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## **Acanthamoeba spp. Found in Freshwater Fishes From Selected Areas of the Philippines - A Preliminary Report**

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*Acanthamoeba* spp. are ubiquitous organisms that have been adapted to different types of habitats and conditions. The study of freshwater fishes has become the interest of many researchers for their ability to harbor parasitic organisms, and they also play a significant role as intermediate hosts in the transmission cycle to humans. This study examines the *Acanthamoebae* spp. present in a variety of edible freshwater fish in the Philippines. A total of 14 different fish species (six fish per species) were collected from major lakes all over the Philippines. Fish intestines were aseptically dissected, pooled, processed, and cultured in non-nutrient agar lawned with *Escherichia coli*. Culture plates were examined for 14 days to determine their response to amoebal growth. Thirty-one percent of the fish species sampled were found to be positive for amoebic growth. Genomic DNAs were extracted and examined by polymerase chain reaction (PCR) using *Acanthamoeba*-specific primers. Further sequencing of PCR amplicons confirmed the presence of four *Acanthamoeba* species (*A. mauritanensis*, *A. polyphaga*, *A. castellanii*, and *A. lenticulata*) from the culture-positive samples. This study shows the presence of *Acanthamoeba* spp. from edible freshwater fishes in the Philippines. The presence of potentially pathogenic free-living amoebae like *Acanthamoeba* in edible freshwater fish may pose a public health risk. Although the effects of direct consumption of *Acanthamoeba*-infected fish are yet to be established, the potential of other means of infection, as discussed previously, needs to be taken into serious perspective.

**Keywords;** *Acanthamoeba*, fish, Free-living-amoeba (FLA), PCR

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## Introduction

*Acanthamoeba* spp. are free-living amoebae (FLA) isolated from a wide variety of habitats worldwide (Schuster & Visvesvara, 2004). They are ubiquitous organisms found in soil and aquatic environments (fresh, brackish, seawater), sewage, beach sands, hospital and dental environments, contact lenses, and even atmospheric dust (Visvesvara et al., 2007; Goldschmidt et al., 2012). Acanthamoebiasis has been reported in a variety of animal species, including fish, due to the presence of *Acanthamoeba* spp. in the aquatic environment. This allows for interaction with a broad range of aquatic resources, making it a primary habitat for these organisms (Schuster & Visvesvara, 2004). According to Chovanec et al. (2003), fish may act as bioindicators for ecotoxicological studies. Moreover, from a public health standpoint, it can be a potential reservoir and transmission host of several parasitic infections in humans. FLA such as *Acanthamoeba* spp. have been known to thrive as facultative parasites in certain species of fish such as *Sarotherodon aureus* (Taylor, 1977), *Astronotus ocellatus* (Laoprasert et al., 2009), *Oreochromis niloticus* (Dykova et al., 1997; Milanez et al., 2017), *Catostomus commersoni* and *Notropis cornutus* (Franke & Mackiewicz, 1982). Despite such findings, studies concerning the diversity of possible amphizoid amoeba infecting fish have not been given enough attention, and they usually conduct studies with other eukaryotic organisms (Dykova & Lom, 2004).

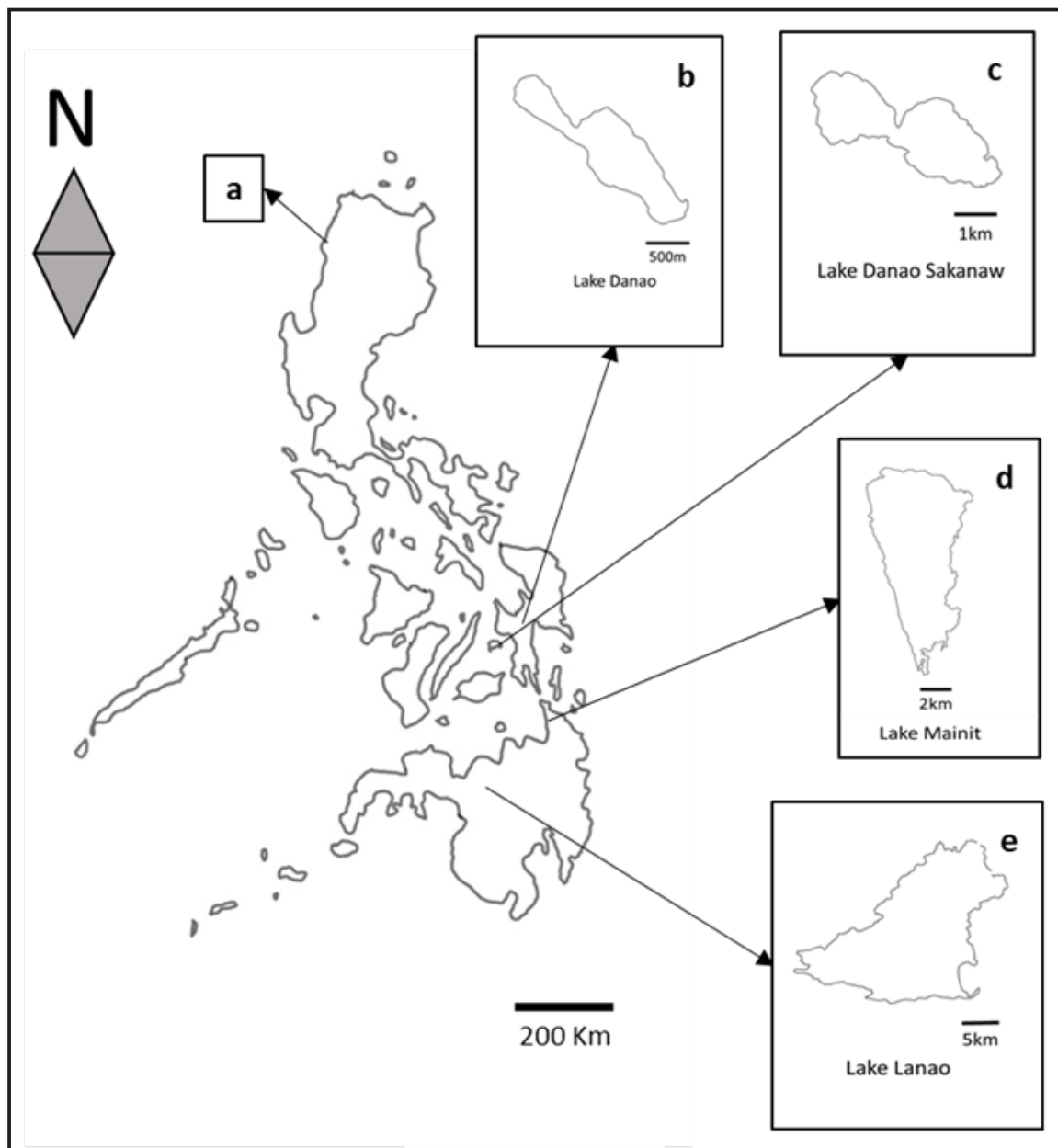
The clinical significance of *Acanthamoeba* species to humans has already been established. It is the frequent cause of *Acanthamoeba* keratitis (AK), a potentially blinding infection of the cornea in non-immunocompromised individuals (Marciano-Cabral & Cabral, 2003). Consequently, it can also cause granulomatous amoebic encephalitis (GAE), a fatal brain infection (Marciano-Cabral & Cabral, 2003; Schuster & Visvesvara, 2004; Khan, 2006;

Visvesvara et al., 2007). In the Philippines, the different eating habits of Filipinos in some parts of the country have proven to be a major risk factor for the transmission of fish-borne diseases and other waterborne protozoan pathogens. Several deaths in Northern and Southern Philippines due to intestinal parasites have been traced to the raw consumption of freshwater fish marinated in vinegar, such as the cases in 1963 in Luzon, 1999 in Mindanao, and 2007 in Siayan Municipality (Belizario et al., 2010). Although certain freshwater fish species in the country have been identified as reservoir/intermediate hosts for parasitic helminths, there is a knowledge gap on fish that serve as hosts for FLAs. This study attempted to investigate the possible types of *Acanthamoeba* spp. from a variety of edible freshwater fish in the Philippines. As a result, pertinent data on the isolation and identification of *Acanthamoebae* spp. were generated.

## Methods

### Sampling Sites, Sample Collection, Processing, and Culture

Eighty-four edible freshwater fish samples were collected from major lakes in the Philippines, namely West Pudoc, Lake Danao, Lake Sakanaw, Lake Mainit, and Lake Lanao (Figure 1). There were 14 unique edible fish species sampled (Table 1). Six samples for each fish species were collected, three of which were used for *Acanthamoeba* identification, and three were used for fish authentication. The representative samples for fish identification and authentication were preserved in plastic containers using 70% ethanol and were sent to the Institute of Biology, University of the Philippines, Diliman. Fish intestines were aseptically harvested, pooled according to fish species, processed, cultured, and subcultured following protocols (Milanez et al., 2017). Fish intestines were aseptically dissected upon arrival in the laboratory. Then, they were placed in 50 ml sterile polyethylene conical tubes with physiologic saline and were vortexed for 10 min. Intestines were removed, and the remaining fluid was centrifuged at 2000 rpm for 15 min, after which the resulting pellets were placed in previously prepared non-nutrient agar (NNA) lawned with *Escherichia coli* and were placed in an incubator set at 33 °C (Milanez et al., 2017).



**Figure 1.** Geographical Representation of Edible Freshwater Fish Sampling Sites in the Republic of the Philippines

**Notes:** (a) Fish ponds, West Pudoc, Ilocos Sur; (b) Lake Danao, Leyte, Eastern Visayas; (c) Lake Sakanaw, Camotes Island, Cebu, Eastern Visayas; (d) Lake Maiinit, Agusan Del Sur, Northern Mindanao; and (e) Lake Lanao, Lanao Del Sur, Mindanao.

**Table 1.** Consolidated Data of Fish Samples Details and *Acanthamoeba* Isolates

Scientific name	Common name	Local names	Average fish size (cm)	Aquatic source	Island source	Coordinates	<i>Acanthamoeba</i> spp. isolate with GenBank accession number
<i>Leiopotherapon plumbeus</i>	Silver perch	Bugaong <sup>2</sup> Ayungin <sup>3</sup>	9.25	West Pudoc	Luzon	16.9224° N, 120.4170° E	None
<i>Ambassis kopsii</i>	Singapore glassy perchlet	Bagsang <sup>4</sup>	16.0	West Pudoc	Luzon	16.9224° N, 120.4170° E	None
<i>Hypseleotris cyprinoides</i>	Tropical carp-gudgeon	Bagsit <sup>4</sup>	5.98	West Pudoc	Luzon	16.9224° N, 120.4170° E	None
<i>Gobiopertus stellatus</i>	Dwarf freshwater goby	Birut <sup>4</sup>	4.75	West Pudoc	Luzon	16.9224° N, 120.4170° E	None
<i>Glossogobius aureus</i>	Golden tank goby	Bukto <sup>4</sup>	8.20	West Pudoc	Luzon	16.9224° N, 120.4170° E	None
<i>Mistichthys luzonensis</i>	Sinarapan	Sinarapan <sup>5</sup>	1.00	Lake Buhi	Luzon	13.4496° N, 123.5168° E	None
<i>Oreochromis niloticus</i> <sup>1</sup>	Nile tilapia	Tilapia <sup>3</sup>	4.80	West Pudoc	Luzon	16.9224° N, 120.4170° E	None
<i>Glossogobius giuris</i>	Tank goby	Bul-A fish <sup>6</sup>	4.20	Lake Danao	Visayas	11.0708° N, 124.6953° E	<i>A. polyphaga</i> MN093961
<i>Stenogobius polyzona</i>	Chinesestripe goby	Gunggong <sup>6</sup>	4.00	Lake Sakanaw	Visayas	11.0708° N, 124.6953° E	None
<i>Bunaka gyrinoides</i>	Greenback gauvina	Buto Bot <sup>4</sup> Bunak <sup>3</sup>	8.00	Lake Bito	Visayas	11.0708° N, 124.6953° E	<i>A. mauritaniensis</i> MN093971
<i>Giuris margaritacea</i>	Snakehead gudgeon	Bugwan <sup>7</sup>	8.00	Lake Mainit	Mindanao	9.4521° N, 125.5111° E	None
<i>Channa striata</i>	Mudfish	Hayuan <sup>8</sup> Haluan <sup>9</sup> Dalag <sup>3</sup>	12.00	Lake Lanao	Mindanao	7.8931° N, 124.2644° E	<i>A. castellanii</i> MN093970
<i>Butis butis</i>	Duckbill sleeper	Pijanga <sup>7</sup>	8.00	Lake Mainit	Mindanao	9.4521° N, 125.5111° E	None
<i>Oreochromis niloticus</i>	Nile tilapia	Tilapia <sup>3</sup>	7.00	Lake Lanao	Mindanao	7.8931° N, 124.2644° E	<i>A. lenticulata</i> MN093972

<sup>1</sup>Juveniles; <sup>2</sup>Cebuano dialect; <sup>3</sup>Tagalog dialect; <sup>4</sup>Ilocano dialect; <sup>5</sup>Bicolano dialect; <sup>6</sup>Lineyte-Samarnon dialect; <sup>7</sup>Butuanon dialect; <sup>8</sup>Surigaonon dialect; <sup>9</sup>Binisaya dialect

**Table 2.** Reference Strains of *Acanthamoeba* spp., GenBank Accession Number and Location

Organism	GenBank Accession Number	Source/Location
<i>Acanthamoeba mauritaniensis</i>	AY351647 [37]	No source given/Korea
<i>Acanthamoeba castellani</i>	U07401 [37]	Corneal scraping/USA
<i>Acanthamoeba polyphaga</i>	AF019052 [38]	Corneal scraping/USA
<i>Acanthamoeba griffini</i>	U07412 [37]	Corneal scraping/USA
<i>Acanthamoeba triangularis</i>	AF316547 [37]	No source given/Korea
<i>Acanthamoeba palestinensis</i>	U07411 [37]	No source given/USA
<i>Acanthamoeba castellani Castellani</i>	U07413 [37]	No source given/USA
<i>Acanthamoeba rhyodes</i>	AY351644 [37]	No source given/Korea
<i>Acanthamoeba polyphaga</i>	AF019061 [38]	No source given/Korea
<i>Acanthamoeba castellani</i> NEFF	U07416 [37]	No source given/Korea
<i>Acanthamoeba castellani</i> Ma	U07414 [37]	No source given/USA
<i>Acanthamoeba divionensis</i>	AY351646 [37]	No source given/USA
<i>Acanthamoeba lenticulata</i> JC	U94739 [38]	No source given/USA
<i>Acanthamoeba lenticulata</i>	U94730 [38]	No source given/USA
<i>Acanthamoeba lenticulata</i> PD2S	U94741 [38]	No source given/USA
<i>Vermamoeba vermiformis</i>	MF716853 [7]	Freshwater fish/Philippines

### Microscopic Analysis

Culture plates were examined for 14 consecutive days using a light microscope (Nikon Eclipse E100) under 400X magnification before declaring negative. Briefly, the agar surface of positive culture plates were observed under a light microscope to identify the area of amoebic growth. After this, the selected area was cut to a block of approximately 1 x 1 cm using a sterile scalpel blade. The agar block was placed upside down onto a new NNA plate lawned with live *Escherichia coli* and incubated at 33 °C.

### DNA Extraction

Amoebic cysts and trophozoites were harvested from the positive culture plates by flooding the agar surface with cold phosphate-buffered saline solution and by gently scraping the agar surface with a sterile scalpel blade to detach adherent cells. A total of 800 microliter of fluid suspension was then aspirated and transferred to microcentrifuge tubes, and DNAs were extracted using (QIAamp) DNA Mini Kit® following the manufacturer's protocol.

### Identification of *Acanthamoebae* spp. using specific primers

DNAs were amplified using PCR (BioRad T100 Thermal Cycler®) with primer sets JDP1

5'GGCCCAGATCGTTTACCGTGAA-3'. Then, the JDP2 5'TCTCACAAGCTGCTAGGGAGTCA-3' for cells that resemble *Acanthamoeba* spp. PCR conditions were set as follows: 95 °C for 7 minutes for initial denaturation, 40 cycles of denaturation at 95 °C for 1 minute, the annealing temperature of 55 °C for 1 minute, extension at 72 °C for 2 minutes, and a final extension of 72 °C for 15 minutes (Booton et al., 2004). *Acanthamoeba* genotype T4 DNA was used as the positive control, which was generously provided by Prof. Patrick Scheid and Lt. Col. Dr. Carsten Balczun of Bundeswehr Central Hospital Koblenz, Germany.

### DNA Sequencing and Phylogenetic Analysis

To further identify the exact speciation of the isolates, PCR amplicons were visualized on a 1.5% agarose gel stained with ethidium bromide and were sent to a gene laboratory (Macrogen, Seoul, South Korea) for further sequencing. Sequences were aligned using ClustalW of BioEdit with careful visual consideration of gaps and ambiguous sequences. Next, they were deposited to the GeneBank database and are available under the following accession numbers: isolate T (MN093972), isolate M (MN093971), isolate H (MN093970), and isolate B2 (MN093961). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 7 (Kumar et al., 2016).

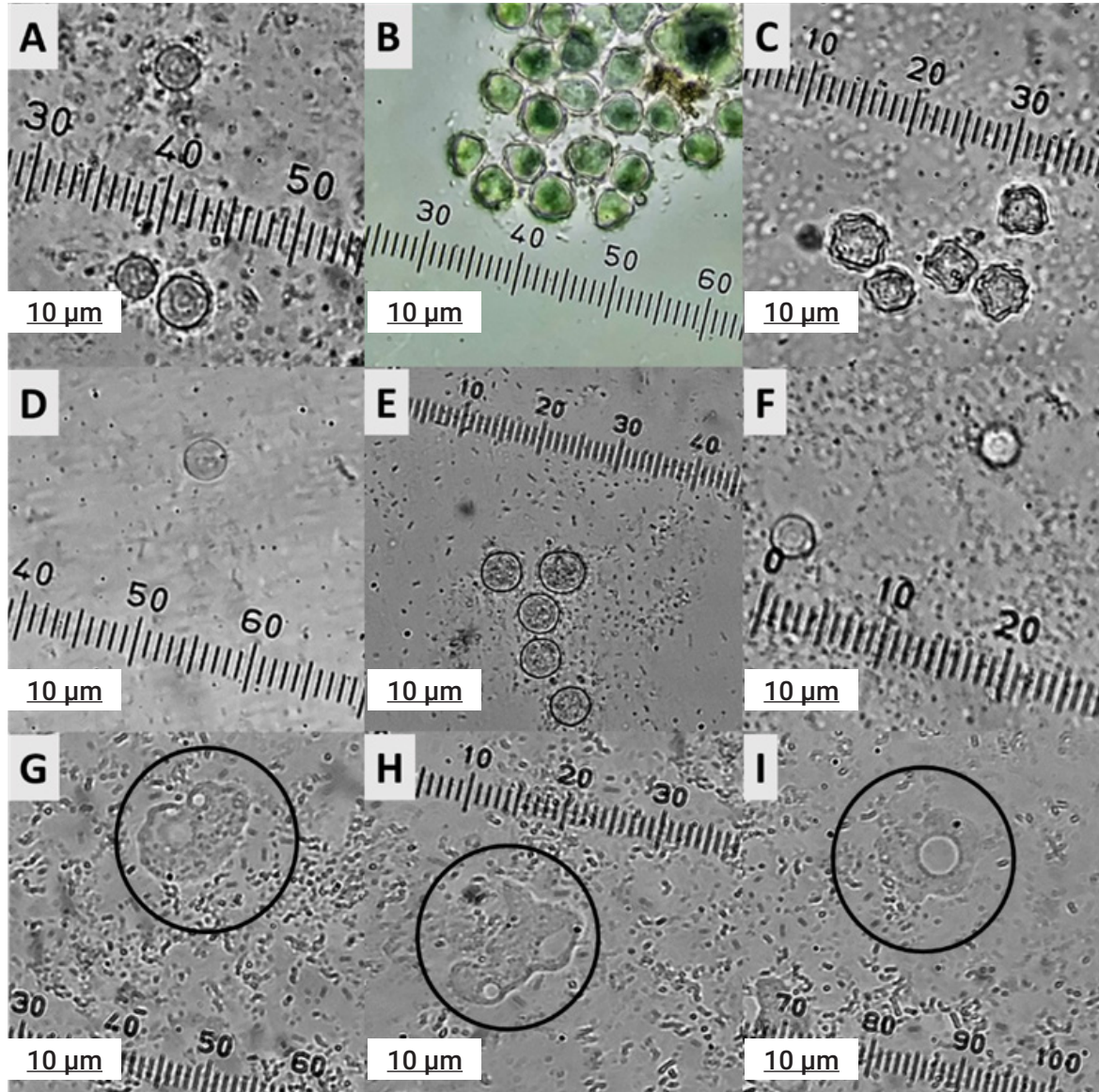


## Results

### Microscopic examination

The positive isolates for amoebic growth from freshwater fishes after 14 days of microscopic evaluation of culture plates were recorded at 28.57% (4/14). Cystic stages observed under the light microscope were irregular and double-walled,

with sizes ranging from 5 to 6  $\mu\text{m}$ . Trophozoites demonstrated singular pseudopodia with sluggish uni-directional motility with a distinct nuclear structure (Figure 4). Presumptive identification of isolates based on morphological criteria provided by PAGE relative to form, size, and shape (Page, 1967) was consistent with *Acanthamoeba* species.

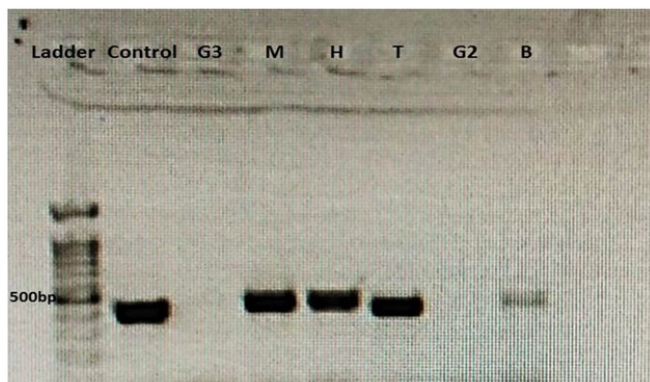


**Figure 2.** Micrographs of Free-Living Amoebae Isolated From the Organs of Edible Freshwater Fishes in the Philippines

Developmental forms showed cystic stages (A-F) and vegetative forms (G-H encircled) grown in non-nutrient agar lawned with *Escherichia coli*. Cystic stages showed irregular double walls with an average size of 5-7  $\mu\text{m}$ , while vegetative forms showed single pseudopodia with sluggish motility with an average size of 10-12  $\mu\text{m}$ . Magnification 400x. Scale bar = 10  $\mu\text{m}$ .

### Molecular Analysis of *Acanthamoeba* Isolates

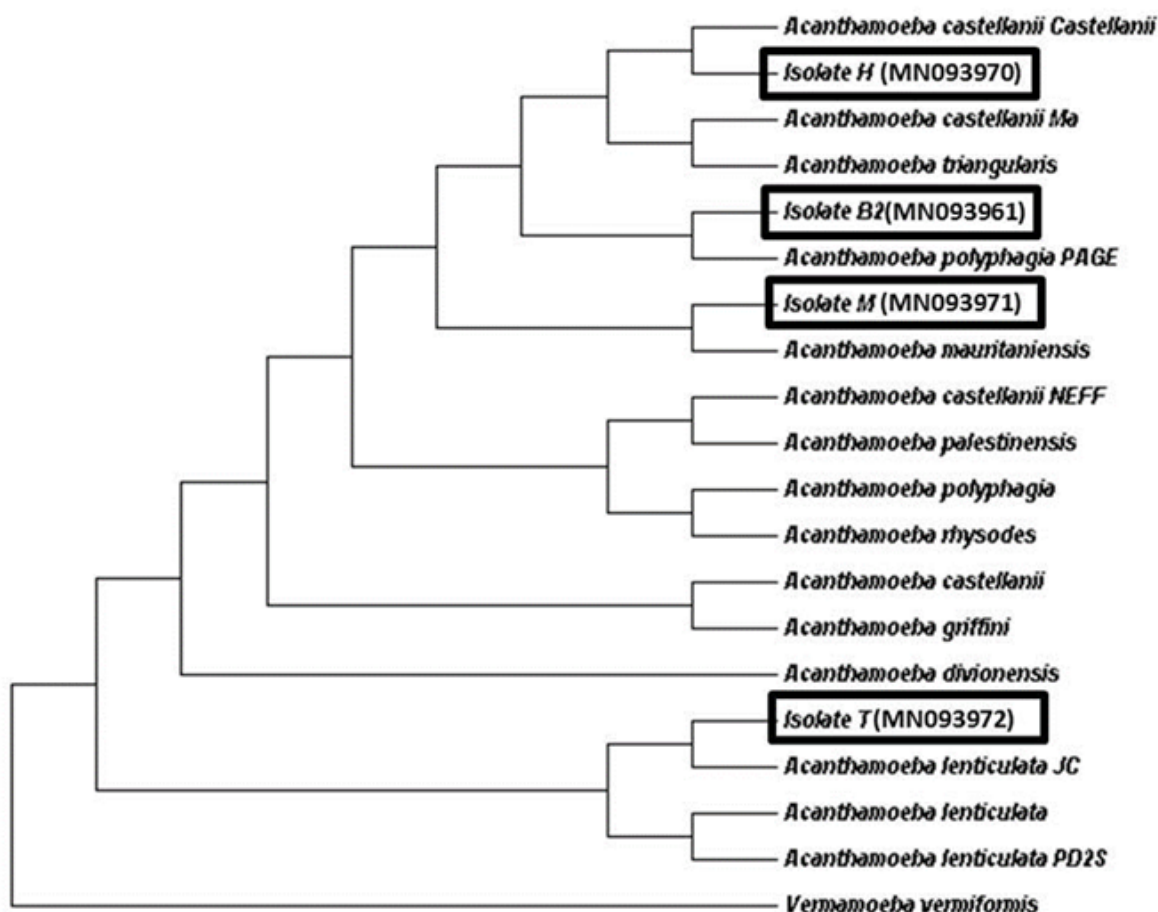
DNAs from presumptively identified *Acanthamoeba* isolates demonstrated distinct band formation between 400 and 500 bp against positive control of *Acanthamoeba* genotype T4 (Figure 3).



**Figure 3.** CR Amplicons Using JDP1 and JDP2 Primers in 1.5% Agarose Gel Stained With Ethidium Bromide

**Note:** Isolates G3, G2, and B showed no bands, while isolates M, H, and T showed bands in the 400 and 500bp regions. Control is *Acanthamoeba* genotype T4.

Further sequencing of PCR amplicons was performed and confirmed the presence of four *Acanthamoeba* species, namely *Acanthamoeba mauritaninsis*, *A. polyphaga*, *A. castellanii*, and *A. lenticulata* from four different edible freshwater fish species (Table 1). DNA sequences obtained from the test were aligned using ClustalW of Bioedit with consideration of gaps and ambiguous sequences. The maximum likelihood tree of isolated *Acanthamoebae* spp. with reference to *Acanthamoebae* strains was constructed using the Kimura 3 Parameter as the best tree model using the MEGA 7 application (Figure 4). Table 2 shows reference strains from GenBank used in this study.



**Figure 4.** Maximum Likelihood Tree of Isolated *Acanthamoeba* spp. (in boxes) With Reference to *Acanthamoeba* Strains

**Note:** Tree was constructed using the Kimura 3 Parameter as the best tree model using the MEGA 7 application.



## Discussion

This study confirmed the presence of potentially pathogenic *Acanthamoeba* spp. from four different species of edible freshwater fish in the country. Furthermore, to the best of our knowledge, this is the first and most comprehensive report of *Acanthamoeba* isolation in fish in the Republic of the Philippines. *Acanthamoebae* spp. has been reported to cause granulomatous amoebic encephalitis (GAE) and *Acanthamoeba* keratitis (AK) in humans, transmitted through several possible routes (Khan, 2006). Systemic acanthamoebiasis is slow to develop, with an insidious, subclinical course from the time of infection. *Acanthamoeba* has been isolated even from the nasal epithelial surfaces of healthy humans (Schuster et al., 2004). AK is a more frequent and acute disease with rapid onset following infection. As there is no treatment specifically approved for AK by the Food and Drug Administration, the early diagnosis of *Acanthamoeba* in improving prognosis and avoiding keratoplasty is of major importance (Juárez et al., 2018).

Four different species of *Acanthamoeba* were isolated, belonging to two different genotypes, namely *A. lenticulata* belonging from genotype T5 (MN093972), *A. castellanii* (MN093971), *A. mauritaniensis* (MN093970), and *A. polyphaga* (MN093961). All belong to genotype T4 and are from four different types of freshwater fish located in different parts of the country. *A. castellanii* and *A. polyphaga* are included in the list of pathogenic species that have been reported as the causative agent of GAE and skin lesions in immunocompromised patients and to cause AK in healthy patients (Visvesvara et al., 2007; Trabelsi et al., 2010; Schuster et al., 2004). Furthermore, *A. lenticulata* belonging to the T5 genotype has been reported to cause disseminated skin infections in humans, which later proved to be fatal (Barete et al., 2007). The environmental isolate *A. mauritaniensis* T4D, which was first characterized as a non-pathogenic amoeba, was proved to be involved in damaging Madin-Darby Canine Kidney (MDCK) cells in cell culture, indicating a potential pathogenic impact (Coronado-Velázquez et al., 2020). Genotype T4 is the *Acanthamoeba* most commonly isolated from the general human-inhabited environment (Booton et al., 2002, 2005; Khan, 2006; Ledee et al., 2009; Prashanth et al., 2011; Magnet et al., 2012). This is also found in human AK and GAE (Booton et al., 2005; Ledee et al., 2009; Magnet et al., 2012; Qvarnstrom et al., 2006). In the Philippines, the two genotypes T4 and T5 have been isolated before from samples from both environmental and

contact lens storage (Rivera & Adao, 2008, 2009). This type of *Acanthamoeba* spp. was isolated from each culture-positive fish sample despite the fact that other studies have shown multispecies infection in fish (Dykova et al., 1997), warranting further investigation relative to host specificity of *Acanthamoeba* suggested by some studies (Taylor, 1977).

Zoonotic fish-borne parasites have been implicated in human infection globally (Shamsi & Sheorey, 2018). Despite the fact that over 40 species of parasites associated with seafood have been reported in humans, diseases caused by parasites are significantly understudied compared to other infectious agents (Shamsi, 2019). A previously reported case of the isolation of the FLA, *Vermamoeba vermiformis*, from the gut of *Oreochromis niloticus* (Milanez et al., 2017) raises important questions about the role of aquaculture and the potential for FLAs to thrive in fish guts. This discovery has significant implications for the proliferation and potential transmission of FLAs to humans. This study has identified freshwater fishes as positive for *Acanthamoeba* spp. through molecular testing. It is important to note that some of the selected fish, such as tank goby (*Glossogobius giurus*), are consumed whole, including gut, after being dipped in vinegar. This raises concerns regarding the safety of consuming small freshwater species that are eaten raw. Proper handling and preparation are crucial to avoid the risk of infection from zoonotic parasites (Adams et al., 1997). After the fish death, parasites like nematodes are known to migrate from the internal organs of the fish into the flesh, increasing the risk they may pose to public health, a fact that can be minimized by the evisceration of fish immediately after capture and appropriate cold storage before consumption (Shamsi & Suthar, 2016; Shamsi & Sheorey, 2018). Although the translation or migration of infection from the fish gut to the muscles has not been established in all cases, the occurrence of *Acanthamoeba* spp. in the gut of the fish in this study should be taken into perspective, and additional testing is suggested. In addition, the possibility of a link between the proliferation of potentially pathogenic FLA in freshwater and the presence of aquaculture resources is likely, to which, hypothetically, the latter acts as a reservoir host. Although there is no solid evidence on this point, this should be taken into perspective.

Parasitic infections in aquaculture are an important cause of economic loss, which requires an integrated pest management strategy to reduce the attendant economic costs of parasitism (Shinn et



al., 2015). These strategies will reduce the impact on fish welfare and overall farm production and minimize economic losses. It will offer the best available preventative treatment and control strategies to minimize the impact of pathogens in fish production, increasing sustainability. The effectiveness of the applied strategies is strongly related to disease monitoring and knowledge of pathogen, host, and environmental risk factors (Sitjà-Bobadilla, 2017). Studies have shown that *Acanthamoeba* has the potential to cause severe infections in fish, leading to mass deaths that could impact the aquaculture industry (De Jonckheere, 1979). However, the risk of fish serving as a reservoir or intermediate host for FLA is of greater concern, particularly in countries where consuming certain species of raw fish is common. Handling and consuming these fish can increase the risk of transmitting potentially pathogenic FLA, such as *Acanthamoeba*. The presence of the T4 genotype in the fish sampled in this study may directly affect fish handlers in the market and may become potential agents of AK infection through hand-to-eye contact. With this in mind, the possibility of *Acanthamoeba* having access to the human system through broken skin or even during the whole consumption of smaller fish such as those included in this study is potentially high. Although it is not a commonly known mode of transmission, FLA can still be contracted through ingestion. Cystic stages have the ability to withstand the gut environment and survive in the host's intestinal tract as trophozoites. This is especially true in cases of immunodeficiency or other physiological abnormalities within the host.

Finally, *A. palestinensis* is known to be a Trojan horse of other pathogenic prokaryotes and is known to promote the growth of *Legionella pneumophila*, which exists as an endosymbiont (Anand et al., 1983). This makes *Acanthamoeba* a Trojan host of a sort or a host within a host in fish that is capable of harboring and transmitting other agents of morbidity or mortality.

The current data on the isolation of FLA in edible fishes in Southeast Asian countries are fragmented and require further exploration. Although there were similar studies, it should be pointed out that these studies are limited to a single species of fish (Laoprasitthet et al., 2009; Milanez et al., 2017). The observation of edible freshwater fishes is paramount to establishing the potential relationship between freshwater fishes and FLAs, both as a reservoir and pathogen, respectively. Conversely, knowledge of the impacts on FLA presence in fishes will provide not only additional perspective on the

capacity of this protozoan to exist in aquaculture but, more importantly, the impact it may bring to the aquaculture industry and consumers, as argued previously.

## Conclusion

This study provides the first documentation of the isolation and identification, through molecular techniques, of *Acanthamoeba* spp. from edible freshwater fish in selected areas in the Philippines. The presence of *Acanthamoebae* in edible fish poses serious public health risks, especially if these freshwater fish are not handled properly. This study provides evidence of the capacity of fish to become reservoir hosts for FLAs. The proper handling and appropriate preparations prior to consumption of the fish in this study are highly encouraged to avoid serious conditions brought about by *Acanthamoeba* infection.

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**Author contribution:** Conceptualisation: GDM. Developing methods: GDM, FRM. Data analysis: GDM, FRM, EG, PK. Preparation of figures and tables: MR, BH. Conducting the research, data interpretation, and writing: GDM, FRM, MR, BH, EG, PK.

## Conflict of interest

The authors declare no conflict of interest.

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