**Panchai *et al*., 2014**
***Achlya* sp. 8**	*Achlya* sp. KJ511776.1	96%	98,68	Thailand	*O. niloticus*	**Panchai *et al*., 2014**
	Achlya sp. KJ511775.1	92%	99,45	Thailand	*O. niloticus*	**Panchai *et al*., 2014**

Achlya sp. KJ511774.1, KJ511775,1, KJ511776.1, KJ511773.1, and KJ511777.1 were isolated from *Oreochromis niloticus* (Thailand) **(Panchai *et al*., 2014)** and *Newbya oblongata* LC 149928.1 from *Lates calcarifer* (Malaysia) **(Lau *et al*., 2018)**. Based on ≥ 99,5% of similarity **(Lucking 2020)**, only *Achlya* sp. isolate 4 had high similarity with *Achlya* sp. KJ511773.1, KJ511777.1 (100%) and *Newbya oblongata* LC 149928.1 (99, 85%). *Achlya* sp. isolates 1, 2, 3, 5, 6, 7, and 8, had low similarity (94% of percent identity) with another *Achlya* that has a specific epithet available from GenBank.

The result of phylogenetic analysis for *Achlya* sp. isolate 1, 2, 3, 4, 5, 6, 7, and 8 were described in the phylogenetic tree (Fig. 5). The phylogenetic tree of the two methods, maximum likelihood (character-based method) and neighbor-joining (distance-based method) showed a similar pattern. *Achlya* sp. isolates 1, 2, 3, 5, 6, 7, and 8 were grouped with *Achly*a sp. KJ511774, KJ511775, and KJ511776, from GenBank. On the other side, *Achlya* sp. isolate 4 was grouped with *Achlya* sp. KJ511773.1, *Achlya* sp. KJ511777.1, *Newbya oblongata* LC149928.1, *Achlya oblongata* DQ324365.1, and *Achlya bisexualis* MT908192.1, MT908917.1 and EU441154.1 from the GenBank. *Saprolegnia parasitica* AM228809.1 as an outgroup, was separated from the two groups significantly.

There were two clades in each group of *Achlya*. Group I consisted of clade A and clade B, and Group II consisted of clade C and clade D. Clade A included *Achlya* spp. isolates 1, 2, 3, 5, 6, and 7, calde B included *Achlya* sp. isolate 8, and three species from GenBank consisted of *Achlya* sp. KJ511774.1, KJ511775.1, and KJ511776.1, clade C included *Achlya*

bisexualis MT908192.1, MT908917.1 and EU441154.1 (from GenBank), and clade D included *Achlya* sp. isolate 4, and four species *Achlya* sp. from GenBank, that consisted of *Achlya* sp. KJ511773.1, *Achlya* sp. KJ511777.1, *Newbya oblongata* LC149928.1, and *Achlya oblongata* DQ324365.1.

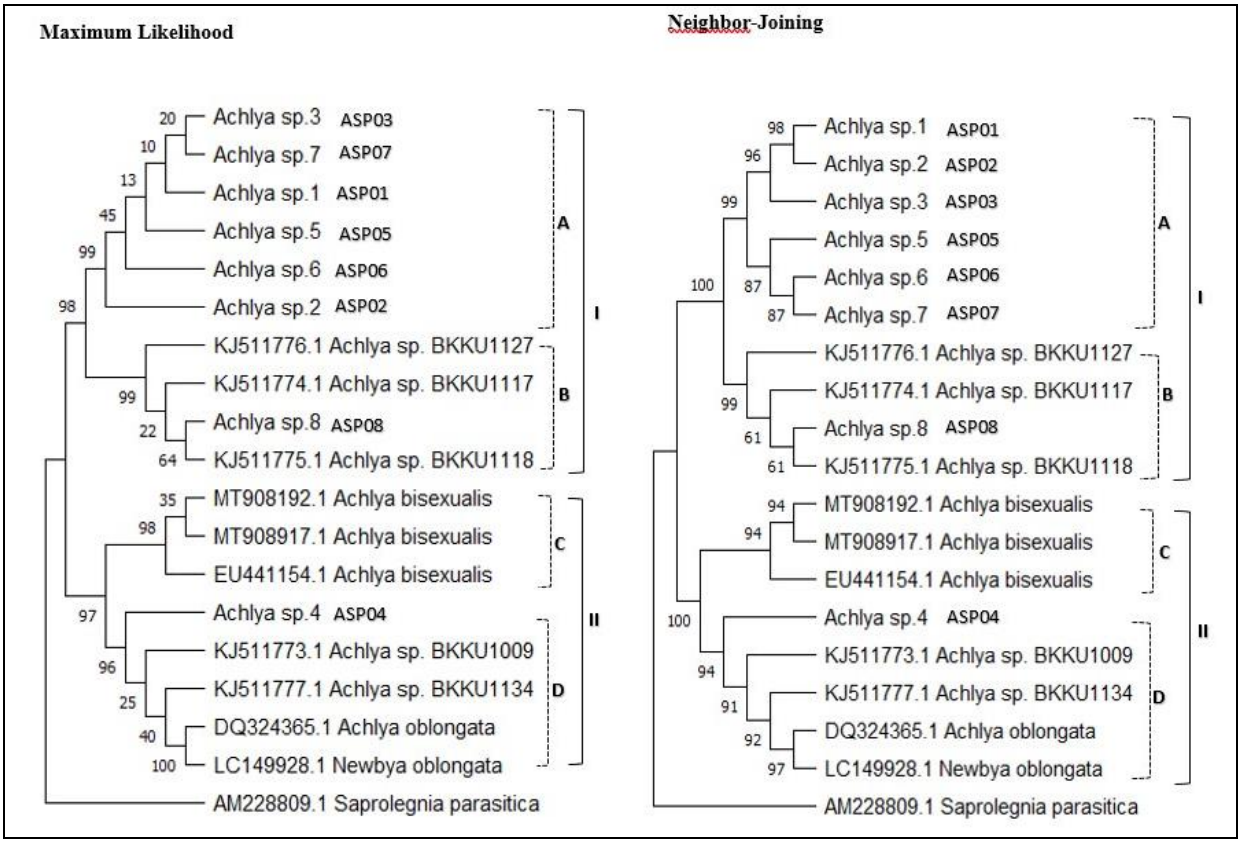

Fig. (5). Phylogenetic tree with maximum likelihood and neighbor-joining method inferred from ITS rDNA sequences of isolates of *Achlya*. The number next to branches indicates bootstrap resampling 1000x and the bar shows the number of substitutions per site

DISCUSSION

Saprolegniasis in Gourami was often found in the early stages of fish development (eggs, larvae, and fry) **(Nuryati *et al*, 2015)**. Fungal-infected Gourami specimens in this study were more commonly found in the early development stages from ponds and fish markets. Temporary rearing of Gourami in holding ponds and poor handling of fish in fish markets can reduce fish immunity due to environmental stress. Environmental stress and decreased fish immunity could increase the risk of infectious diseases including saprolegniasis **(Irianto, 2005; Jeney, 2017)**.

Achlya caused death more often in tropical fish with saprolegniasis than *Saprolegnia* **(Roberts, 2012)**. Research on Gourami with saprolegniasis in this study resulted in eight isolates identified as *Achlya*. *Achlya* was characterized by monomorphic spores that release sed from thick primary sporangium with an achlyoid spore release pattern. The fungi exhibited monomorphic spores, with only one type of motile spore, which was a secondary zoospore (lateral or reniformis flagellated spore). Thick primary sporangium had cylindrical, clavate, fusiform, or navicular in shape, with more than one row of sporangium inside it **(Johnsons Jr. *et al*, 2002)**. Sporangium with achlyoid pattern in spore release characterized

the primary spore did not swim away from sporangium, but cluster and encyst as a hollow ball (spore ball) at the orifice (mouth of the sporangium) **(Webster & Weber, 2007; Roberts, 2012)**.

The character of releasing spores with an achlyoid pattern was the main character of the genus *Achlya*. This character on direct examination could be confused with other characters that arise from the release of spores by secondary sporangium. Secondary sporangium could release spores with a thraustothecoid, dictyuchoid, or aplanoid pattern, in addition to an achlyoid pattern. This was the reason why it was important to characterize the pattern of spore release from the primary sporangium **(Johnsons Jr. *et al*., 2002)**. Primary sporangium from all Gourami's isolates *Achlya* spp. released spores in an achlyoid pattern and secondary sporangium released spores in an achlyoid pattern, and rarely in an aplanoid pattern.

The achlyoid pattern was also shown by other genera, including *Aphanomyces* and *Protoachlya*. *Aphanomyces* were different from *Achlya* because even though they had an achlyoid pattern, the spores released from the thin sporangium were only in one row as the filiform sporangium **(Hughes, 1994)**. *Protoachlya* also had the character of releasing spores with an achlyoid pattern, but some of the primary spores swimming away when released from sporangium and encyst at a distance from the sporangium orifice/exit pore **(Johnsons Jr. *et al*., 2002)**.

There are similarities in morphological characters between *Achlya* spp. isolates from Gourami and *Achlya oblongata*, as described by **Johnsons Jr. *et al*. (2002)**, especially in the diclinous antheridial branch, catenulate oogonium, aborted oospore, and subcentric oospore. *Achlya* spp. isolates 1, 2, 3, 5, 6, 7, and 8 differ from *A. oblongata* in hypogynous antheridial branch and short projection of diclinous antheridial branch to the oogonium. Compared with *A. oblongata*, *Achlya* sp. 4 had a shorter oogonial stalk, more mature oospores in one oogonium, and two types of oospores (subcentric and centric).

The genus *Achlya* was described previously by **Johnsons Jr. *et al.* (2002)** and was then proposed by **Beakes *et al*. (2014)** to be divided into two genera based on the character of their oospores. **Beakes *et al.* (2014)** proposed a new family, *Achlyaceae*, to place species with predominantly single-oospore oogonia and very eccentric oospores. Species in the genus *Achlya* that had these characteristics retain their genus name as *Achlya* and were placed in the family *Achlyaceae*. Another genus *Achlya* which had species with oogonia that were predominantly multi-oospored oogonia and had centric or subcentric oospores was maintained in the family *Saprolegniaceae* and used a new genus name, *Newbya*. The isolate *Achlya* spp. from Gourami in this study had subcentric and centric oospores with predominantly multi-oospores in oogonia. Based on the criteria proposed by **Beakes *et al.* (2014)**, all *Achlya* isolates from Gourami in this study were characterized as genus *Newbya* in the family *Saprolegniaceae.*

Achlya spp. produces clinical signs similar to general saprolegniasis when infecting fish. The presence of cottony mycelium in the infected fish was a common sign of fish saprolegniasis **(Robert, 2012)**. The mycelium of *Achlya* spp. grew thickly and bound tightly to the body tissue of the Gourami. According to **Andersson (2001)**, zoospores from oomycetes could be secreted by the glycoprotein compounds as an adhesive material to attach to their hosts. After the zoospores germinate, they release enzymes that lyse the body cells of the host and then grow penetrated in the fish integument. The reddish color and erosion of the Gourami integument around the mycelium are signs indactive of an advanced *Achlya* infection in Gourami. Advanced infection signs in saprolegniasis, according to **Johnson, Jr. *et al*. (2002)**, were characterized by tissue damage to open wounds due to necrosis of

connective tissue, rapid growth of hyphae into the dermis tissue, loss of scales, necrosis of muscle tissue, and disintegration of superficial areas of the melanophore.

Isolates of *Achlya* spp. from Gourami in this study were compared to isolate *Achlya* spp. from **Panchai *et al*. (2014)** and **Lau *et al*. (2018)**, but they only used the asexual characteristics to identify the isolate of *Achlya* spp., and they did not report the sexual character from their *Achlya* spp. isolates. The absence of oogonium and oospore characteristics means that *Achlya* isolated from Gourami cannot confirm these characteristics with their *Achlya* spp. They used the molecular characters and phylogenetic analysis based on ITS rDNA sequence for further identification.

Panchai *et al*. (2014) reported that the *Achlya* spp. KJ511773.1, KJ511774.1, KJ511775.1, KJ511776.1, and KJ51177.1 were collected from infected *Oreochromis niloticus* (Thailand), positioned as unidentified species of *Achlya*. They were positioned as unidentified *Achlya* based on molecular and phylogenetic analysis in which they did not match with another species of *Achlya* in the GenBank database. Moreover, they also formed an independent phylogenic cluster. **Lau *et al*. (2018)** reported that the *Achlya* sp. IPMB was collected from infected Asian seabass (*Lates calcifer*) in Malaysia and identified as *Achlya oblongata* based on molecular analysis in the ITS region sequence. This isolate has 99% similarity to *Achlya* sp. KJ511773.1, KJ511777.1, and *Achlya oblongata* DQ324365 and were formed as a cluster in the phylogenetic tree. This isolate was registered in GenBank as *Newbya oblongata* with the accession number LC149928.1.

Determining a similarity, threshold was necessary for the grouping approach, but there was no fixed threshold for species delimitation. Sister species in the phylogenetic analysis based on ITS had similarities of around 99.5%. Similarity assessment through pairwise alignment for BLAST-based identification also poses limitations. In BLAST analysis, the result relies on query cover, e- value, and percentage identity **(Lucking *et al*., 2020)**. The BLAST results of *Achlya* sp. isolate 4 of the Gourami in this study placed *A. bisexualis* in the top ten on its sequence alignment list. *A.bisexualis* had a higher query cover percentage value (≥90%) than *Newbya oblongata*, even though the percent identity value was lower (98,69- 99,19%). *Newbya oblongata* was at number 81 in the sequence alignment list, with a query cover of 82% and a percent identity of 99.85%. All the BLAST results of *Achlya* spp. on Gourami had an e- value of 0,0.

Pairwise similarity with BLAST or similar methods of sequence alignment did not accurately reflect taxonomic entities. The BLAST algorithm could be inappropriate, depending on the length of the query and the underlying sequence marker reference set. Verification needs to be carried out to strengthen the identification results with BLAST. Verification could be carried out on phenotypic and molecular diagnostic characteristics. Sequence-based verification could be carried out using phylogenetic analysis with multiple alignments **(Lucking *et al*., 2020)**. Verification in BLAST result of *Achlya* spp. isolates from Gourami in this study were carried out using phylogenetic analysis. *Achlya* sp. 4 grouped with *Newbya oblongata* as a clade that were separated from *A.bisexualis* as another clade. Another set of *Achlya* spp. isolates (isolate 1, 2, 3, 5, 6, 7, and 8) consistently matched their BLAST result, and they were grouped together.

Achlya spp. isolated from Gourami that was constructed with two different phylogenetic trees, maximum likelihood and neighbor-joining, had similar results for a pattern of grouping the species. According to **Munjal *et al*. (2018)**, maximum likelihood was a character-based phylogenetic tree method and neighbor-joining was a distance-based phylogenetic tree method. The character-based method compared all sequences

simultaneously considering one character/ site at a time. The distance-based method used the dissimilarity (the distance) between the two sequences to construct trees.

The similar grouping patterns of the two construction methods had established the position of each *Achlya* spp. isolated from Gourami in Indonesia among other *Achlya* species based on sequences of ITS1, 5,8S, and ITS2 region in GenBank. *Achlya* sp. isolate 4 clustered with *Newbya oblongata* from Malaysia and *Achlya* sp. KJ11773.1 and KJ 11777.1 from Thailand. *Achlya* sp. 4 was close to the *Achlya bisexualis* group. *Achlya* spp. isolates 1, 2, 3, 5, 6, 7, and 8 were not clustered with another of the *Achlya* that has a species epithet name available in GenBank. *Achlya* spp. isolates 1, 2, 3, 5, 6, 7, and 8 were close to *Achlya* sp. KJ511774.1, KJ511775.1, and KJ511776.1 from Thailand. Based on morphological characteristics, the similarity of ITS sequence DNA, and clustering sequence on phylogenetical analysis, *Achlya* spp. group 1 was potentially positioned as a novum species in *Achlya* or *Newbya* genera. **Jeewon and Hyde (2016)** in their study recommended some phylogenetic and morphological data for appropriate fungal species delineation, especially related to the novum species. Species taxonomy based on gene phylogeny must be determined with a strong phylogenetic signal (eg. ITS region). If the ITS region is used, there should be at least 450 base pairs. A new species may be indicated by a minimum nucleotide difference >1.5% in the ITS region. The phylogenetic relationship of a new taxon must be involved at least 4- 5 related taxa closely within the same genus. Additionally, the descriptions of new species must be supported on at least 2- 3 different phenotypic characters.

CONCLUSION

Morphological characteristics of *Achlya* spp. isolated from Gourami were monoecious thallus, achlyoid spore release from primary sporangium, multi-oospores and non-ornamented oogonia, sub-centric oospore, diclinous antheridial branches, and long projections antheridial branch. *Achlya* spp. isolates 1, 2, 3, 5, 6, 7, and 8 also had hypogynous and short projections antheridial branches. Moreover, *Achlya* sp. isolate 4 also had centric oospore. The clinical signs included the hyphal mass (mycelium) growth in the integument of fins, operculum, mouth, and the body of infected Gorami with a reddish lesion and integument erosion around the mycelium. The molecular characterization and phylogenetic analysis resulted in *Achlya* spp. isolates 1, 2, 3, 5, 6, 7, and 8 not being identified with another *Achlya* that has species epithet name in the GenBank (<94% in percent identity). Furthermore, the isolates grouped independently as separated clusters in phylogenetic analysis. The *Achlya* sp. isolate 4 was identified as *Achlya oblongata/Newbya oblongata* (99,84% in percent identity) and phylogenetically clustered with *Achlya* sp. sequences KJ511773.1, KJ511777.1, as well as *Newbya oblongata* sequence LC149928.1.

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