Molecular detection of *Acanthamoeba* spp. in Seven Crater Lakes of Laguna, Philippines

Lea D. Ballares, Frederick R. Masangkay, Joseph Dionisio, Oliver Villaflores, Maria Ruth Pineda-Cortel and Giovanni D. Milanez

ABSTRACT

Acanthamoeba spp. are ubiquitous free-living amoeba with genotypes that cause severe pathology of the eyes, central nervous systems, and rare reports of cutaneous infections. The Seven Crater Lakes are freshwater water resources in Laguna, Philippines primarily used for aquaculture and tourism. A total of 16 surface water samples were collected from different sampling areas per Crater Lake and placed in sterile plastic containers. Samples were filtered using 1.2 μm pore size, glass microfiber filter. Filtered sediments were placed on non-nutrient agar lawned with *Escherichia coli* and incubated aerobically at 35 °C for 14 days. Six out of 16 water samples exhibited amoebic growth. Cystic stages revealed circular to stellate morphology under light microscopy which were initially classified as *Acanthamoeba* spp. DNA from positive isolates were made to react with polymerase chain reaction using *Acanthamoeba* specific primers JDP1 5′-GGCCCAGATCGTTTACCGTGAA-3′ and JDP2 5′-TCTCACAAGCTGCTAGGGAGTCA-3′confirmed the presence of several *Acanthamoeba* species. Phylogenetic analysis revealed the presence of seven isolates belonging to *Acanthamoeba* genotypes T4, T5, and T9. The presence of potentially pathogenic *Acanthamoeba* genotypes in the Seven Crater Lakes of Laguna signifies risk to human health which necessitates the development of programs, policies, and guidelines on the understanding, prevention, and management of potential human infections.

Key words | *Acanthamoeba*, free-living amoeba, genotype, lakes, PCR, Philippines

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HIGHLIGHTS

- This study is the first to provide data on the presence of *Acanthamoeba* spp. on the surface waters of the Seven Crater Lakes of Laguna, Philippines.
- High prevalence of *Acanthamoeba* spp. in Seven Crater Lakes may be attributed to water temperature and anthropogenic activity.
- Phylogenetic analysis of *Acanthamoeba* spp. isolates revealed four genotypes: T9, T5, T4, and T18.
- Bunot Lake, identified with the most number of fish pens/cages demonstrated positive results in 2 out 2 (100%) SW samples.
- T4 and T5 genotypes known to cause AK infections have been identified in Bunot Lake.

INTRODUCTION

Lakes are one of the most important water resources especially in rural areas and have been used as a source of both food and water and also as a site for recreation and livelihood (Vasistha & Ganguly 2020). As an important resource, ensuring safety and security of these water reservoirs is necessary to maintain its

doi: 10.2166/wh.2020.146

usefulness and protect the human end users and the ecosystem.

A common neglected threat in lakes is the presence of opportunistic pathogens living ubiquitously in the system, such as the *Acanthamoeba* species (Siddiqui & Khan 2012; Visvesvara 2013; Walochnik *et al.* 2014; Bunsuwansakul *et al.* 2019). *Acanthamoeba* species are free-living amoeba (FLA) known to cause *Acanthamoeba* keratitis (AK), granulomatous amoebic encephalitis (GAE), disseminated sinusitis cutaneous lesions, and other serious organ infections (Marciano-Cabral & Cabral 2003; Booton *et al.* 2004; Lorenzo-Morales *et al.* 2015; Parija *et al.* 2015; Morrison *et al.* 2016; Brondfield *et al.* 2017; Orosz *et al.* 2019; Kalra *et al.* 2020). *Acanthamoeba* species survive in various environments, such as swimming pools, hot springs, cold waters, lakes, and other ecological water resources facilitating its easy access to infect human beings (Guimaraes *et al.* 2016; Kalra *et al.* 2020). Studies suggest its wide distribution in aquatic habitats such as lakes, rivers, ponds, hot springs, swimming pools, and recreational fountains (Caumo & Rott 2010; Di Filippo *et al.* 2015; Fabres *et al.* 2016; Karamati *et al.* 2016; Lass *et al.* 2017; Haniloo *et al.* 2017; Dendana *et al.* 2018; Ghaderifar *et al.* 2018; Mahmoudi *et al.* 2012; Nuprasert *et al.* 2010; Solgi *et al.* 2012; Kao *et al.* 2012; Paknehad *et al.* 2020; Reyes-Batlle *et al.* 2017). Tap and drinking water have also been reported for the presence of *Acanthamoeba* (Bagheri *et al.* 2010; Kao *et al.* 2012; Coskun *et al.* 2013; Yousuf *et al.* 2013; Behniafar *et al.* 2015; Haniloo *et al.* 2017). Similarly, studies have shown the isolation of *Acanthamoeba* spp. from soil, dust, bentonite deposits, contact lenses, haemodialysis, and dental treatment units and air conditioning systems (Chan *et al.* 2011; Hassan *et al.* 2012; Tanveer *et al.* 2015; Mohaghegh *et al.* 2016; Casero *et al.* 2017; Xuan *et al.* 2017; Saberi *et al.* 2019; Shyrobokov *et al.* 2020).

In the Philippines, limited studies on the isolation of *Acanthamoeba* spp. have been performed over the past decade. Philippines, being known to have numerous water resources, is in need of constant isolation studies to detect such species that may affect the end users of these water resources. The majority of isolation studies were done on clinical samples, in particular, from contact lenses (Rivera & Adao 2009; Buerano *et al.* 2014; Martín-Pérez *et al.* 2017) and nasal swabs (Cruz & Rivera 2014). Point source isolation from main FLA habitats such as freshwater systems is considered fragmented and is mainly focused on fish biodiversity (Papa & Briones 2017; Milanez *et al.* 2019). Only a few studies conducted on point source isolation from freshwater systems have been conducted (Hagosojos *et al.* 2020; Milanez *et al.* 2020).

A good example of water resources of high utility in the Philippines is the Seven Crater Lakes of Laguna. The Seven Crater Lakes of Laguna are primarily utilized for aquaculture, tourism, and recreational activities. Since the early 1980s, these lakes have been known for its tilapia (*Oreochromis niloticus*) farming industry (Brillo 2017), which serves as primary means of livelihood for the locals. Aside from being the main supplier of fish for the suburbs and Metro Manila, it has also become a tourist destination and an avenue for recreational activities. This has led to urban settlements, as well as the emergence of businesses and commercial infrastructures within the area. Among the studies conducted on the Seven Crater Lakes were water quality assessment, fish diversity (Briones *et al.* 2016), and zooplankton characterization (Coronado *et al.* 2011). To date, only studies on parasites primarily affecting freshwater fish are available. Here we investigate the presence of *Acanthamoeba* spp. on the surface waters of the Seven Crater Lakes of San Pablo City, Laguna through microscopic and molecular methods in order to contribute to the pioneering investigation of their bionomics and identify its potential risk to public health.

METHODS

Study setting

The Seven Crater Lakes (Figure 1) of Laguna, namely Sampaloc (S1), Bunot (S2), Palakpakin (S3), Mohicap (S4), Yambo (S5), Pandin (S6), Kalibato (S7) Lake, are maars or low relief craters found in the City of San Pablo Laguna, Philippines (Paller *et al.* 2017). These craters were formed as a result of the interaction of ground water and hot magma coming from Mount San Cristobal as a result of phreatomagmatic eruptions, creating a crater-like depression, which, over a period of time was filled with rain water. The lakes

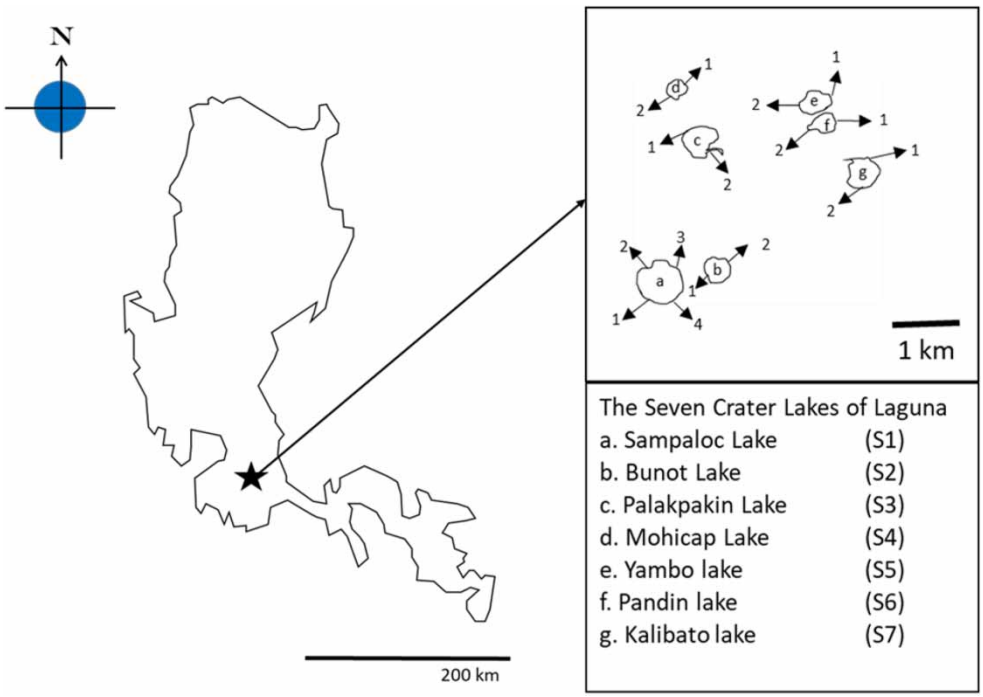

Figure 1 | Geographic representation of Luzon Island, Philippines and the Seven Crater Lakes of Laguna showing sampling sites (arrows) and lake locations (a–g).

are specifically located alongside the rift area between Mount Banahaw and San Cristobal, and Mount Makiling, with depths ranging from 7 to 156 m (LLDA 2008). As mentioned previously, the Seven Crater Lakes of Laguna are primarily utilized for aquaculture, tourism, and recreational activities (Table 1).

Sample collection, processing, and culture

A total of 16 surface water (SW) samples were collected in April 2019 from different sampling areas in the Seven Crater Lakes of Laguna. Two sampling areas were identified for Bunot Lake, Palakpakin Lake, Mojicap Lake, Yambo Lake, Pandin Lake, and Kalibato Lake, while four sampling areas for Sampaloc Lake were identified (Figure 1 and Table 1). Sampling sites were selected based on accessibility, proximity to the community, and presence of aquaculture (fish pens and floating cages). SW (250 mL) were collected at approximately 10–20 cm depth from the surface and placed in sterile plastic containers (Milanez *et al.* 2019). The samples were transported to the Medical Technology Department of Far Eastern University-Manila and processed within 24 h. SW samples were vacuum-filtered through a 47 mm diameter, 1.2 μm pore size glass microfiber filter

Table 1 | Consolidated details of study sites in the Seven Lakes of San Pablo City, Laguna

Study site	Coordinates	Temperature (°C)	Precipitation (cm)	Surf area (sq. km)
S1 Sampaloc Lake	14.075049, 121.326063	29.5	0.44	1.04
S2 Bunot Lake	14.078221, 121.344350	28.5	0.44	0.31
S3 Palakpaking Lake	14.112496, 121.336559	31.00	0.44	0.43
S4 Mohicap Lake	14.120757, 121.334565	27.0	0.44	0.14
S5 Yambo Lake	14.116834, 121.366797	31.0	0.28	0.29
S6 Pandin Lake	14.112748, 121.364742	30.0	0.28	0.21
S7 Kalibato Lake	14.106304, 121.376221	29.0	0.28	0.42

(Whatman™) using a simple Buchner funnel and electric-operated ILMVAC diaphragm pump (Fisher Scientific Pte Ltd) set-up. Filters containing sediments were placed sediment side down on non-nutrient agar (NNA) lawned with live *Escherichia coli* and incubated aerobically at 35 °C for 14 days. NNA plates were examined microscopically for amoebic growth using a light compound microscope (Nikon Eclipse E100) under 400× magnification. The agar surface was scanned for the presence of cysts and trophozoite forms (Figure 2). Positive plates were examined and subcultured following previously established protocols (Milanez *et al.* 2019). Briefly, approximately 1 × 1 cm of agar block from an identified area with the abundant growth was cut using a sterile scalpel blade and then placed culture-side-down onto a fresh NNA plate lawned with *E. coli*. These steps were repeated until a homogenous culture was obtained.

DNA extraction and molecular analysis

Acanthamoeba spp. trophozoites and cysts were harvested from culture plates by flooding the agar surface with cold phosphate-buffered saline solution (pH 7) and were gently scraped with a sterile scalpel blade (Milanez *et al.* 2019). The fluid was then aspirated and transferred to microcentrifuge tubes and DNA was extracted using QIAmp® DNA ministool kit, following the manufacturer's instructions. Primer pair JDP1 5′-GGCCCAGATCGTTTACCGTGAA-3′ and JDP2 5′-TCT CAAGCTGCTAGGGGAGTCA-3′ were used to amplify the ASA1.S1 region (Schroeder *et al.* 2001; Booton *et al.* 2004). Thermal cycling conditions were set as initial denaturation of 95 °C for 7 min, 40 cycles at 95 °C for 1 min, annealing temperature at 55 °C for 1 min, extension at 72 °C for 2 min, and a final extension of 72 °C for 15 min (Booton *et al.* 2004). *Acanthamoeba* spp. genotypes were identified by further DNA sequencing and phylogenetic analysis. In detail, a 1.5% agarose gel stained with ethidium bromide was used to visualize polymerase chain reaction (PCR) amplicons. PCR amplicons were sent to a commercial sequencing company (Macrogen, Seoul, South Korea) for further sequencing. Sequences were aligned using ClustalW of BioEdit with careful visual consideration of gaps and ambiguous sequences and were deposited in GenBank. Phylogenetic analysis of isolate sequences along with reference strains from GenBank (Table 2) was performed using the maximum-likelihood (ML) tree constructed using MEGA7 application.

Culture and microscopy results

Six out of 16 (37.5%) SW samples were positive for amoebic growth. Specifically, positive samples came from four lakes namely: Sampaloc Lake (A1), Bunot Lake (A1 and A2), Palakpakin Lake (A1) and Kalibato Lake (A1). Cysts observed under light microscopy exhibited varying morphology; some exhibited circular inner and outer cystic

Table 2 | Isolated *Acanthamoeba* spp. in the Seven Crater Lakes of San Pablo City, Laguna with assigned accession numbers from GenBank

Location	Isolated genotype	GenBank accession number
Sampaloc Lake	T9	MT328623
Bunot Lake	T5	MT449072
	T4	MT328628
Palakpakin Lake	T4	MT328648
Kalibato	T5	MT328655
	T9	MT328666
		MT328670

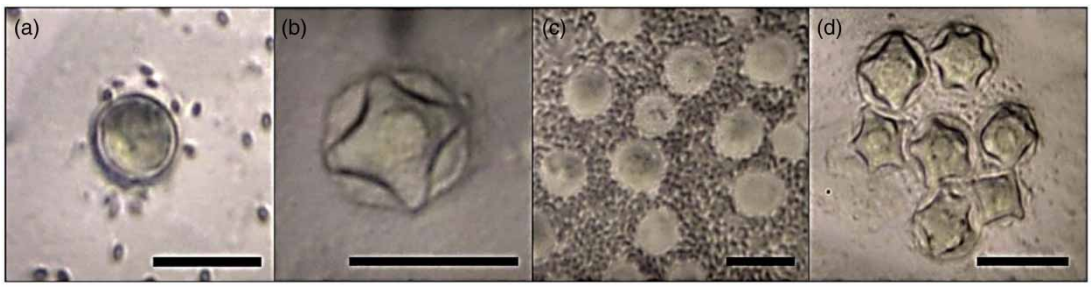

Figure 2 | Photomicrographs of *Acanthamoeba* spp. isolated from the Seven Crater Lakes of San Pablo City, Laguna showing morphological variation. 400× magnification; Scale bars set at 8–10 μm.

walls while some were observed as having stellate appearance with an irregular inner cystic wall (Figure 1). Amoebic cysts measure approximately 8–10 μm. Morphological classification of cysts by size and general morphology were based on Page's established morphological criteria (Page 1967).

Molecular results

PCR results using primer set JDP1 and JDP2 demonstrated distinct band formation between 400 and 500 bp region in agarose gel electrophoresis confirming the presence of seven isolates of *Acanthamoeba* spp. namely, S1A4, S2A1, S2A2, S3A1, S7A1, S7A1.1, and S7A1.2. *Acanthamoeba* spp. genotype T5 DNA was used as positive control which was generously provided by Prof. Dr Patrick Scheid and Dr Carsten Balczun of Bundeswehr Central Hospital in Koblenz, Germany. Aligned sequences of isolates were deposited in GenBank and were assigned with accession numbers MT328623, MT449072, MT328628, MT328648, MT328655, MT328666, and MT328670, respectively (Table 2). Phylogenetic analysis confirmed the presence of *Acanthamoeba* spp. belonging to genotype T4, T5, and T9 (Figure 3).

DISCUSSION

The Seven Crater Lakes of Laguna are primarily used for fishing and venues for tourism and recreational activities. Mojicap, Yambo, Pandin, and Kalibato Lakes offer activities for tourists while Palakpakin, Bunot, and Sampaloc Lakes provide aquaculture set-ups. In addition, all of the Seven Crater Lakes are used for recreational activities by the local inhabitants and as water sources used for drinking and domestic purposes. The present study is the first to provide data on the presence of *Acanthamoeba* spp. on the surface waters on these lakes that have an abundance of anthropogenic activities. The presence of potentially pathogenic *Acanthamoeba* spp. along with anthropogenic activities within the area signifies a potential risk factor for possible infection through swimming by inhalation of contaminated water (Arance-Gil *et al.* 2014), bathing (Casero *et al.* 2017), or via skin lesions (Megha *et al.* 2018).

The isolation of a single type of *Acanthamoeba* species in one sample setting has been observed in other similar studies done in the country (Hagosojos *et al.* 2020; Milanez *et al.* 2020). Although the physio-chemical properties of the water samples were not established in this study, such factors may be an important predictor of the type of FLA, in this case, *Acanthamoeba*, present in a given body of water (Milanez *et al.* 2019). Further, the occurrence may have been due to water temperature and anthropogenic activity, which could have possibly affected the quality of water in the sampling sites. The temperature of SW samples ranges from 27 to 31 °C at the time of collection, which is ideal for the growth of *Acanthamoeba* spp. FLA normally proliferates at temperatures between 10 and 30 °C (Rodriguez-Zaragoza 1994) which further validates the existence of *Acanthamoeba* spp. in some parts of the lakes. Another possible cause for the occurrence of *Acanthamoeba* spp. is the increased anthropogenic activity within the lakes, brought about by the excessive expansion of aquaculture resulting in congested fish pens/cages. Bunot Lake, identified with the most number of fish pens/cages (Brillo 2015), demonstrated positive results in 2 out 2 (100%) SW samples. On the contrary, only Yambo and Pandin Lakes abide by the 10% area limit in terms of the number of fish pens/cages, which may be accounted for its oligotrophic water (Brillo *et al.* 2019) described as low nutrient, low productivity, high drinking quality and usually found in colder regions.

The occurrence of *Acanthamoeba* spp. T9 genotype in Sampaloc and Kalibato Lakes poses a great concern for both the aquaculture industry and public health. Both lakes are considered the main sources of freshwater fish produce that supplies the community and neighbouring cities (LLDA 2008). The proliferation of *Acanthamoeba* spp. in these lakes may potentially trigger large fish kills and affect the economic aspect of the lake as the pathogenic capacity of *Acanthamoeba* spp. in freshwater fishes has long been established by researchers in previous studies (Dyková *et al.* 1999). In a public health aspect, *Acanthamoeba* belonging to genotype T9 is considered an emerging pathogen linked to cause AK infection in recent studies (Hajialilo *et al.* 2016). Fisher folks and individuals engaged in traditional spear fishing may contract the infection through exposure and inhalation of contaminated water. The isolation of *Acanthamoeba* genotype T5 and T4 in Bunot Lake have greater implications for public health rather than aquaculture. Among the Seven Crater Lakes,

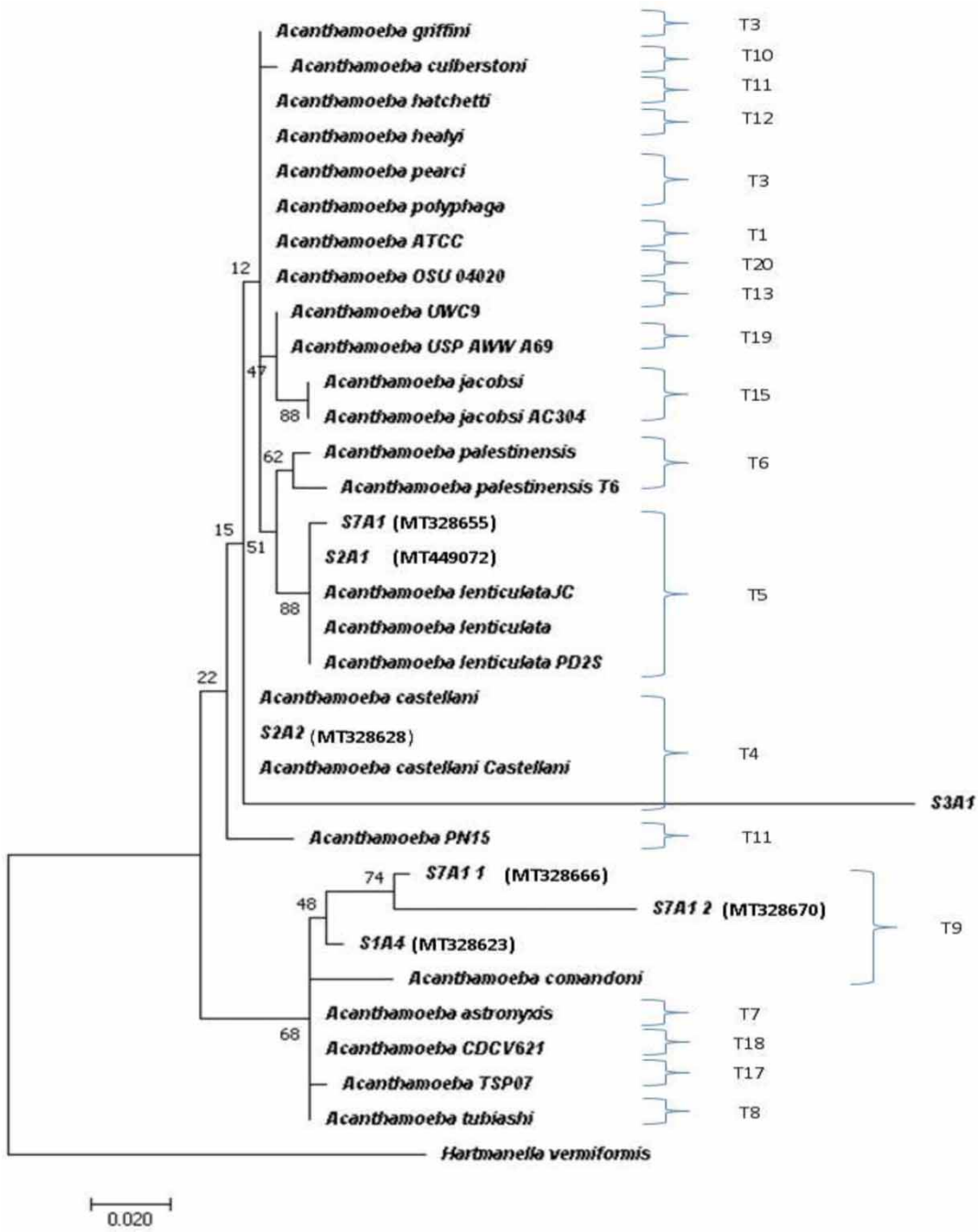

Figure 3 | Maximum-likelihood tree of *Acanthamoeba* spp. isolates (with accession numbers) from the Seven Crater Lakes of San Pablo City, Laguna along with reference strains from GenBank. Phylogenetic tree was constructed using MEGA 7 application. Bootstrap was set for 1,000 replicates.

Bunot Lake stands out in having the most number of human settlements around its shoreline. To add, the lake, despite its small area, have been congested with several fish cages, this translates to the potential increase of anthropogenic activity of nearby settlers engaged in fishing in the lake. This may consequently lead to potential infection of *Acanthamoeba* spp. by inhalation/contact of contaminated waters from the lake. Although the pathogenic capacity of *Acanthamoeba* spp. belonging to genotype 5, like that of T9 have not been well established, several studies would speculate

otherwise since it has been implicated to have caused rare types of disseminated cutaneous infection (Barete *et al.* 2007) and corneal infection in the USA (Leede *et al.* 2009).

This study has provided evidence on the presence of potentially pathogenic *Acanthamoeba* spp. in the Seven Crater Lakes of Laguna. Being known for its aquaculture industry and venues for tourism, this study elucidates possible modes of transmission of *Acanthamoeba* spp. and its health implications to humans promoting public awareness. Maintaining water quality through the regulation of the number of fish pens/cages may be an important step in mitigating the proliferation of *Acanthamoeba* spp. and its subsequent transmission to humans.

CONCLUSION

The present study reports data on the presence of potentially pathogenic and pathogenic genotypes of *Acanthamoeba* spp. from SW samples in the Seven Crater Lakes of Laguna, Philippines. PCR and phylogenetic analysis revealed isolates belonging to T4, T5, and T9 genotypes. This study contributes to the expansion of literature on the local distribution of *Acanthamoeba* spp. which will aid in the formulation of both private and governmental policies in the prevention, mitigation, and management of *Acanthamoeba* human infections.

ACKNOWLEDGEMENTS

The authors would like to thank the Department of Medical Technology, Far Eastern University-Manila for facilities assistance. Gratitude is extended to Romel Solomon, Virgilio Bitangcol, Ma. Lourdes Policarpio, Arnel Lopez, and Ivy Fababier for providing technical support.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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First received 27 May 2020; accepted in revised form 31 July 2020. Available online 1 September 2020