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Freshwater oomycete isolated from net cage cultures of *Oreochromis niloticus* with water mold infection in the Nam Phong River, Khon Kaen Province, Thailand

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Abstract. Water mold-infected Nile tilapia (*Oreochromis niloticus*) from cultured net cages along the Nam Phong River, Khon Kaen Province, northeast Thailand, were collected from September 2010 to August 2011. The 34 obtained water mold isolates belonged to the genus *Achlya* and were identified as *Achlya bisexualis*, *A. diffusa*, *A. klebsiana, A. prolifera* and unidentified species of *Achlya*. Isolates of *A. bisexualis* and *A. diffusa* were the most abundant (35%), followed by the unidentified species of *Achlya* (18%) and then, *A. klebsiana* and *A. prolifera* (6% each). The ITS1-5.8S-ITS2 region of the unidentified isolates was sequenced for phylogenetic analysis. Three out of 6 isolates were indicated to be *A. dubia* (BKKU1005), *A. bisexualis* (BKKU1009 and BKKU1134), and other 3 out of 6 isolates (BKKU1117, BKKU 1118 and BKKU1127) will be an as-yet unidentified species of *Achlya*. The biological characteristics of the isolates showed optimum temperatures for vegetative growth of 25–35ºC. All of the *Achlya* isolates were able to grow under up to 1.5% sodium chloride. The isolates grew well, and their zoospores were able to germinate at pH 4.0–11.0. Microscopic examination of the skin lesions of the infected tilapia revealed bacteria and hyphae. Some of the hyphae penetrated into the epidermis, and numerous small blood vessels and scattered macrophages could be observed throughout the infected area.
Key Words: *Achlya*, biological characteristic, histopathology, identification, Nile tilapia.

Introduction. Freshwater fish play a particularly important role as a traditional protein source for local consumption of the family diet in the northeastern region of Thailand (El-Sayed 2006). The numbers of naturally occurring fish have declined markedly due to an increasing human population and the impact of aquatic environmental degradation. The average fish consumption per capita in this region was 33.5 kg in 2001 (ADB 2005). As the demand for low-priced protein has become greater, intensive fish culture systems with high stocking densities should be implemented (Chinabut 2002). Hence, fish culture is valuable for the aquaculture industry (Chukanhom & Hatai 2004). The Nile tilapia (*Oreochromis niloticus*), which belongs to the family Cichidae, is one of the most economically important cultured fish in Thailand, generating production of 9,664.4 million Baht (Fisheries Information Technology Center 2012). Fish farmers have succeeded in achieving large-scale fish production without good management (Sreevatana 1993). Therefore, these fish cultures are occasionally affected by several different diseases. There have been reports of infectious diseases, including bacteria such as *Streptococcus agalactiae* and *S. iniae* (Yuasa et al 2008), fungi such as *Achlya* (Yuasa et al 2000), and protozoa such as *Ichthyoptherius* (Panchai et al 2013), among cultured tilapias in the northeastern region. Water mold infections have been a problem in intensively cultured

fish during the cool season in Thailand (Willoughby & Lilley 1992; Chinabut et al 1995; Chukanhom & Hatai 2004). The genera *Achlya*, *Aphanomyces*, and *Saprolegnia* are members of the family Saprolegniaceae in the class Oomycota that have been reported as being responsible for water mold infections in fish and their eggs, affecting both wild and farmed fish (Bruno et al 2011). Studies on the occurrence of oomycetes in many species of fish have been conducted by a number of researchers. The first report of an oomycete infection was attributed to *Aphanomyces* spp. infecting snakehead (*Ophicephalus* spp.) and carp (*Puntius* spp.) (Tonguthai 1985). *Achlya*, *Aphanomyces*, and *Saprolegnia* have been isolated from dead fish (Willoughby & Lilley 1992). Striped catfish (*Pangasius hypophthalmus*), walking catfish (*Clarias batrachus*), sepat siam (*Trichogaster pectoralis*) (Chinabut et al 1995), striped snakehead (*Channa striata*), and three spot gourami (*Trichogaster trichopterus*) (Lilley & Roberts 1997) were found to be severely infected by *Aphanomyces* spp. Lawhavinit et al (2002) reported the presence of *Achlya bisexualis* associated with a *Tetrahymena corlissi* infection in guppy (*Poecilia reticulata*). Chukanhom & Hatai (2004) isolated *Saprolegnia diclina*, *Achlya klebsiana* and *Allomyces arbuscula* from eggs of the common carp (*Cyprinus carpio*). Panchai et al (2007) identified *Achlya bisexualis* in infected eggs from Nile tilapia. Hanjavanit et al (2012) isolated *S. diclina*, *Achlya ambisexualis* and *Achlya* spp. from eggs of African catfish (*Clarias gariepinus*). Although freshwater oomycetes occur consistently in Thailand, no scientific reports on water molds or histopathological studies of infected Nile tilapia are currently available.

In this study, we isolated water molds from net cage-cultured infected Nile tilapias from the Nam Phong River in Khon Kaen Province to study the morphological and biological characteristics of the molds. Sequencing of the internal transcribed spacer (ITS1-5.8S-ITS2; ITS) region of ribosomal RNA from unidentified oomycete that did not produce reproductive organs was employed as an alternative method of identification based on morphological characteristics (Lilley et al 2003; Phadee et al 2004; Muraosa et al 2009, 2012). The histopathological characteristics of the infected fish were also examined.

Material and Method

Isolation and identification of water molds. Oomycete-infected tilapias were randomly collected from private culture net cages at Ban Hua Sua–tent, Nam Phong district (latitude 16° 44' 23.165'' N, longitude 102° 46' 10.945'' E) and Ban Dong Phong, Muang district (latitude 16° 28' 21.295'' N, longitude 102° 54' 8.086'' E) along the Nam Phong River in Khon Kaen Province, northeast Thailand from September 2010 to August 2011. For determination of the presence of aquatic oomycetes, the following procedure of oomycetes isolation was employed to obtain pure single colonies (Hatai & Egusa 1979), and sexual organs were induced via inoculation in cultures of sterilized hemp seeds (*Cannabis sativa*) (Kitancharoen et al 1995). The classification and identification of these oomycetes are traditionally based on morphological characteristics related to asexual and sexual reproduction according to the criteria of Johnson (1956) and Johnson et al (2002). They were routinely maintained on glucose yeast extract (GY) agar (Hatai & Egusa 1979) at 25°C and transferred to fresh GY agar every month. All isolates were deposited at the Department of Biology, Faculty of Science, Khon Kaen University, and remarked as BKKU1001 to BKKU1134.

DNA sequencing and molecular phylogenic analysis. The ITS1-5.8S-ITS2 (ITS) regions of 6 unidentified *Achlya* isolates (*Achlya* spp. BKKU1005, 1009, 1117, 1118, 1127, and 1134) were subjected to molecular analysis. According to the methods of Lilley et al (2003), Phadee et al (2004) and Muraosa et al (2009, 2012), the edges of hyphae that had been growing for 3 days, incubated at 25°C in GY broth, were cut and frozen at –85°C. Then, the ITS region was amplified via PCR using the primer pair ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White et al 1990). Amplifications were performed using the TaKaRa PCR Thermal Cycler Personal (TaKaRa Shuzo, Kyoto, Japan), with an initial denaturation step (94°C for 5 min),

followed by 40 cycles of denaturation (94°C for 30 s), annealing (63°C for 30 s) and extension (72°C for 30 s), and final extension step (72°C for 7 min). The PCR products were analyzed via 2% agarose gel electrophoresis, and a 100-bp DNA molecular marker was included in each electrophoresis run (EZ Load™, BIO-RAD, Hercules, CA, USA). The bands obtained through electrophoresis were visualized and photographed under ultraviolet (UV) illumination (FAS III, TOYOBO, Osaka, Japan). Subsequently, the PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced via the direct sequencing method using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems), according to the instructions of the manufactures. The obtained sequences were assembled and submitted to the NCBI GenBank database for retrieved of accession numbers.

Finally, the similarity of sequences was analyzed. The sequences were edited and assembled between complementary strands by the Basic Local Alignment Search Tool (BLAST) (Altschul et al 1990) function of GenBank. Consensus sequences were then aligned using ClustalW (Thompson et al 1994) and compared with other known sequence in Genbank using BLASTN algorithm. The ITS1-5.8S-ITS2 region of the sequences was identified by using ITSx software (Bengtsson-Palme et al 2013). Phylogenetic analysis was performed using the Phylip software (Felsenstein 1989), including other 15 sequences from *Achlya* species available in the GenBank database and using four sequences of *Saprolegnia* spp. as the outgroup (Table 1). UPGMA tree robustness was determined in terms of bootstrap values and evaluated through 1,000 replications.

Table 1

Lists of accession numbers of the internal transcribed spacer (ITS) region of *Achlya* spp. and *Saprolegnia* spp. obtained from GenBank

Species	*References*	*Origin*	*GenBank accession no.*
Achlya spp.			
A. ambisexualis	Leclerc et al (2000)	France	AF218147
A. ambisexualis	Unpublished	USA	FJ545256
A. ambisexualis	Robideau et al (2011)	USA	HQ643083
A. bisexualis	Leclerc et al (2000)	France	AF218151
A. bisexualis	Leclerc et al (2000)	France	AF218153
A. bisexualis	Robideau et al (2011)	USA	HQ643087
A. bisexualis	Unpublished	Iran	KF225573
A. caroliniana	Robideau et al (2011)	Nigeria	HQ643089
A. caroliniana	Mélida et al (2013)	Spain	JX418018
A. dubia	Leclerc et al (2000)	France	AF218155
A. dubia	Robideau et al (2011)	Nigeria	HQ643093
A. flagellata	Robideau et al (2011)	UK	HQ643095
A. flagellata	Robideau et al (2011)	UK	HQ643096
A. prolifera	Unpublished	Japan	AY647196
A. prolifera	Unpublished	France	EU849169
Outgroup			
Saprolegnia diclina	Unpublished	Japan	AY455775
S. parasitica	Unpublished	USA	FJ545238
S. ferax	Robideau et al (2011)	France	HQ643987
S. salmonis	Unpublished	Japan	AB219399

Effects of various temperatures, sodium chloride (NaCl) concentrations and pH levels on vegetative growth. The advancing edges of growing colonies that had been incubated at 25°C for 2–3 days were cut with a No. 2 cork borer prior to being subjected to further analyses. For determination of the effects of temperature, GY agar blocks with young mycelia were placed on 20 mL of GY agar and incubated at the following temperatures: 5, 10, 15, 20, 25, 30, 35 and 40°C. To assess the effects of salinity, agar blocks were placed on plates containing 20 mL of GY agar with the following

concentrations of NaCl: 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0%, then incubated at 25°C. To evaluate the effects of various pH levels, GY broth was adjusted to pH levels of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 by adding 1 N HCl or 1 N NaOH and filtered through 0.2 µm millipore filter paper (Sartorius, Hannover, Germany). Then, the blocks were transferred to 10.0 mL of GY broth and incubated at 25°C. The diameters of the resultant colonies and growth of hyphae were measured daily with a Vernier caliper for 7 days. Three replicates of each experiment were conducted. Due to *A. bisexualis* BKKU1001, *A. diffusa* BKKU1006, *A. klebsiana* BKKU1003 and *A. prolifera* BKKU1124 grew well and produced numerous zoospores. Therefore, they were selected as representative species and all six unidentified of *Achlya* (BKKU1005, BKKU1009, BKKU1117, BKKU1118, BKKU1127 and BKKU 1134) were also used in this study.

Effects of various pH levels on zoospore germination. The mycelia of colonies that had been growing in GY broth, incubated at 25ºC for 48 h were washed several times with sterilized tap water (STW) and then incubated in STW at 25ºC for 48 h to obtain zoospores. A 100 µL aliquot of a $1x10^3$ zoospores mL^{-1} mixture of each isolate was transferred to 10 mL of GY broth with various pH levels, as indicated above. The plates were incubated at 25ºC for 24 h to observe zoospore germination and hyphal growth. Four isolates of identified and six unidentified *Achlya* spp. as described above were also used in this study.

Preparation procedures for light microscopy. Necropsy of tilapias with oomycete infections was performed. Materials from the skin, gills, pancreas, kidney, liver, spleen and intestinal tract of individual fish were preserved in a 10% phosphate-buffered formalin solution and processed through the paraffin method. Small pieces of the tissue samples (0.5 x 0.5 cm) were dehydrated through a graded series of ethanol and embedded in paraffin. Then, serial sections were cut at a thickness of 5 µm and stained with hematoxylin and eosin (H & E). Some sections were also stained via the Periodic Acid-Schiff (PAS) reaction and with Giemsa, Gram stain and Uvitex 2B (Wada et al 2003). The histological sections were examined and photographed microscopically at magnifications of 40x, 100x and 400x.

Results

Isolated oomycetes. A total of 34 infected Nile tilapias were collected from cultured net cage at Ban Hua Sua–tent and Ban Dong Phong along the Nam Phong River, Khon Kaen Province, northeast Thailand. The total lengths and body weights of the fish ranged from 8.3 to 15.6 cm and 161.0 to 498.0 g, respectively. All samples exhibited clinical signs of oomycete infection in the head region and on the body surface and fins. As shown in Figure 1, severe lesions observed on the fish consisted of deep, open ulcers, associated with degeneration of muscular tissue. Some areas of the ulcers were covered with numerous white or grey cotton-like mycelia. In addition, swollen and hemorrhagic lesions were also recorded. No abnormal clinical signs were observed in the visceral organs.

Thirty-four isolates were collected from the infected fish belonging to the Saprolegniales, which were classified as members of the genus *Achlya* based on the mode of zoospore release, which presented an achlyoid type. The isolates were designated as BKKU1001-1134. Table 2 illustrates the distribution of *Achlya* isolates, which was as follows: 5 isolates (14.7%) were obtained from farm A, 12 isolates (35.3%) from farm B, 13 isolates (38.2%) from farm C and four isolates (11.8%) from farm D. *Achlya* were identified, with *A. bisexualis* and *A. diffusa* showing the highest prevalence (35% each), followed by *Achlya* spp. (18%) and then *A. klebsiana* and *A. prolifera* (6% each). The obtained *Achlya* spp. consisted of six unidentified isolates, which were divided into three groups according to morphological differences in their colony shape and hyphal diameter.

Figure 1. Gross appearance of a Nile tilapia with an oomycete infection, showing degeneration of muscle in severe ulcers, with cotton–like mycelia on the body surface and caudal tail.

Table 2

The 34 oomycete isolates obtained from freshwater oomycetic infections of Nile tilapia in the Nam Phong River, Khon Kaen Province, northeast Thailand

Date	*Location*	*Identification*	*Numbers of isolates*	*Remark*
23 Sep 2010	Farm A, Ban Hua Sua–tent, Nam Phong district	*A. bisexualis*	1	BKKU1001
		A. diffusa	1	BKKU1002
		A. klebsiana	2	BKKU1003, 1004
		Achlya sp.	1	BKKU1005
	Farm B, Ban Hua Sua–tent, Nam Phong district	*A. bisexualis*	2	BKKU1006, 1007
		A. diffusa	1	BKKU1008
		Achlya sp.	1	BKKU1009
	Farm C, Ban Hua Sua–tent, Nam Phong district	*A. bisexualis*	2	BKKU1010, 1011
		A. diffusa	2	BKKU1012, 1013
19 Jan 2011	Farm B, Ban Hua Sua–tent, Nam Phong district	*A. diffusa*	3	BKKU1114, 1115, 1116
		Achlya spp.	2	BKKU1117, 1118
	Farm C, Ban Hua Sua–tent, Nam Phong district	*A. bisexualis*	3	BKKU1119, 1120, 1121
		A. diffusa	1	BKKU1122
12 Feb 2011	Farm C, Ban Hua Sua–tent, Nam Phong district	*A. bisexualis*	2	BKKU1123, 1124
		A. prolifera	2	BKKU1125, 1126
		Achlya sp.	1	BKKU1127
10 Jul 2011	Farm B, Ban Hua Sua–tent, Nam Phong district	*A. bisexualis*	1	BKKU1128
		A. diffusa	2	BKKU1129, 1130
	Farm D, Ban Dong Phong, Muang district	*A. bisexualis*	1	BKKU1131
		A. diffusa	2	BKKU1132, 1133
		Achlya sp.	1	BKKU1134

Phylogenetic analysis. The six sequences data presented in this study were deposited in GenBank as accession numbers KJ511772 to KJ511777, respectively (Table 3). The approximately 750-bp partial rRNA gene fragment amplified from genomic DNA extracted from the six unidentified *Achlya*. The phylogenetic tree inferred from the sequences of the ITS1-5.8S-ITS2 (ITS) region is presented in Figure 2, showing that the sequences of the six unidentified *Achlya* isolates were separated from the *Saprolegnia* outgroup with a 100% bootstrap value. The sequences of all of the unidentified isolates of *Achlya* were

similar to sequences of associated *Achlya* originating from different countries, that have been submitted to GenBank. It was found that the subclade consisting of the sequence from *Achlya* sp. BKKU1005 was similar to *A. dubia* (accession numbers HQ643093 and AF218155), presenting 58 and 98% similarity, respectively. The ITS sequences of the subclade composed of *Achlya* spp. BKKU1009 and 1134 (presenting 100% similarity within this clade) were similar to *A. bisexualis* (accession number KF225573, AF218153, AF218151 and HQ643087), exhibiting a similarity value of 65%. In addition, the ITS sequences of the subclade comprised of *Achlya* spp. BKKU1117, 1118 and 1127 formed a separate, independent phylogenic cluster with 100% similarity being observed within this group. It still remains unidentified in the species.

Table 3

Ribosomal *Achlya* spp. sequences determined in phylogenetic analysis

Strains	*Total size (bps)*	*GenBank accession number*
BKKU1005	758	KJ511772
BKKU1009	756	KJ511773
BKKU1117	758	KJ511774
BKKU1118	721	KJ511775
BKKU1127	757	KJ511776
BKKU1134	714	KJ511777

Biological characteristics of Achlya species. According to our biological analyses (Table 4), all of the *Achlya* isolates were able to grow at a temperature range of 5–35ºC, presenting optimum temperatures of 25–35ºC and rapid, maximal growth at 30ºC. *A. klebsiana* exhibited the highest vegetative growth rate among the isolates. All of the *Achlya* isolates showed maximal growth in GY agar without NaCl (0%) and were able to grow at concentrations up to 1.5% NaCl. All of the isolates also grew in GY broth over a wide range of pH levels from 4.0–11.0, displaying an optimal pH of 6.0–8.0. As shown in Table 4, the zoospores from all of the isolates were able to germinate in GY broth over a wide range of pH levels from 4.0–11.0 after 24 h of incubation. In addition, zoospores in GY broth at pH 4.0 showed irregular, flaccid mycelia, and abnormal branching of their hyphae, while zoospores at pH 11.0 exhibited swollen mycelia with dense cytoplasm and abnormal branching of their hyphae after incubation at 25ºC for 3 days.

Histopathology. Microscopic examination of the skin lesions of infected tilapia showed cellular debris and necrosis of epidermal cells associated with the invasion of aseptate hyphae. These hyphae were thick with a diameter of approximately 11.7–30.2 µm (n = 30). The epidermal tissues were covered by the hyphae and some hyphae penetrated into the epidermis. The hyphae were not easily visible following H & E staining, but were clearly recognizable using both PAS, based on a pink color and Uvitex 2B, based on blue-white fluorescence (Figure 3A). In severe cases, some of the overlying epidermis and scales had sloughed off. It was appeared that the stratum spongiosum disintegrated, numerous blood congestion occurred, and macrophages scattered (Figure 3B). Some germinated and fragmented hyphae were also found in the disintegrated dermis (Figure 3C). Moreover, some area of underlying musculature tissue was degenerated, which enclosed by inflammatory cells and scattered melanomacrophages were also occurred (Figure 3D). There was no evidence of granulomas observed. Giemsa-and Gram-stained sections of skin lesions showed the presence of bacteria (Figure 3E). No hyphae or bacteria were detected in the visceral organs.

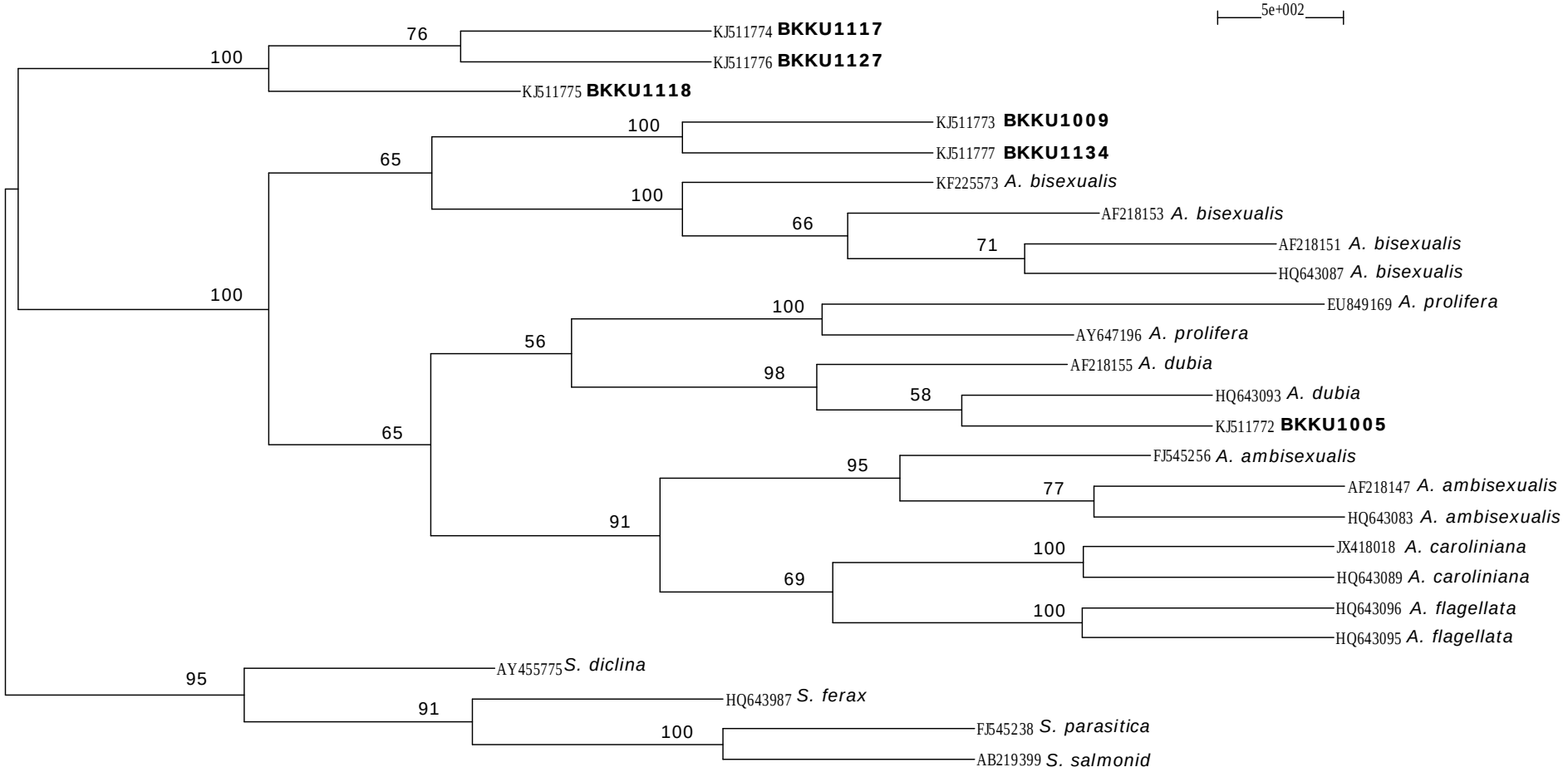

Figure 2. UPGMA tree for six unidentified species of *Achlya* (note in bold) and related oomycetes (n = 15) obtained from GenBank based on the internal transcribed spacer (ITS) region. Clusters were supported by 1,000 bootstrap-resembled data sets and bootstrap value less than 50% are not shown. *Saprolegnia* spp. were used as outgroup sequences.

Table 4

Biological characteristics of *Achlya* isolates

Strains	*Mean colony radius (mm)*										*Mean hyphal length (mm) / Germination of zoospores*									
	Temperature[a] (°C)						*NaCl[b] (%)*				*pH*									
	10	*15*	*20*	*25*	*30*	*35*	*0.5*	*1.0*	*1.5*	*2.0*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
A. bisexualis BKKU1001	4	13	20	24	36	35	18	9	2	0	0[c]/-[d]	6/+	21/+	25/+	29/+	28/+	24/+	8/+	4/+	0/-
A. diffusa BKKU1006	4	12	18	23	33	32	16	9	2	0	0/-	6/+	21/+	25/+	29/+	27/+	22/+	8/+	4/+	0/-
A. klebsiana BKKU1003	7	18	23	31	42	37	23	14	5	0	0/-	12/+	21/+	28/+	31/+	29/+	25/+	9/+	4/+	0/-
A. prolifera BKKU1124	3	10	18	21	33	34	16	7	1	0	0/-	9/+	18/+	25/+	28/+	25/+	19/+	7/+	5/+	0/-
A. dubia BKKU1005	6	16	21	28	37	33	18	12	4	0	0/-	11/+	26/+	30/+	32/+	27/+	16/+	7/+	0/+	0/-
A. bisexualis BKKU1009	2	10	18	22	33	36	16	7	3	0	0/-	8/+	12/+	14/+	17/+	18/+	15/+	6/+	5/+	0/-
Achlya sp. BKKU1117	3	10	18	21	32	35	15	9	2	0	0/-	6/+	19/+	27/+	29/+	26/+	22/+	9/+	1/+	0/-
Achlya sp. BKKU1118	3	10	18	22	32	34	14	8	1	0	0/-	7/+	17/+	25/+	28/+	26/+	16/+	9/+	5/+	0/-
Achlya sp. BKKU1127	3	9	17	20	31	33	14	8	1	0	0/-	4/+	17/+	26/+	30/+	24/+	16/+	9/+	4/+	0/-
A. bisexualis BKKU1134	2	10	16	21	31	32	13	7	1	0	0/-	5/+	17/+	18/+	24/+	20/+	18/+	7/+	0/+	0/-

[a] various temperatures on colonial growth after 3 days of incubation;
[b] various concentrations of NaCl on day 3 of incubation at 25°C;
[c] various pH levels on hyphal growth on day 3 of incubation at 25°C;
[d] various pH levels on zoospore germination after 1 day of incubation at 25°C: "-" no germination and "+" germination.

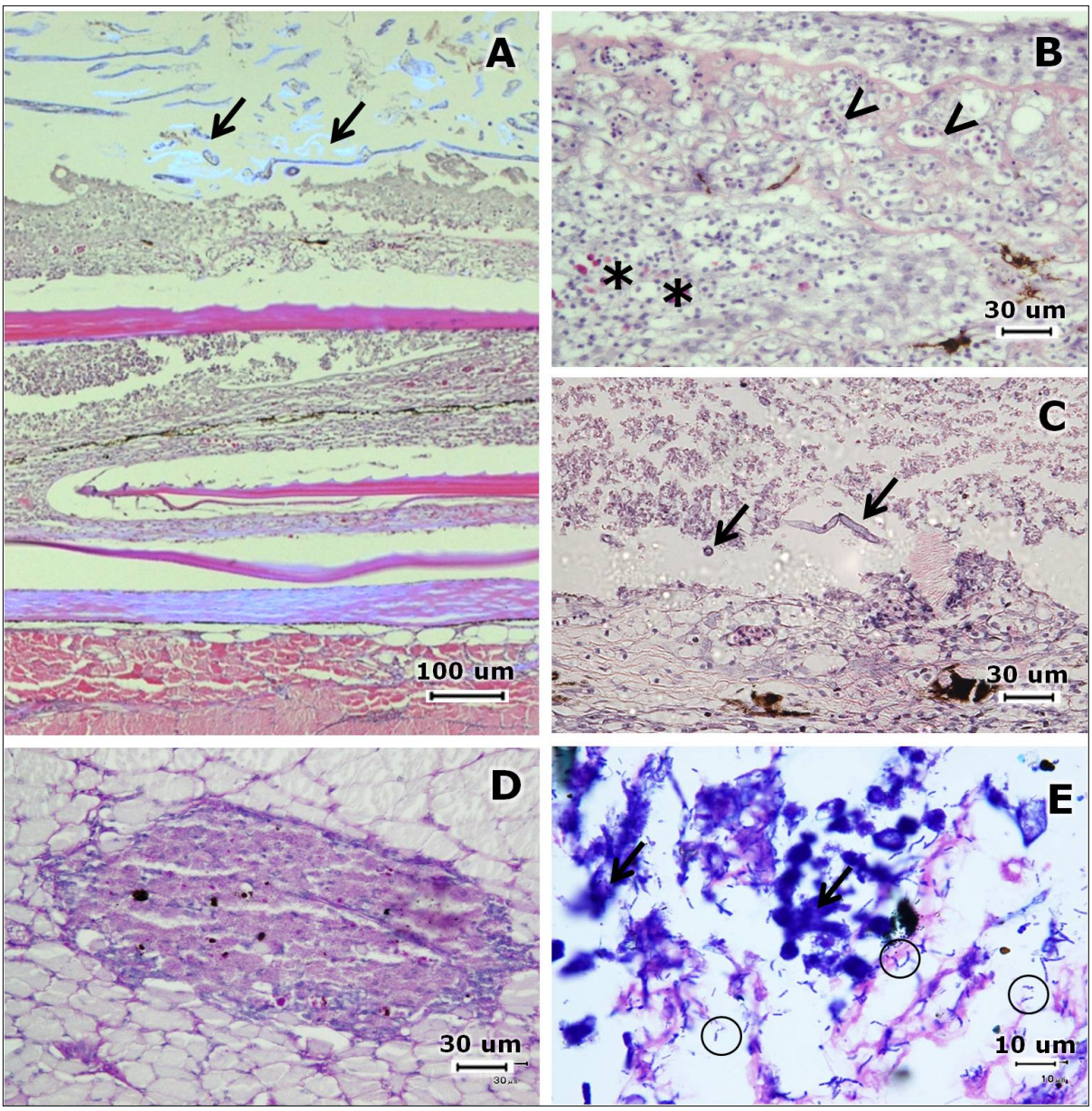

Figure 3. A - Cross-section of Nile tilapia skin showing aseptate hyphae (arrows) (Uvitex 2B–H&E); B - numerous small blood vessels (<) and scattered macrophages (*) in dermis (Giemsa); C - germinated and fragmented hyphae (arrows) found in the disintegrated dermis (Giemsa); D - the underlying musculature tissue degenerated, enclosed by inflammatory cells and scattered melanomacrophages (PAS); E - scattered rod-shaped bacteria (◯) among hyphae (arrows) (Giemsa).

Discussion. *Achlya* species are commonly reported in the aquatic environment and have been found in infections of fish from tropical regions for many years (Srivastava 1980; Willoughby & Lilley 1992; Kitancharoen et al 1995). These molds cause great losses in aquaculture production and continue to be a problem for culturists (Chukanhom & Hatai 2004). In the present study, only isolates from the genus *Achlya* were obtained from the infected Nile tilapia, which similar to the results of El-Hissy et al (1989), El-Sharouny & Badran (1995) and Hussein et al (2013), who isolated *Achlya* from infected tilapia, but contrasting with the finding of El-Atta (2008), who observed *Saprolegnia* infections in infected tilapia. *Achlya* species also occur opportunistically in other fish and their eggs year-round in the tropics, as reported by many researchers in Thailand (Willoughby & Lilley 1992; Roberts et al 1993; Lawhavinit et al 2002; Chukanhom & Hatai 2004; Hanjavanit et al 2012), Myanmar (Kitancharoen 1995) and India (Srivastava 1980; Sati

1991). In the present study, the obtained members of this genus consisted of *A. bisexualis*, *A. diffusa*, *A. klebsiana*, *A. prolifera*, and unidentified *Achlya* species. During the collection period, *A. bisexualis* and *A. diffusa* were the most dominant species and were found frequently at all sampling sites. According to sequences from the ITS1-5.8S-ITS2 (ITS) region, the six unidentified *Achlya* isolates were grouped and separated from the *Saprolegnia* outgroup. Two subclades of unidentified *Achlya* isolates showed similarity to a known *Achlya* species in the BLAST search results for the ITS sequences. This result was supported by the findings of Leclerc et al (2000), who found that ITS sequence analysis provided individual characteristics of nucleotides among different *Achlya* species. The molecular-based groupings of the ITS region sequences from the oomycetes generally agreed with established classical morphological criteria (Cooke & Duncan 1997; Daugherty et al 1998). It may be concluded that molecular-based phylogenetic analysis was successfully used as an alternative approach for the identification of unidentified *Achlya* sp. BKKU1005 as *A. dubia* (accession numbers HQ643093 and AF218155), which showed similar morphological characteristics to *A. dubia* as described by Johnson (1956), Yuasa et al (2000) and Johnson et al (2002). While *Achlya* spp. BKKU1009 and 1134 were identified as *A. bisexualis* (accession number KF225573, AF218153, AF218151 and HQ643087) and their morphological characteristics corresponded to *A. bisexualis* as described by Johnson (1956), Johnson et al (2002) and Lawhavinit et al (2002). In contrast, the sequences of *Achlya* spp. BKKU1117, 1118, and 1127 did not match those of oomycetes in the GenBank database and formed a separately independent phylogenic cluster (100% similarity within this group). It may be indicated that *Achlya* spp. BKKU1117, 1118, and 1127 were the same species and it will be an as-yet unidentified species of *Achlya*.

According to Schreck et al (1993) and Rach et al (1997, 2005), aquatic oomycetes are ubiquitous in the natural water supplies of fish hatcheries and often cause considerable negative impacts due to disease in fish aquaculture. Based on the finding of the present study, it may be stated that *Achlya* is the most common genus distributed throughout the freshwater ecosystems of the Nam Phong River, Khon Kaen Province, Thailand, and this genus appears to occur in many parts of the world (Riethmüller et al 1999; Leclerc et al 2000; Lilley et al 2003; Phadee et al 2004).

Gupta & Mehrota (1989) noted that environmental factors play an important role in controlling the occurrence of aquatic fungi. In the present study, all isolates of *Achlya* were able to grow at a temperature range of 5–35ºC, showing optimum temperatures of 25–35ºC, similar to the findings of Oláh & Farkas (1978) and Chukanhom & Hatai (2004). These isolates were able to tolerate in higher temperatures up to 35ºC, which appears to indicate that they are well adapted to high temperatures, which are characteristic of the aquatic environmental conditions in tropical regions (Chukanhom & Hatai 2004) and they are mesophillic water molds (Griffin 1994). The pH of freshwater plays a critical role in the growth of oomycetes (Oláh & Farkas 1978). Even though the hyphae of *Achlya* were able to grow and their zoospores could germinate over wide pH range of 4.0–11.0, abnormalities of the hyphae were observed at both pH 4.0 and 11.0. According to Griffin (1994), there may be effects on metabolically active components of the cell wall and outer membrane surface of the hyphae associated with pH conditions. In this study, the *Achlya* isolates were able grow on GY agar containing 0–1.5% NaCl, which was in accord with the findings of Chukanhom & Hatai (2004). From the results of biological studies, it may be concluded that the freshwater ecosystems of the Nam Phong River are suitable for the growth of *Achlya*.

The microscopic analysis of the skin lesions of infected tilapia showed that numerous hyphae covered the epidermis and some penetrated through the epithelial layer. No granulomas around the hyphae were observed, similar to the results of Hussein & Hatai (2002), who recorded *Saprolegnia* in infected salmonids. These findings may be due to rapid growth of the oomycete hyphae (Hatai & Hoshiai 1992). According to Howe et al (1998) and Roberts (2012), skin lesions arising from minor injury due to parasitic infections or handling may increase the susceptibility of open sores on the skin to water mold infections. An interested finding of the present study was the presence of scattered bacteria adjacent to skin lesions, which may be associated with the *Achlya* infections of

the tilapia. This finding is in agreement with the results of Bruno et al (2011), who considered Saprolegniaceae to be a secondary pathogenic agent in infections arising from, for example, bacterial infection. However, oomycete infection can cause massive problems in the epidermis and infected fish fail to control their osmoregulatory systems, which directly causes the death of fish (Richards & Pickering 1979; Fontenot & Neiffer 2004). Based on our findings, it may be concluded that *Achlya* is a secondary, opportunistic, saprophytic pathogen of Nile tilapia, which is supported by the findings of Lilley & Roberts (1997) and Sosa et al (2007).

This is the first report of oomycete infections in net cage-cultured Nile tilapia caused by *Achlya* in the Nam Phong River, Khon Kaen Province, Thailand. The pathogenicity of these isolates and their virulence to fish will be investigated in the future.

Conclusions. From the present study, the morphological characteristics of the isolates are classified as family *Saprolegniaceae*. It is composed of *A bisexualis*, *A. diffusa*, *A. klebsiana, A. prolifera* and unidentified species of *Achlya*. *A. bisexualis* and *A. diffusa* are common species, which were found from almost net-cage culture of Nile tilapia. For molecular study of the ITS1-5.8S-ITS2 region of the unidentified isolates, it was found that three groups of *Achlya* spp. were divided according to their morphological characteristic differences. Therefore, the molecular-based phylogenetic analysis of the ITS1-5.8S-ITS2 region of *Achlya* was successfully used as alternative approached for identification of species. For the biological characteristic study, all *Achlya* grew well at 25-35°C and were able to grow under up to 1.5% sodium chloride. They grew well and their zoospores were able to germinate at pH 4.0–11.0. Microscopic examination of the skin lesions of the infected tilapia revealed numerous hyphae covered epidermis and some penetrated into the epidermis. Bacterial infection could be observed adjacent to the infected area. It may be stated that *Achlya* is the secondary, opportunistic pathogen of Nile tilapia.

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