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# Molecular detection of free-living amoebae from Namhangang (southern Han River) in Korea

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The free-living amoebae *Naegleria* spp. and *Acanthamoeba* spp. exist in the natural environment and are sometimes causal agents of lethal primary amoebic meningoencephalitis (PAM), amoebic keratitis (AK) and granulomatous amoebic encephalitis (GAE) in humans, respectively. To ascertain the existence of free-living amoebae in Korea, water samples were collected from the Korean hydrosphere, Namhangang (southern Han River), an active location for water skiing and recreation. Samples underwent two-step filtration and were cultured on non-nutrient agar medium with inactivated *E. coli*. The remaining samples were subjected to PCR for primarily the 18S small ribosomal RNA gene and gene sequencing. Similarities in 18S rDNA sequences, in comparison with various reference amoebae in GenBank, showed 86–99% homology with *N. gruberi*, *N. philippinensis*, *N. clarki*, *A. polyphaga*, *A. castellanii*, and *Hartmannella (Vermamoeba) vermiformis*. Therefore, this study will be useful for seasonal detection of free-living amoebae from various Korean hydrospheres in future studies.

The free-living amoebae (FLA) *Naegleria* spp. and *Acanthamoeba* spp. are mainly distributed in ponds, rivers, and fresh waters worldwide. Their existing stages are trophozoite and cyst and additional biflagellate form in case of *Naegleria*. Trophozoites shows moving, feeding, and proliferation activity. However, cysts are formed in poor environments, such as under abrupt temperature changes, drying, and food depletion, and can survive for long periods<sup>1,2</sup>. *N. fowleri* is a pathogenic agent that causes primary amoebic meningoencephalitis (PAM), which is fatal to humans and laboratory animals<sup>2,3</sup>. *Acanthamoeba* spp. and *Balamuthia mandrillaris* cause chronic granulomatous amoebic encephalitis (GAE)<sup>4–6</sup>. Further, *A. castellanii* and *A. polyphaga* can infect the eye, resulting in acanthamoeba keratitis (AK)<sup>2,7,8</sup>.

PAM is mainly associated with activities in amoeba-contaminated water (swimming or water leisure activity), use of Neti-pots in rhinitis treatment, and religious ceremonies in some Asian countries<sup>5,9,10</sup>. The amoeba enters through the nasal cavity to invade the mucosal membrane. Subsequently, it moves into the olfactory bulb and meninges via the nasal nerve system, leading to development of meningoencephalitis<sup>11–13</sup>. Symptoms of PAM include acute headache, anorexia, nausea, vomiting, high fever (38–40 °C), and limb dysfunction symptoms. It also progresses acutely, with a mortality rate of over 95%. Amphotericin B is mainly used as a therapeutic agent, as a combination treatment by mixing with micronazole, rifampin, and doxycycline; however, only limited therapeutic effects have been demonstrated<sup>1,2,14–16</sup>.

AK usually occurs after wearing contaminated contact lenses, improper ophthalmic surgery, or corneal injury in various cases. With the popularization of contact lenses and careless lens management, the number of AK patients continues to increase<sup>2,7,17,18</sup>.

PAM due to *N. fowleri* occurs annually worldwide. In the United States, 143 cases of PAM occurred from 1962 to 2017, of which 80% were in males. Further, infection was most reported in adolescents<sup>19–22</sup>. In Pakistan, 22 patients in 2012 were found to be infected with *N. fowleri* after washing their nostrils with tap water as a religious ceremony<sup>10,23</sup>. Additional PAM patients are expected worldwide. In Korea, there has been no case of PAM caused

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**Figure 1.** Location of Namhangang River (nearby Yeosu city and Yangpyeong) in South Korea. Water sampling from Namhangang, location where water skiing and recreation are actively performed. Maps made by H-J Shin in Adobe photoshop (version 7.0.1, <https://www.adobe.com/>).

by *N. fowleri*; yet, one case of GAE due to *Acanthamoeba* sp. has been reported<sup>24</sup>. Conversely, multiple AK patients have been identified and reported<sup>25–28</sup>.

The occurrence of PAM cases continues to increase in the tropics and subtropics, with increasing risk worldwide due to global warming. In addition, as many people enjoy water-related sports, PAM occurrence has been a social issue through the broadcast media. While it has been reported in many countries, there has not yet been reported in South Korea. There, we wanted to find out what kinds of free living amoeba, especially *N. fowleri*, exist in South Korea. In addition, various FLAs are expected to reside in the Korean hydrosphere. Therefore, a distribution survey was conducted to identify *Naegleria* and *Acanthamoeba* species in southern Han River, an active location for water skiing and recreation.

## Materials and Methods

**Water sample collection.** To investigate the distribution of free living amoeba in Yeosu city and Yangpyeong-gun, near southern Han River (Namhangang), which are geographically close to Seoul, we collected 1-L surface water samples with a 20-cm diameter scoop from 10 sites periodically from August 2015 to August 2016 (over 120 water samples) (Fig. 1). Sample collection sites where carries out water recreational activities were shown in Fig. 2. The atmospheric temperature at the water sampling sites was between  $-11^{\circ}\text{C}$  and  $35^{\circ}\text{C}$ , and the water temperature ranged from  $0.1^{\circ}\text{C}$  to  $29^{\circ}\text{C}$ , especially  $26\text{--}29^{\circ}\text{C}$  in August (Table 1).

**Harvesting of FLA.** To concentrate FLA from water samples, we used a two-step filtration system. The collected surface water was first filtered using a Whatman filter, followed by filtration (with a  $0.4\ \mu\text{m}$  pore-size bottle top filter (Thermo Fisher Scientific Inc., USA) (Fig. 3). After leaving about 30 mL of solution and thorough washing of the filter, the mixture was centrifuged at 1,500 rpm for 5 min. A portion of the pellet and filter paper were placed on a non-nutrient agar (NN-agar) plate for the amoeba culture, and the remaining pellet was subjected to PCR for amplification of the 18S RNA gene.

**Culturing of FLA.** To culture FLA, the NN-agar plate was prepared with NN-agar medium. Briefly, 15 g of nutrient agar medium and 0.1 g of yeast extract were dissolved in 1,000 mL of distilled water. After sterilization and solidification, the agar medium was poured into 10 petri dishes. *Escherichia coli* was killed at  $60^{\circ}\text{C}$  for 30 min, and then evenly coated on the surface of the prepared agar plate. The prepared specimen was dropped onto the medium, following which the culture dish was covered and incubated at  $27^{\circ}\text{C}$  for 2–4 days. The amoebae were sub-cultured on fresh NN-agar plates two or three times. Further, *N. fowleri*, *A. castellanii*, and *A. polyphaga* used as positive control amoebae were cultured on Nelson's and PYG medium according to previous reports<sup>29,30</sup>. The morphological identification of cultured amoebae was observed with an inverted microscope (Olympus CKX 31, Japan).



**Figure 2.** Water collection sites in Yeoju city (①~⑧) and Yangpyeong (⑨,⑩). The collection was repeated at the same sites for one year. Maps made by H-J Shin in Adobe photoshop (version 7.0.1, <https://www.adobe.com/>). Photograph by H-J Sohn.

**PCR identification of FLA.** DNA was extracted from environmental sample according to the manufacturer's protocol (Qiagen, USA). Next, molecular identification of FLA has been performed as described below.

Briefly, 2  $\mu$ L of the extracted DNA was used as a template, and mixed with 10  $\mu$ L of Noblzyme™ PCR Plus Premix (Noble Bioscience Inc., Korea) in a PCR tube. Primer pairs to amplify the 18S rRNA gene of various FLAs, called pan primers (P-FLA) (2pi, Bioneer Inc., Korea), were according to previous studies<sup>31,32</sup>. The recommended amplicon sizes were as follows: *Acanthamoeba* spp., 1080–1500 bp; *Vahlkampfia* spp., 950 bp; *N. fowleri*, 900 bp; and *Vermamoeba (Hartmannella) vermiformis*, 800 bp. Amplification was performed with an initial polymerase activation step (5 min at 95 °C), followed by 35 cycles of denaturation (1 min at 94 °C), primers hybridisation (1 min at 60 °C), and extension (3.5 min at 74 °C) in a G-Storm thermocycler (Genetechnologie, UK). All PCR experiments were performed with the inclusion of positive controls (DNA extracted from *N. fowleri*, *A. polyphaga*, and *A. castellanii*) to ensure correct functionality of the reaction.

After completion of the reaction, the PCR products were electrophoresed at 120 V for 20 min using a 1% agarose gel stained with ethidium bromide (0.005%) and then analyzed by Gel-doc (Bio-rad, USA).

**18S rDNA sequencing and phylogenetic analysis.** The FLA nucleotide sequences of the PCR-amplified 18S rRNA gene were obtained from the direct sequencing (Genotech, Daejeon, Korea), and homology against registered FLAs in GenBank was analyzed. Based on this, we conducted FLA phylogenetic analysis by estimating the neighbor-joining distance using the MEGA6 program<sup>33</sup>.

## Results

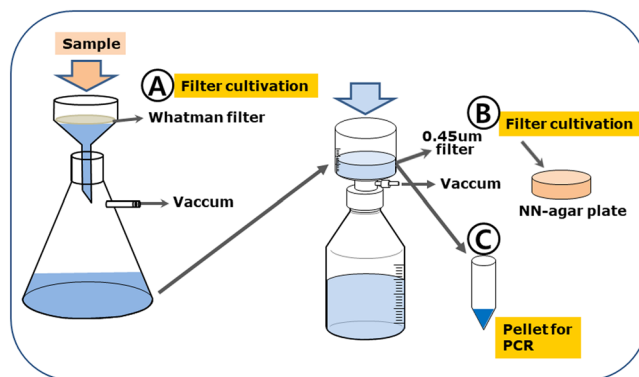
**Morphology of cultured FLA.** FLA cultured from water samples showed a resemblance to the presumed genus *Naegleria* or *Acanthamoeba*, as trophozoites showing round pseudopodia or acanthopodia; cysts were also observed in the colony (Fig. 4).

**18S rRNA gene sequence and alignment.** Based on the PCR results using P-FLA primers, the main PCR reaction bands were 700–900 bp for Yeoju water samples collected throughout the year (Fig. 5). Further, a homology search of DNA sequences from 18S rRNA genes of FLA isolates indicated matches with *N. gruberi* (99%), *N. philippinensis* (99%), *N. clarki* (97%), *A. polyphaga* (98%), *A. castellanii* (99%), and *Hartmannella (Vermamoeba) vermiformis* (97%), as which one Yangpyeong sample showed the lowest homology with *N. gruberi* (86%), and one

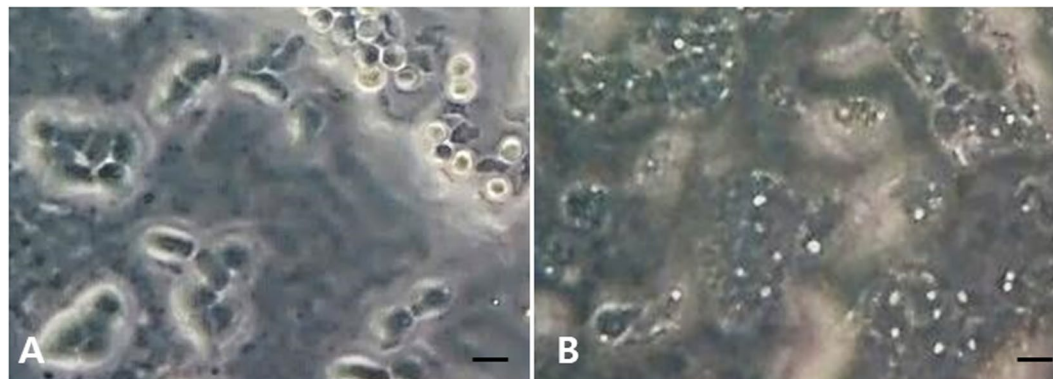
Collection date	Identified free-living amoebae and microorganisms	Homology (%)	Ambient temp. (°C) (Mini/Max)	Water temp. (°C)
August 2015	<i>Naegleria clarki</i>	97%	20/35	26~29
	<i>Naegleria gruberi</i>	99%		
	<i>Acanthamoeba castellanii</i>	99%		
	<i>Acanthamoeba polyphaga</i>	98%		
	<i>Naegleria philippinensis</i>	99%		
	<i>Naegleria australiensis</i>	95%		
	<i>Acanthamoeba lugdunensis</i>	99%		
	<i>Hartmannella (Vermamoeba) vermiformis</i>	97%		
	<i>Scapholeberis mucronata</i>	—		
	Uncultured fungus	—		
September	<i>Tetmemena</i> sp.	—	11/27	23~24
	<i>Stichotrichia</i> sp.	—		
	<i>Brachionus plicatilis</i>	—		
	<i>Oxytricha ferruginea</i>	—		
	Uncultured ciliate	—		
	Uncultured eukaryote	—		
October	<i>Thalassiosira gravida</i>	—	7/25	17~19
	<i>Scapholeberis mucronata</i>	—		
	<i>Choreotrichia</i> sp.	—		
	Uncultured metazoan	—		
	Uncultured eukaryote	—		
	Uncultured fungus	—		
November	<i>Naegleria gruberi</i>	86%	6/14	13~14
	<i>Naegleria clarki</i>	98%		
	<i>Paramecium pytrinum</i>	—		
	<i>Vorticella fusca</i>	—		
	<i>Choreotrichia</i> sp.	—		
	Uncultured ciliate	—		
	Uncultured eukaryote	—		
December	<i>Stephanodiscus parvus</i>	—	-10/-2	0.1~4.2
	<i>Skeletonema subsalsum</i> strain	—		
	<i>Skeletonema costatum</i>	—		
	Uncultured eukaryote	—		
January 2016	<i>Stephanodiscus parvus</i>	—	-11/-1	0.8~2.1
	<i>Paramecium putrinum</i>	—		
	<i>Synura petersenii</i>	—		
	<i>Synura glabra</i>	—		
	Uncultured eukaryote	—		
February	<i>Spumella</i> sp.	—	-5/8	3.3~4.7
	Uncultured eukaryote	—		
March	<i>Mallomonas cratis</i>	—	-2/17	8.6~12
	<i>Mallomonas tonsurata</i>	—		
	<i>Odontella rostrata</i>	—		
	<i>Synura</i> sp.	—		
	Uncultured eukaryote	—		
	Uncultured ciliate	—		
April	<i>Stephanodiscus</i> sp.	—	9/19	12~18
	Uncultured eukaryote	—		
May	<i>Peridiniopsis penardii</i>	—	13/25	17~19
	<i>Discostella stelligera</i> strain	—		
	<i>Hatschekia japonica</i>	—		
	<i>Thalassiosira</i> sp.	—		
	Uncultured eukaryote	---		
Continued				

Collection date	Identified free-living amoebae and microorganisms	Homology (%)	Ambient temp. (°C) (Mini/Max)	Water temp. (°C)
June	<i>Lacinularia flocculosa</i> isolate	—	17/30	24~26
	<i>Selaginella wildenowii</i>	—		
	Uncultured ciliate	—		
	Uncultured eukaryote	—		
July	<i>Tintinnidium balechi</i> isolate	—	23/33	26~28
	<i>Cricetulus griseus</i>	—		
	<i>Choreotrichia</i> sp.	—		
	Uncultured eukaryote	—		

**Table 1.** Summary of free-living amoebae and other microorganisms from Yeosu and Yangpyeong water samples identified by the homology analysis with 18s-rRNA gene sequence.



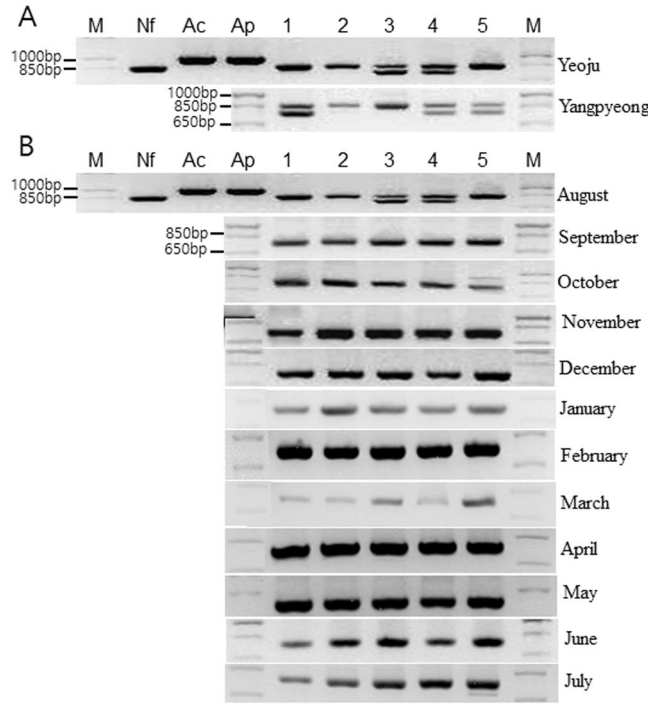
**Figure 3.** Free-living amoebae collection methods using two-filtration system. After the second filtration, a filter and some suspended water were cultured on NN-agar medium (B), and the remaining water pellet was subjected to PCR for 18s-rRNA gene amplification. The figure was prepared by H-J Shin using Adobe photoshop (version 7.0.1, <https://www.adobe.com/>).



**Figure 4.** Free-living amoebae cultured on non-nutrient agar plate, predicted as *Naegleria* sp. (A) and *Acanthamoeba* sp. (B). Bars, 10 µm.

Yeosu sample showed the highest homology with *N. clarki* (99%) by sequence alignment (Figs. 6 and 7). And then, summary of results was showed in Table 1.

**Phylogenetics of FLA 18S rDNA sequences.** A neighbor-joining distance tree was constructed based on phylogenetic analysis of the DNA sequences from the amplified FLA 18S rRNA gene. Many Yeosu specimens clustered mainly with *N. clarki* and *N. gruberi*, whereas Yangpyeong samples were closely related to *N. gruberi* and *N. australiensis* (Fig. 8). However, one of the Yeosu samples clustered with *A. castellanii* and *A. polyphaga* (Fig. 8).



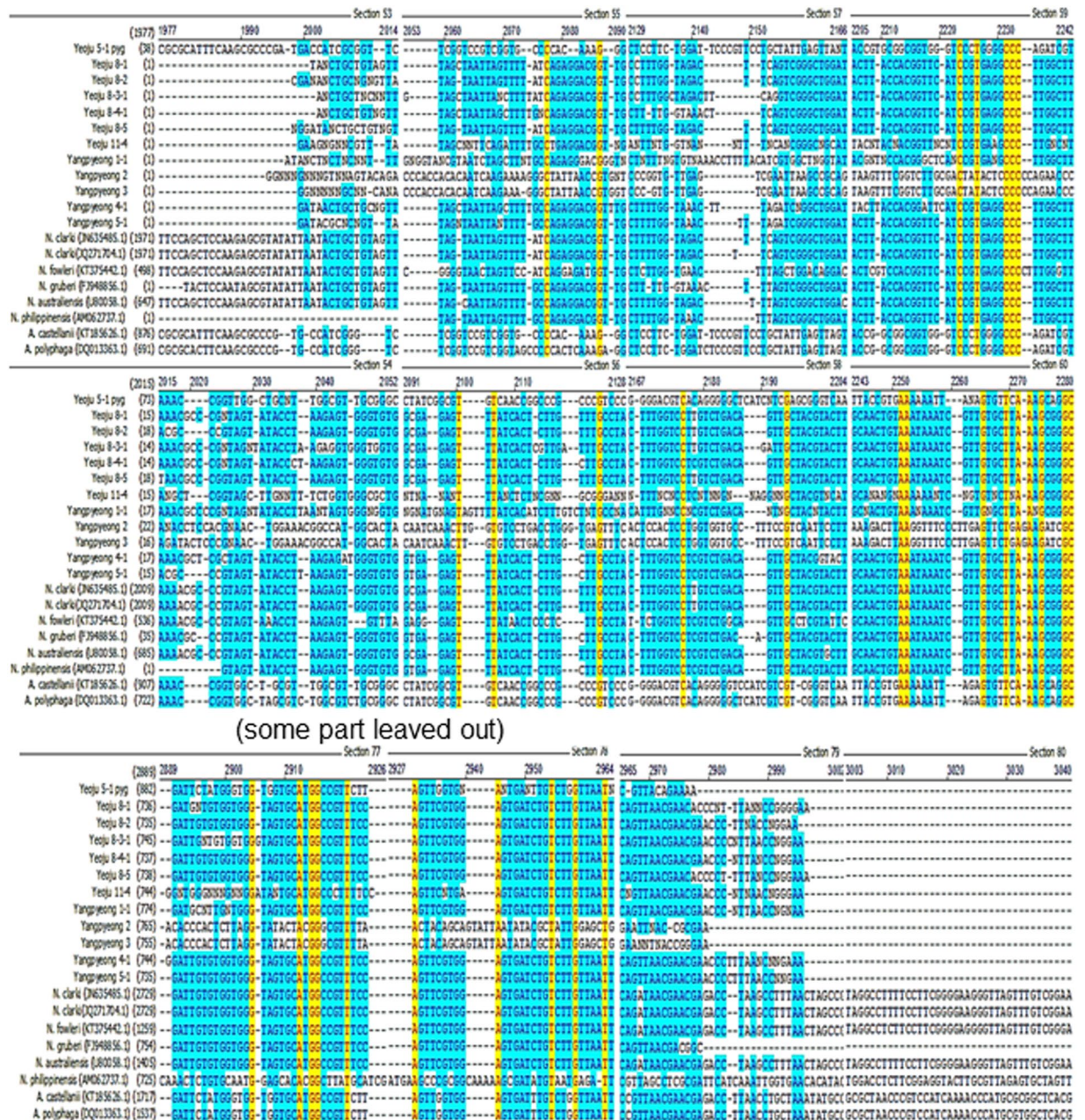
**Figure 5.** Amplified PCR products using P-FLA primers in water samples collected from Nanhangan, Yeoju city, and Yangpyeong (A), and a year in Yeoju city (B). Nf; *N. fowleri*, Ac; *A. castellanii*, Ap; *A. polyphaga*; lane 1–5, water sampling sites. M; PCR molecular marker.



**Figure 6.** 18S rDNA sequences and homology search of FLA isolates in Yeoju city and Yangpyeong.

### Discussion

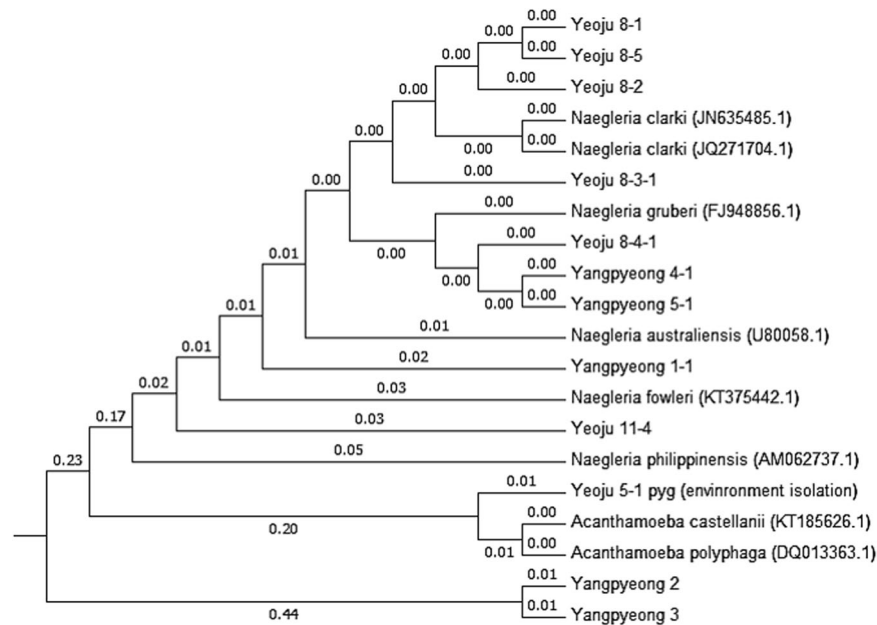
Epidemiological studies of PAM have mainly occurred in the southeastern part of the United States, through swimming and watering activities in *N. fowleri*-contaminated freshwater, such as lakes and ponds, during the summer months<sup>20,34,35</sup>. Subsequently, in other countries, various survey have been conducted on ponds, lakes, rivers, swimming pools, hot springs, and contaminated sewage, which are known as FLA habitats.



**Figure 7.** Alignment of 18S-rDNA sequences of free-living amoebae isolated from Namhangang against the reference amoeba.

In Thailand and Japan, *Naegleria* and *Acanthamoeba* species were detected in hot springs frequented by tourists and local residents<sup>36,37</sup>. In Italy, New Zealand, and California of the United States, *N. fowleri* was detected in a river area used for swimming and in a swimming pool<sup>38–40</sup>. In Taiwan, the nearest Asian country, *N. fowleri* was isolated from hot springs, and *Acanthamoeba* and *Naegleria* spp. from recreational water<sup>41</sup>. *Acanthamoeba* spp. was detected in water in neighboring China<sup>42</sup>. In Korea, *Acanthamoeba* spp. has been detected in amoeba-contaminated tap water in damaged water pipes and in several AK patients<sup>43,44</sup>.

We investigated only water samples where water sports or leisure activities were active, because we have the interest in public health which is caused by pathogenic free-living amoebae, especially *Naegleria* spp. and *Acanthamoeba* spp. Based on this survey on FLA distribution in the Korean hydrosphere, especially Namhangang, various species of *Naegleria* and *Acanthamoeba* were found in Yeoju and Yangpyeong samples, especially in August. Notably, the highly virulent *N. fowleri* (which favors high temperatures) was not found in this survey, possibly because the temperature of the water system was inadequate (measured as 26–29°C). A future detailed and extensive survey will be required to determine whether virulent *N. fowleri* inhabits this area. We conducted the survey over the course of a year to observe seasonal variations as well. However, the temperature in Korea



**Figure 8.** Estimation of neighbor-joining distance tree by phylogenetic analysis of free-living amoebae based on 18s-rDNA sequences.

sharply decreased in September, and the river surface water was frozen in December. As such, we could not observe FLA from September to June, although some bacteria and fungi were detected (data not shown).

Because many studies have not described the detailed process of FLA collection in an environmental water system, especially in rivers containing many floats, the culture protocol using NN-agar medium and the PCR protocol were difficult to conduct. By implementing the two-filtration system, we were able to overcome this limitation. After two filtrations, we were able to readily perform the amoeba culture and obtain PCR results. In addition, we attempted aseptic culture with Nelson's and PYG medium, which are commonly used in *Naegleria* and *Acanthamoeba* culture protocols. However, much time and effort are required for successful aseptic culture. Several *Acanthamoeba* species were successfully cultured; however, no *Naegleria* spp. could be cultured aseptically. These results will be published in a future report.

On the results of PCR-based DNA amplification, amoebic 18S rRNA sizes amplified with PAN primer, especially *Acanthamoeba* species, from water samples and reference amoebae were smaller than that suggested in previous paper<sup>31,32</sup>. Although there were differences in size, sequencing results were consistent and complete in this study. This issue is worth further study. Another primer, Nfa1 and ITS primer for the amplification of *Naegleria* spp.<sup>29</sup>, and 18s-rDNA primer for *Acanthamoeba* spp.<sup>27</sup>, was applied in the preliminary experiment, but various amoebae were not amplified (data not shown). Otherwise, PAN primer amplified various species.

Considering that FLA was isolated in many countries worldwide, especially around the Korean peninsula that PAM cases or *N. fowleri* were detected in the natural environment in China and Japan, the survey of FLA distribution in the Korean hydrosphere was required. As suggested in this study, the discovery of pathogenic *Acanthamoeba* sp. in southern Han River poses a potential risk to Korea. Therefore, future studies are necessary to investigate its wide distribution in various water environments, such as rivers with frequent water activities, hot springs, and swimming pools, in Korea.

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## Author contributions

H.K.K., H.-J.S. and H.-J.S. conceived and designed the experiments. H.K.K., A.-Y.P. and G.-E.S. performed the experiments. H.K.K., H.-J.S., G.-S.S., S.-Y.J. and A.-J.H. Collection of water sample and analyzed the data. H.-J.S., S.-E.L. and S.-H.C. helping to many discussions. H.-J.S. contributed reagents/materials/analysis tools and illustration preparation. H.K.K. and H.-J.S. wrote the paper.

## Competing interests

The authors declare no competing interests.

### Additional information

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