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RESEARCH

Characterizations of four freshwater amoebae including opportunistic pathogens newly recorded in the Republic of Korea

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Background: Free-living amoebae (FLA) are widely distributed in freshwater, seawater, soil, and extreme environments, and play a critical role as feeders on diverse preys in the ecosystem. Also, some FLA can become opportunistic pathogens in animals including humans. The taxa Amoebozoa and Heterolobosea are important amoeboid groups associated with human pathogens. However, the identification and habitat of amoebae belonging to Amoebozoa and Heterolobosea remain poorly reported in the Republic of Korea. This study highlights the first record for identification and source of four amoebae including putative pathogens in the Republic of Korea.

Results: In the present study, four previously reported FLA were isolated from freshwaters in Sangju Gonggeomji Reservoir (strain GO001), one of the largest reservoirs during the Joseon Dynasty period, and along the Nakdong River, the largest river in the Republic of Korea (strains NR5-2, NR12-1, and NR14-1) for the first time. Microscopic observations and 18S rDNA phylogenetic trees revealed the four isolated strains to be *Acanthamoeba polyphaga* (strains NR5-2 and NR12-1), *Tetramitus waccamawensis* (strain GO001), and *Naegleria australiensis* (strain NR14-1). Strains NR5-2 and NR12-1 might be the same species and belonged to the morphological Group 2 and the T4 genotype of *Acanthamoeba*. Strain GO001 formed a clade with *T. waccamawensis* in 18S rDNA phylogeny, and showed morphological characteristics similar to previously recorded strains, although the species' flagellate form was not observed. Strain NR14-1 had the typical morphology of *Naegleria* and formed a strongly supported clade with previously recorded strains of *N. australiensis* in phylogenetic analysis of 18S rDNA sequences.

Conclusions: On the bases of morphological and molecular analyses, four strains of FLA were newly observed and classified in the Republic of Korea. Three strains belonging to the two species (*A. polyphaga* and *N. australiensis*) isolated from the Nakdong River have the potential to act as opportunistic pathogens that can cause fatal diseases (i.e. granulomatous amoebic encephalitis, *Acanthamoeba* Keratitis, and meningoencephalitis) in animals including humans. The Nakdong River in the Republic of Korea may provide a habitat for potentially pathogenic amoebae, but additional research is required to confirm the true pathogenicity of these FLA now known in the Republic of Korea.

Keywords: *Acanthamoeba*, amoeba, classification, habitat, *Naegleria*, Nakdong River

Introduction

Free-living amoebae (FLA), including some of the main feeders of bacteria, are found in a variety of environments, such as freshwater, seawater, soil, and extreme environments (De Jonckheere et al. 2011; Lee et al. 2022; Park et al. 2009, 2012; Rodríguez-Zaragoza 1994). Amoebozoa Lühe, 1913 (emend. Cavalier-Smith, 1998), Rhizaria Cavalier-Smith, 2002, and Heterolobosea Page and Blanton, 1985 include amoeboid group, and some amoebozoan or heterolobosean amoebae are amphizoic (living in natural environments but having the potential to parasitize animals, including humans: Martinez and Visvesvara 1997; Smirnov and Brown 2004). The amoebozoan genera *Acanthamoeba* Volkonsky, 1931 and *Balamuthia* Visvesvara et al., 1993 and the heterolobosean genus *Naegleria* Alexeieff, 1912 (emend. Calkins, 1913) are well-known and opportunistically pathogenic FLA that can cause corneal infections such as *Acanthamoeba* Keratitis (AK) and fatal amoebic meningoencephalitis in humans (De Jonckheere 2014; Marciano-Cabral and Cabral 2003; Visvesvara et al. 1993). In addition to their own pathogenicity, some FLA can also serve as reservoirs for pathogenic bacteria such as *Legionella pneumophila* (Brown and Barker 1999; Greub and Raoult 2004; Winiecka-Krusnell and Linder 2001).

Acanthamoeba has been classified into three morphological groups proposed by Pussard et al. (1977): Group 1, which has a larger cyst (>18 μm) than other groups, with a wrinkled or smooth ectocyst and stellate endocyst; Group 2, with a wrinkled ectocyst and variously shaped endocysts (stellate, polygonal, triangular, or oval); and Group3, typically with a thin and smooth ectocyst and rounded endocyst. The molecular phylogeny of *Acanthamoeba* has shown that some species were classified based on morphology are polyphyletic. Stothard et al. (1998) proposed 12 genotypes based on 18S rDNA sequences, and regrouped the *Acanthamoeba* spp.; more recently, 21 genotypes (T1–T21) have been reported (Corsaro 2020). Most pathogenic *Acanthamoeba* causing AK belong to morphological Group 2 and the T4 genotype.

Heterolobosea is an important group for FLA research. This group includes diverse FLA with a distinct eruptive pseudopodial movement; some heterolobosean species are amoeboflagellates, having distinct amoeba and flagellate stages (Pánek et al. 2017). The heterolobosean genus *Naegleria*, includes more than 47 species, some of which can cause disease in humans and other animals (De Jonckheere 2014; Visvesvara et al. 2007). The genus *Tetramitus* comprises over 12 species with diverse morphological types. Some species of *Tetramitus* have mistakenly been assigned to other heterolobosean genera, such as *Singhamoeba*, *Learamoeba*, *Paratetramitus*, and *Vahlkampfia*, under morphology-based classification (Brown and De Jonckheere 1999; De Jonckheere and Brown 2005b). Molecular phylogenetic analyses using the 18S rRNA gene and ITS sequences has resulted in the reclassification of these species as members of *Tetramitus* (Brown and De Jonckheere 1999; De Jonckheere and Brown 2005b). Thus, accurate species identification and classification requires studies integrating morphological analysis for general group placement, and molecular phylogeny for species identification.

In the present study, four strains of FLA were isolated from freshwater samples in the Republic of Korea. Based on microscopic observations and molecular phylogeny, two isolates were identified as *A. polyphaga*, one isolate as *Tetramitus waccamawensis*, and one isolate as *Naegleria australiensis*. The morphology and 18S rRNA gene sequences of these three species were recorded for the first time in the Republic of Korea, two of which (i.e., *A. polyphaga* and *N. australiensis*) are known to be opportunistic pathogens in humans and other animals.

Materials and Methods

Sampling, isolation, and cultivation

The Nakdong River plays a crucial role as a drinking water source for almost 13 million people, which accounts for 26% of the total population of the Republic of Korea. Due to its significance as one of the most important freshwater resources, continuous monitoring of potential pathogenic amoebae is essential to safeguard the health of the Korean people. To find such amoebae, field surveys were continuously conducted from May 2015 to July 2017 along the Nakdong River, the largest river of the Republic of Korea, and in the Gonggeomji Reservoir, the largest reservoir during the Joseon Dynasty period. Four amoebae were isolated from freshwater samples collected from the Nakdong River and Gonggeomji Reservoir (NR5, NR12, GO, and NR14; Fig. 1). Collection data are shown in Table 1. Monoprotistan cultures of amoebae were obtained by using a plate-cultivation method. Briefly, 100 μL of each freshwater sample was inoculated onto plates of Page's amoeba saline (PAS; Page 1988) with 2% agar (w/v, final concentration), after spreading *Escherichia coli* as a food source. The cultures were maintained at 25°C until clearance zones formed. Cysts inside clearance zones were gently scraped off and inoculated into 15 mL conical flasks (50015; SPL Life Sciences Co., Ltd., Pocheon, Korea) containing 10 mL sterile PAS medium with 0.5% Luria-Bertani Lysogeny Broth (LB; v/v, final concentration), and incubated at 25°C for 1 week. After confirming active growth of the four isolates, each isolate was transferred into 50 mL tissue flasks (70125; SPL Life Sciences Co., Ltd.) containing 10 mL sterile PAS with autoclaved barley grains and incubated at

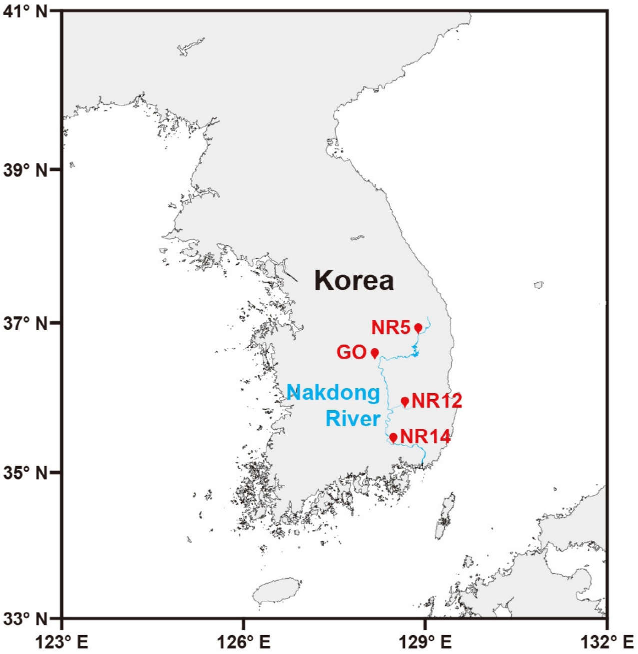

Fig. 1 Location of sampling sites (NR5, NR12, GO, and NR14). NR: Nakdong River; GO: Gonggeomji Reservoir.

Table 1 Collection data for the four freshwater samples

Sampling site	Sampling point coordinates (latitude; longitude)	Date (d/mo/y)	Temperature (°C)	pH
NR5	36°50′44.5″ N; 128°53′22.8″ E	28/May/2015	15.6	9.3
NR12	35°52′17.8″ N; 128°40′44.1″ E	29/May/2015	26.6	8.8
GO	36°30′46.8″ N; 128°09′41.4″ E	03/July/2017	30.7	9.1
NR14	35°22′58.8″ N; 128°28′34.4″ E	26/July/2017	20.1	9.1

NR: Nakdong River; GO: Gonggeomji Reservoir.

25°C. Subculturing was carried out every 2 weeks. Fixed specimens of each strain were deposited at the National Institute of Biological Resources (NIBR), Incheon, the Republic of Korea.

Microscopy

Live amoebae, flagellates, and cysts were observed using a differential interference microscope (Leica DM5500B; Leica Microsystems, Wetzlar, Germany) equipped with a DFC550 digital camera, and dimensions were measured using the open-source software FIJI (Schindelin et al. 2012).

For scanning electron microscopy, cultures were collected by centrifugation for 5 minutes at 4,000 × g and fixed with glutaraldehyde (final concentration 2.5% v/v; electron microscopy grade, Sigma-Aldrich, St. Louis, MO, United States) overnight at 4°C. Fixed cells were washed three times with sterile PAS medium by centrifuging for 5 minutes at 4,000 × g. Concentrated cells were then postfixed with OsO_4 (final concentration 2% w/v; SPI Supplies, West Chester, PA, United States) for 1 hour at room temperature while settling on glass coverslips coated with 1% poly-L-lysine. After rinsing with sterile PAS medium, cells were dehydrated using a graded ethanol series (30%, 50%, 70%, 80%, 90%, 95% × 2, and 100% × 3 dry, 10 minutes each step). Samples were dried using a critical point dryer (HCP-2; Hitachi, Tokyo, Japan) and coated with platinum using an ion-sputter system. Specimens were examined under a SU8220 field emission scanning electron microscope (Hitachi).

Molecular sequencing and phylogeny

Nucleic acids (DNA) from the four amoeba cultures were extracted using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. 18S (small subunit) rRNA gene sequences were amplified using the EukA/EukB primer set (EukA: 5'-AACC TGGTTGATCCTGCCAGT-3'; EukB: 5'-TGATCCTTCTGCAGGTTCACCTAC-3', Medlin et al. 1988). Polymerase chain reaction (PCR) amplification was performed in a 20 μL reaction mixture using TaKaRa Taq^{TM} polymerase (TaKaRa Bio Inc., Shiga, Japan). The PCR was performed on a Biometra TRIO thermal cycler (Analytik Jena, Jena, Germany) with an initial denaturing step at 94°C for 5 minutes, followed by 35 cycles of 45 seconds at 94°C, 1 minute at 55°C, and 3 minutes at 72°C, with a final extension step for 20 minutes at 72°C. Amplicons were isolated after gel electrophoresis using the QIAquick gel extraction kit (Qiagen) and sequenced using the Sanger method (Macrogen Inc., Seoul, Korea). Sequences were deposited in GenBank under accession codes OQ534278, OQ534279, OQ534280, and OQ520223.

We constructed two datasets of 18S rRNA gene sequences for phylogenetic analysis: one for the *Acanthamoeba* group, and one for the *Naegleria-Tetramitus* group. The *Acanthamoeba* dataset included sequences from 62 representative species from the family Acanthamoebidae, including two sequences from the isolated amoebae (strains NR5-2 and NR12-1), 55 species from members of the genus *Acanthamoeba*, and sequences from five other genera as an outgroup; the seed alignment originated from Corsaro (2020). The *Naegleria-Tetramitus* dataset included 52 representative *Naegleria* and *Tetramitus* sequences including two sequences from isolated amoebae (strains NR14-1 and GO001), 31 from *Naegleria* species, 19 from *Tetramitus* species, and two sequences of *Acrasis* species as an outgroup; the seed alignment originated from Khwon and Park (2018) and Park (2017). The datasets were aligned using MAFFT v.7 (Katoh and Standley 2013) and masked by eye. Maximum likelihood trees were estimated using IQ-TREE v.1.6.12. The GTR + F + I + G4 model was selected through the best-fit model test option (-m TEST, Flouri et al. 2015; Minh et al. 2013; Nguyen et al. 2015). Statistical support was assessed using ultrafast bootstrapping with 1,000 replicates. Bayesian analysis was conducted using MrBayes 3.2.7a (Ronquist et al. 2012) with two independent runs, each with four chains running for 5,000,000 generations with the default heating parameter (0.1) and sampling frequency (0.01). A conservative 30% burn-in was used; the average standard deviation of split frequencies for the last 70% of generations was <0.01.

Results

Amoeba strains NR5-2, NR12-1, GO001, and NR14-1 were each isolated from freshwater samples collected from separate sites in the Republic of Korea (Fig. 1 and Table 1). The three amoebae strains marked with "NR" were isolated from the Nakdong River, while one strain was isolated from Sangju Gonggeomji Reservoir (strain GO001). Strains NR5-2 and NR12-1 exhibited typical morphology of the genus *Acanthamoeba*, and the other two strains NR14-1 and

GO001 had eruptive pseudopodia, which is a typical characteristic of heterolobosean amoebae (see below). These four strains grew actively in PAS medium at 25°C. Most of the cells transformed into cysts within 1 week of cultivation; upon subculturing, most of the amoebae excysted within 2–3 days. Interestingly, viable cysts were found in cultures that were approximately 4 years old, demonstrating the remarkable survival and recovery capability of the cysts.

Acanthamoeba polyphaga strains NR5-2 and NR12-1

Morphological characteristics

The strain NR5-2 (Fig. 2) was very similar to the strain NR12-1 (Fig. 3) based on light and scanning electron microscopic observations, suggesting the same species. The shapes of the amoebae of strains NR5-2 and NR12-1 were irregular and ovoid, ellipsoid, or fan-shaped, with a broad, hyaline anterior lobopodium (i.e., a hyaline cap), and numerous short, slender, flexible, and tapering acanthopodia (Figs. 2A-H and 3A-K). Their mean lengths were 25.8 μm (range: 16.5–39.6 μm; n = 50) and 21.0 μm (range: 8.4–35.9 μm; n = 50), respectively, and their mean widths were 19.1 μm (range: 10.4–25.9 μm; n = 50) and 14.0 μm (range: 6.4–26.4 μm; n = 50), respectively, for length-to-width ratios averaging 1.4 (range: 1.1–1.9) and 1.5 (range: 1.1–3.0), respectively. Sometimes amoebae showed fine and short uroidal filaments at their posterior ends (“U” in Figs. 2D, E and 3A-G, I). A single vesicular nucleus with a central nucleolus was observed in most cells of both strains (Figs. 2A-G and 3A-K), but occasionally two nuclei were observed instead, implying the dividing cell (Fig. 2H). Cells of both strains contained one or more contractile vacuoles and numerous food vacuoles (Figs. 2A-H and 3A-K).

Cysts of strains NR5-2 and NR12-1 were highly refractile, having irregular, spherical, or oval shapes, with mean diameters of 12.8 μm (range: 10.7–15.3 μm; n = 50) and 12.9 μm (range: 11.4–18.1 μm; n = 50), respectively (Figs. 2I-V and 3L-Z). Several folds and ripples were observed in the ectocysts of both strains. Endocysts having different shapes, such as polygonal, triangular, or oval, were observed in both of the strains (Figs. 2I-V and 3L-Z). The cysts of both strains contained a single nucleus with a nucleolus (“N” in Figs. 2I and 3Z) and lipid droplets within the endocyst (“LD” in Figs. 2K and 3Z). Occasionally, spike-like structures were observed on the ectocysts of strain NR5-2 (arrowhead in Fig. 2R, S, V). One side of the ectocyst was occasionally open in strain NR12-1 (Fig. 3R, Y). A few cysts of each strain had no cytoplasm (Figs. 2U, V and 3X, Y). Scanning electron micrographs of the strains NR5-2 and NR12-1 showed that the cysts had wrinkled outlines, and prominent opercula and ostioles (Figs. 2W and 3AA, AB). Ectocysts of strain NR12-1 were occasionally observed to be open on one side, and amoebae with numerous acanthopodia were also observed in the same strain (Fig. 3AA, AB).

Molecular phylogeny

The length obtained for the 18S rRNA gene sequences from strain NR5-2 (2,125 bp) was notably shorter than the NR12-1 (2,295 bp). A BLASTN search of 18S rRNA gene sequences returned near-maximal identity with strains from the *Acanthamoeba* group. Strain NR5-2 showed the highest identity to *A. hatchetti* strain 2HH (99.9% identity: 2,123 of 2,125 bp), whereas strain NR12-1 showed the highest identity to *A. polyphaga* (99.3% identity: 2,286 of 2,294 bp). Phylogenetic analyses of 18S rDNA sequences placed both strains in the genus *Acanthamoeba* with maximal ultrafast bootstrap support (UFBS) and a Bayesian posterior probability (PP) of 1 (Fig. 4). Both strains NR5-2 and NR12-1 were assigned to the T4 genotype of *Acanthamoeba*, specifically with the T4E subgenotype with *A. polyphaga* Page-23, the type strain of that species, with good UFBS (97%) and a PP of 1 (Fig. 4).

Tetramitus waccamawensis strain GO001

Morphological characteristics

Amoebae of strain GO001 were monopodial limax or polypodial fan-shaped amoebae with eruptive pseudopodia (Fig. 5A-H). The average length and width of the limax amoebae was 24.8 μm (range: 16.3–32.2 μm) and 12.7 μm (range: 6.7–17.6 μm), respectively (n = 30; Fig. 5A-D); that of the fan-shaped amoebae was 21.8 μm (range: 16.9–27.5 μm) and 16.3 μm (range: 10.6–20.8 μm), respectively (n = 30; Fig. 5E-H). The average length-to-width ratio of the limax amoebae was approximately 2.0 (range: 1.5–2.8). Most amoebae exhibited the monopodial limax form; fan-shaped amoebae also transformed into limax amoebae over time. Some trophozoites had fine trailing uroidal filaments extending from the posterior part of their cells (“U” in Fig. 5D, H). A large, subcircular nucleus with a large central nucleolus and one or more contractile vacuoles were observed in the cells (“N” and “CV”, respectively, in Fig. 5C). Flagellate forms were not observed in liquid cultures.

A spherical to oval shape with a smooth outline was observed in the cysts of the strain GO001 (Fig. 5I-L), and sometimes the ectocyst was found collapsed at one point (arrow in Fig. 5J). The average diameter of the cysts was 10.6 μm (range: 8.4–13.6 μm; n = 30). The ectocyst wall was easily distinguishable from the endocyst. A single nucleus with a distinct nucleolus, but no cyst pores, was observed in the cysts (Fig. 5I). Some cysts lacked cytoplasm (Fig. 5L). Scanning electron micrographs of the strain GO001 revealed cysts with smooth outlines (Fig. 5M). Sometimes one point of the ectocyst wall was collapsed (arrow in Fig. 5N).

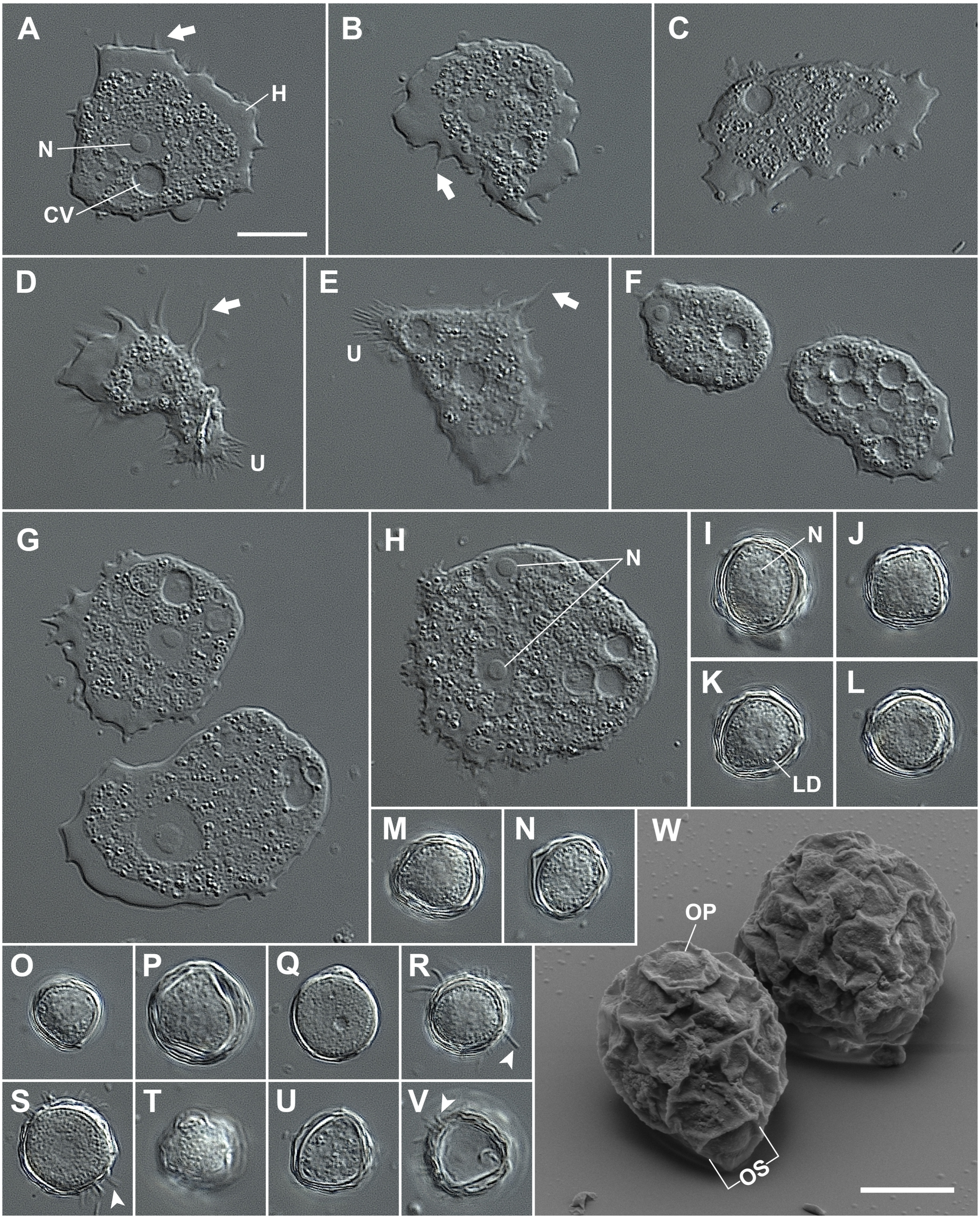

Fig. 2 Differential interference contrast micrographs (A-V) and scanning electron micrographs (W) of *Acanthamoeba polyphaga* strain NR5-2. (A-H) Amoebae (trophozoites) showing size, shape, and pseudopod formation. (H) Amoebae with two nuclei. (I-W) Cysts with various shaped and double wall. (U, V) Empty cysts. Arrow and arrowhead denote acanthopodia and spike-like structure on the ectocyst wall, respectively. N: nucleus; CV: contractile vacuole; H: hyaloplasm; U: uroid; LD: lipid droplet; OP: operculum; OS: ostiole. Scale bar in A = 10 μm, for A-V. Scale bar in W = 5 μm.

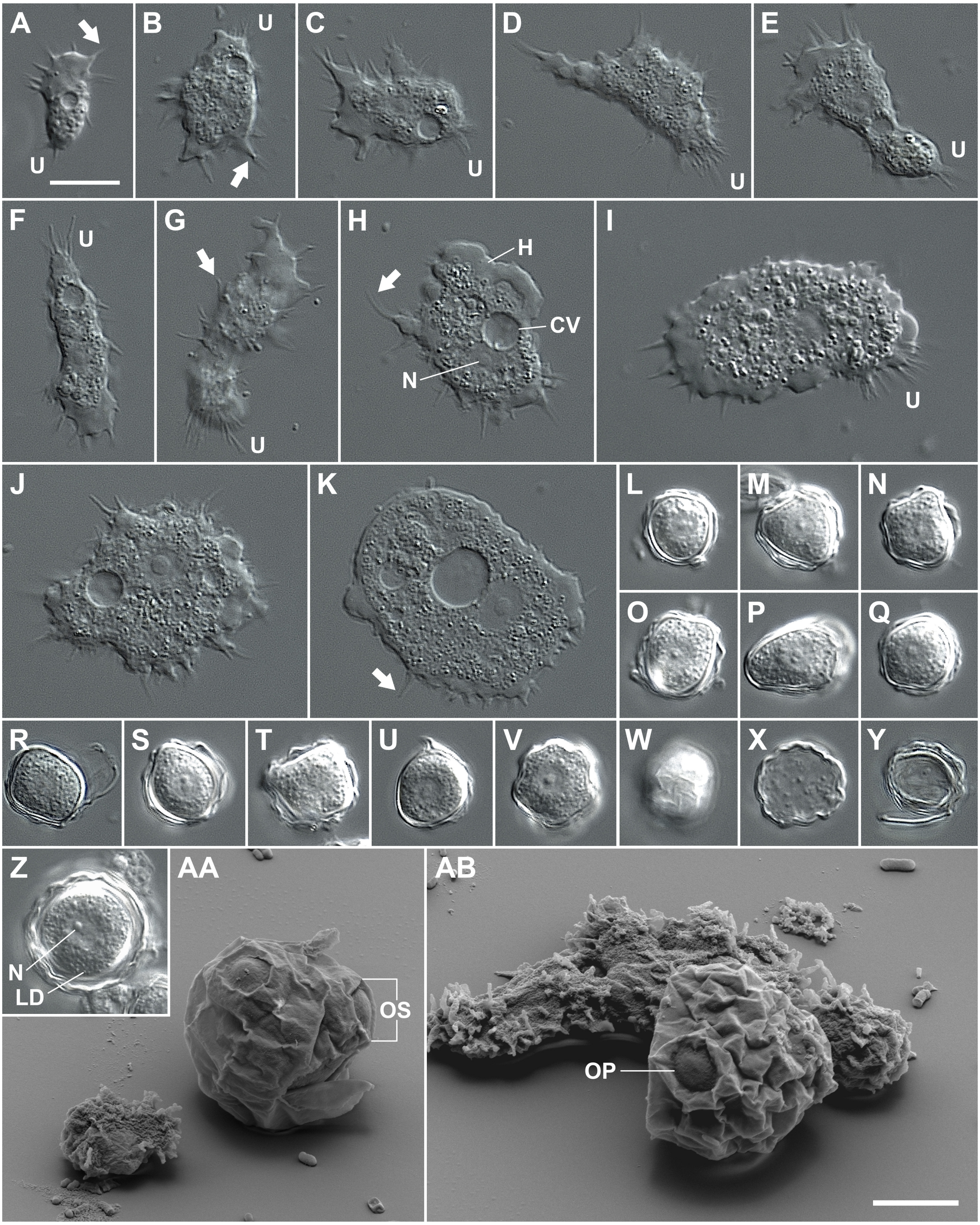

Fig. 3 Differential interference contrast micrographs (A-Z) and scanning electron micrographs (AA, AB) of *Acanthamoeba polyphaga* strain NR12-1. (A-K) Amoebae (trophozoites) showing size, shape, and pseudopod formation. (L-Z) Cysts with various shaped and double wall. (X, Y) Empty cysts. (AA, AB) Amoebae and cysts. Arrow denotes acanthopodia. U: uroid; N: nucleus; CV: contractile vacuole; H: hyaloplasm; LD: lipid droplet; OS: ostiole; OP: operculum. Scale bar in A = 10 μm, for A-Z. Scale bar in AB = 5 μm, for AA, AB.

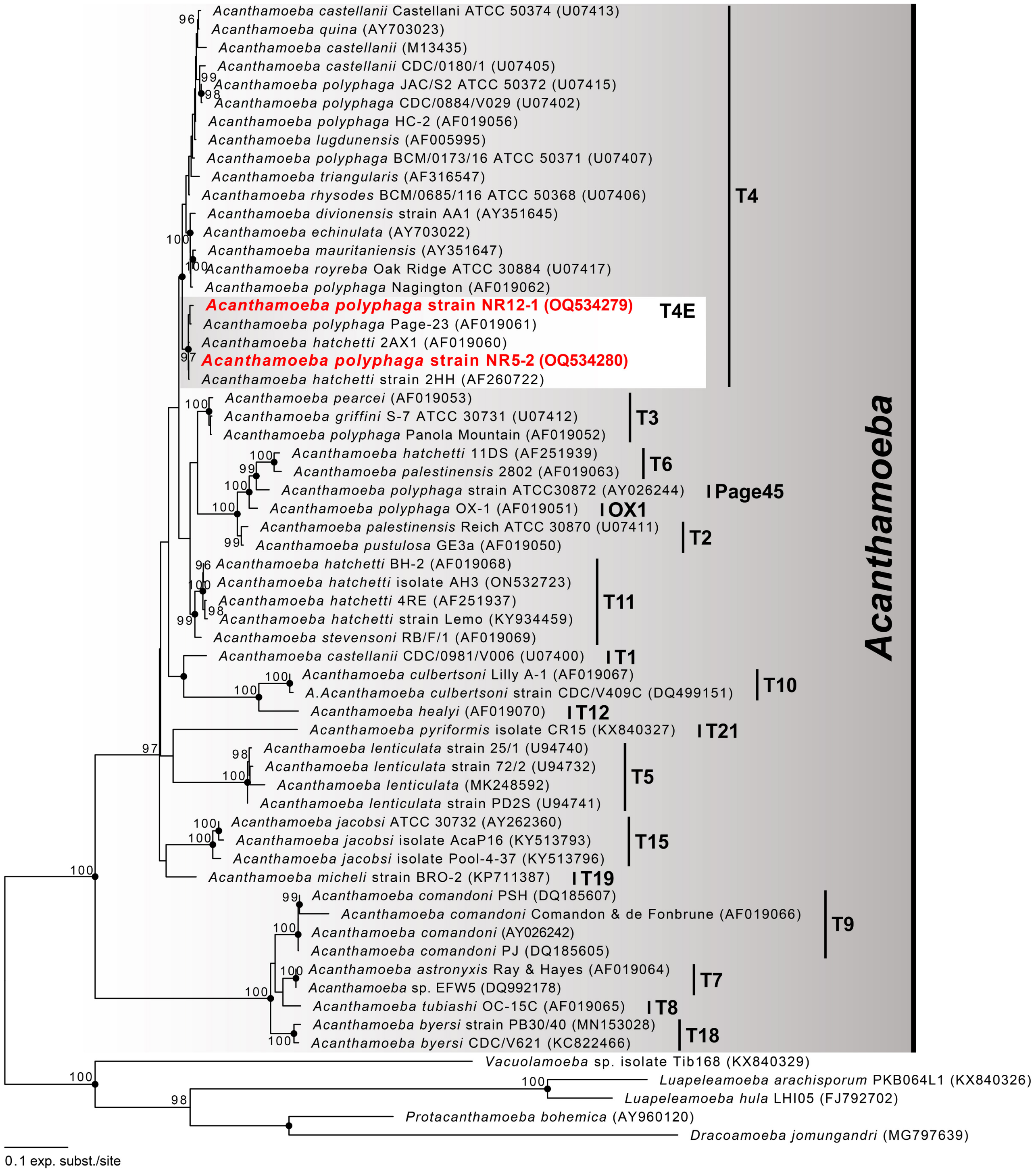

Fig. 4 Maximum likelihood phylogenetic tree of 18S rRNA gene sequences showing the phylogenetic position of *Acanthamoeba polyphaga* strains NR5-2 and NR12-1 relative to 62 Acanthamoebidae taxa. Ultrafast bootstrap support values (≥95%) are shown at the nodes. Solid circles represent a Bayesian posterior probability (PP) of 1 (PP < 0.95 not shown).

Molecular phylogeny

The 18S rRNA gene sequence obtained from strain GO001 was 2,116 bp long. A BLASTN search returned *T. waccamawensis,* formerly *Learamoeba waccamawensis*, as the top hit, with 99.7% identity. The phylogenetic tree of the 18S rDNA sequences placed GO001 within the genus *Tetramitus* with maximal UFBS and a PP of 1 (Fig. 6). Within that genus, strain GO001, formed a monophyletic group with all included strains of *T. waccamawensis* with maximal UFBS and a PP of 1 (Fig. 6).

Naegleria australiensis strain NR14-1

Morphological characteristics

Amoebae of strain NR14-1 were amoeboid, exhibiting monopodial limax or polypodial fan-shaped forms with

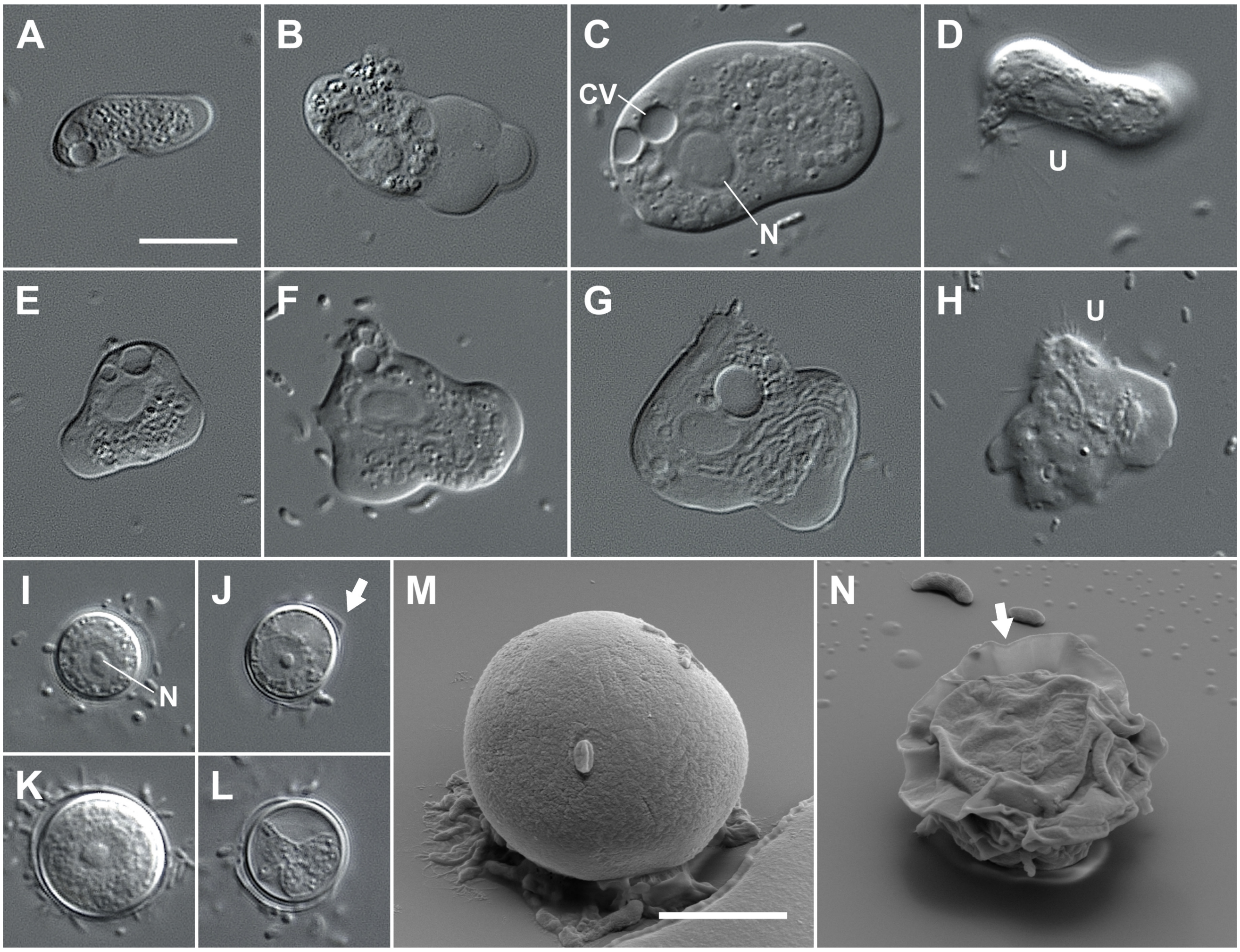

Fig. 5 Differential interference contrast micrographs (A-L) and scanning electron micrographs (M, N) of *Tetramitus waccamawensis* strain GO001. (A-H) Amoebae (trophozoites), showing size, shape, and pseudopod formation. (I-N) Cysts with a round shape, and double wall. (J, N) Cysts with one-point collapsed ectocyst (arrow). (L) Empty cyst. N: nucleus; CV: contractile vacuole; U: uroid. Scale bar in A = 10 μm, for A-L. Scale bar in M = 5 μm, for M, N.

eruptive movements (Fig. 7A-H). The average length and width of the limax amoebae was 23.9 μm (range: 16.0–33.4 μm) and 9.7 μm (range: 6.9–14.3 μm), respectively (n = 30; Fig. 7A-D); that of the fan-shaped amoebae was 19.8 μm (range: 14.8–26.6 μm) and 13.9 μm (range: 10.9–19.3 μm), respectively (n = 30; Fig. 7E-H). The average length-to-width ratio of the limax amoebae was approximately 2.5 (range: 1.5–3.5). Most of the cells exhibited a monopodial limax form; fan-shaped cells gradually transformed into the limax form. Short and fine uroidal filaments at the posterior end of the cells were observed in some trophozoites; however, long and trailing filaments was also observed in some cells ("U" in Fig. 7A-F, H). A single nucleus with a conspicuous central nucleolus, and one or more contractile vacuoles, were also observed in the cells ("N" and "CV", respectively, in Fig. 7C).

A flagellate form was occasionally observed in liquid cultures of this strain (Fig. 7N-P). These flagellates had spherical to elliptical shapes (average length: 10.2 μm, average width: 8.0 μm; n = 10) with two flagella that were approximately 18.4 μm long. The body of the flagellates sometimes extended pseudopodia (arrow in Fig. 7O, P). Flagellate cells were mononucleate.

Cysts of strain NR14-1 had a spherical shape with a smooth outline (Fig. 7I-M). The average diameter of the cysts was 11.4 μm (range: 8.6–14.5 μm; n = 30). The ectocyst and endocyst walls were in close proximity. A single nucleus with a central nucleolus was observed in the cysts, as were 1–4 cyst pores with thickened rims (arrowheads in Fig. 7I-L). Some cysts had no cytoplasm or a ruptured ectocyst wall, presumably due to excystment (Fig. 7K-M). Scanning electron micrographs of the strain NR14-1 showed that the amoeba had an amoebostome (double-arrow in Fig. 7Q) and the cyst showed the presence of cyst pores (arrowhead in Fig. 7R).

Molecular phylogeny

The 18S rRNA gene sequence obtained from strain NR14-1 was 1,847 bp long. A BLASTN search returned near-maximal identity with *N. australiensis* strains. Phylo-

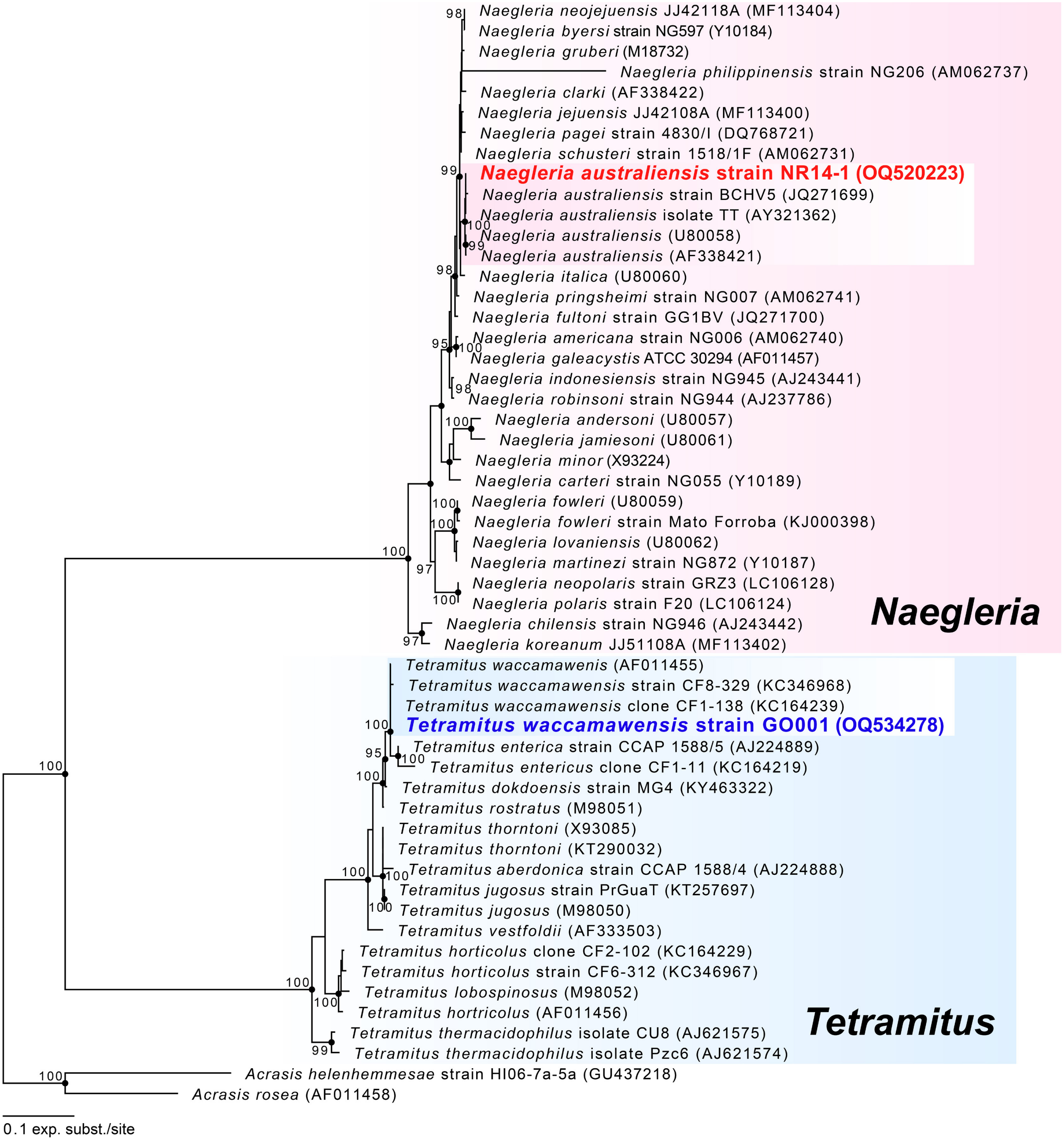

Fig. 6 Maximum likelihood phylogenetic tree of 18S rRNA gene sequences showing the phylogenetic position of *Naegleria australiensis* strain NR14-1 and *Tetramitus waccamawensis* strain GO001 relative to 52 *Naegleria*-*Tetramitus* taxa and 2 *Acrasis* species outgroup. Ultrafast bootstrap support values (≥95%) are shown at the nodes. Solid circles represent a Bayesian posterior probability (PP) of 1 (PP < 0.95 not shown).

genetic analyses placed this strain NR14-1 in the genus *Naegleria* with maximal UFBS and a PP of 1 (Fig. 6). In particular, NR14-1 was placed within *N. australiensis* with maximal UFBS and a PP of 1 (Fig. 6).

Discussion

Classification of *Acanthamoeba*

Prior to the proposed classification of *Acanthamoeba* based on the phylogenetic anlyasis of 18S rDNA sequences by Stothard et al. (1998), *Acanthamoeba* was classified based on only morphological differences. *Acanthamoeba* species were classified into three morphological groups of

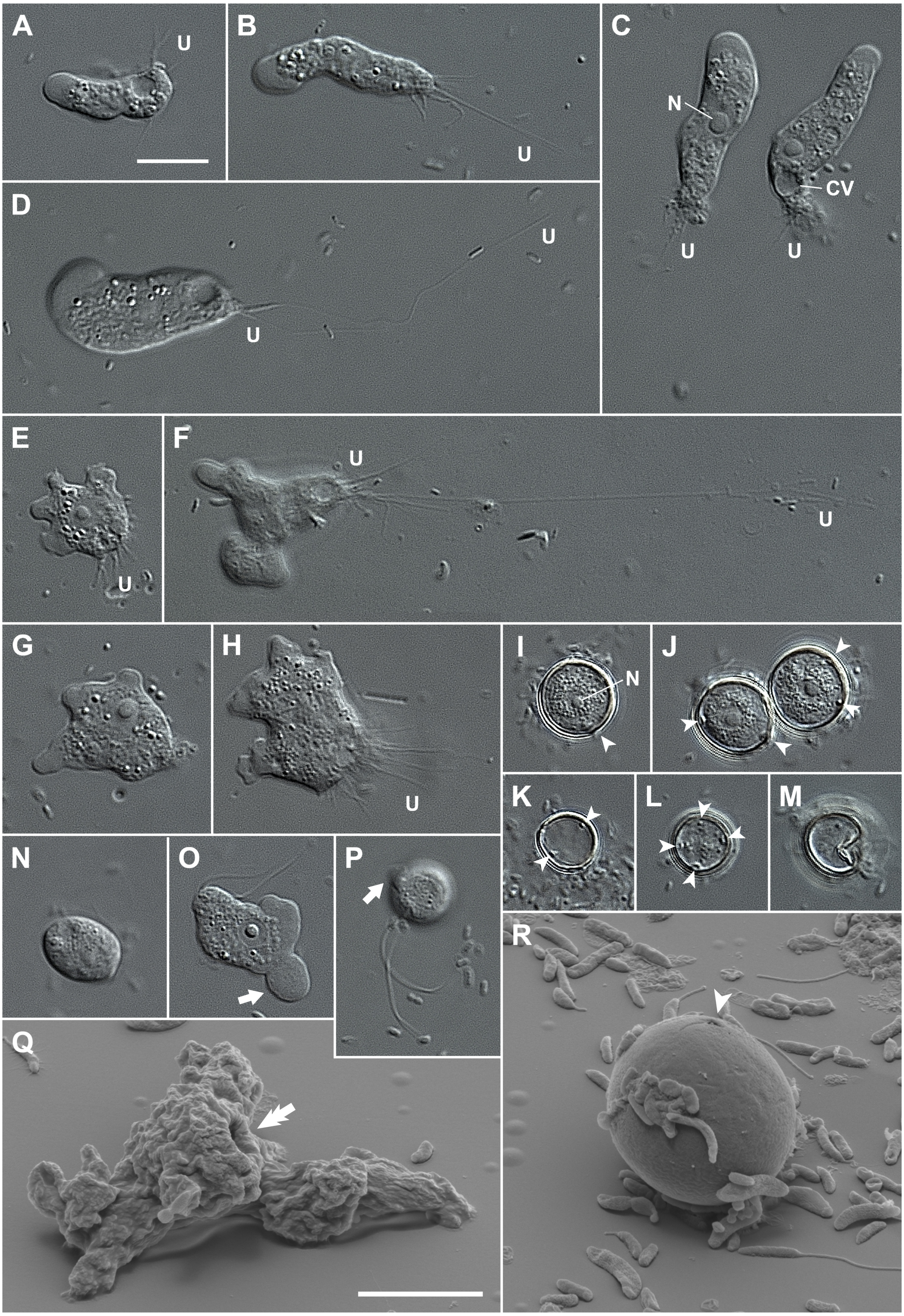

Fig. 7 Differential interference contrast micrographs (A-P) and scanning electron micrographs (Q, R) of *Naegleria australiensis* strain NR14-1. (A-H) Amoebae (trophozoites), showing size, shape, and pseudopod formation. (I-M) Cysts with a round shape, one to four cyst pores, and smooth-walled outline. (K-M) Empty cysts. (N-P) Flagellate phase, with two flagella. (Q) Amoebae with amoebostome (double-arrow). (R) Cyst with cyst pore (arrowhead). Arrow and arrowhead denote the pseudopod of flagellate and cyst pore, respectively. U: uroid; N: nucleus; CV: contractile vacuole. Scale bar in A = 10 μm, for A-M. Scale bar in Q = 5 μm, for Q, R.

cysts proposed by Pussard et al. (1977): Group 1, which has a larger cyst (>18 μm) than other groups, with a wrinkled or smooth ectocyst and stellate endocyst; Group 2, with a size smaller than 18 μm, a wrinkled ectocyst wall, and endocysts with various shapes, such as polygonal and triangular; and Group 3, typically with a thin and smooth ectocyst and rounded endocyst. In spite of this, since the morphology of cysts can change depending on culture conditions, it can be challenging to identify *Acanthamoeba* species based solely on their morphology (Sawyer 1971). Indeed, phylogenetic analysis based on 18S rDNA sequences revealed that many *Acanthamoeba* species, classified based on morphology, were polyphyletic. As a result, a recent reclassification has been proposed, based on 21 genotypes (T1–T21: Corsaro 2020; Stothard et al. 1998). Specifically, among the 21 genotypes, the T4 genotype, also termed the *A. castellanii* complex, includes the largest number of *Acanthamoeba* sequences, and is subsequently divided into seven subgroups (T4A–T4G; Corsaro 2020). Prior to this study, the T4E subtype was composed of three strains, which were the 18S rDNA sequences of *A. polyphaga* strain Page-23, and two *A. hatchetti* strains 2HH and 2AX1. However, given that *A. polyphaga* strain Page-23 (Page 1967a) is the type strain, and the type strain of *A. hatchetti* (BH-2) actually belongs to the T11 genotype, it becomes evident that strains 2HH and 2AX1 were wrongly assigned to *A. hatchetti*, and T4E represents the *A. polyphaga* group (Corsaro 2020). Thus, molecular phylogenetic analysis of 18S rDNA sequences clealy shows that our strains NR5-2 and NR12-1 are members of *A. polyphaga* group, rather than *A. hatchetti*.

The morphology of strains NR5-2 and NR12-1 is consistent with typical *Acanthamoeba* species, with amoebae displaying numerous acanthopodia and with well-defined ectocysts and endocysts, ectocysts having corrugated walls, and endocysts having varying shapes. This cyst morphology is consistent with *Acanthamoeba* morphological Group 2 (Table 2). Our molecular phylogeny of the 18S rDNA sequences of strains NR5-2 and NR12-1 shows that they are closely related to *A. hatchetti* (in fact, *A. polyphaga*) strain 2HH and *A. polyphaga* Page-23, respectively, all belonging to the subtype T4E. *Acanthamoeba hatchetti*, first de-

Table 2. Morphological characteristics of *Acanthamoeba polyphaga*, *Tetramitus waccamawensis*, and *Naegleria australiensis*

Species	Strain	Amoeba morphology			Cyst morphology		References
		Features	Length (μm)	Width (μm)	Features	Diameter (μm)	
Acanthamoeba polyphaga	Page-23	Irregular ovoid or ellipsoid-shape; hyaline lobopodia and acanthopodia; no uroidal filaments	13–42	NA	Ectocyst with folds and ripples; endocyst with irregular polyhedral-shape; appearance of ostiole-like configuration	8–21	Page (1967a)
	NR5-2	Irregular ovoid, ellipsoid, or fan-shape; hyaline lobopodia and acanthopodia; short and fine uroidal filaments	16.5–39.6	10.4–25.9		10.7–15.3	This study
	NR12-1		8.4–35.9	6.4–26.4		11.4–18.1	This study
Tetramitus waccamawensis	NA	Limax; flattened flabellate forms; eruptive pseudopodia; flagellates with four flagella	32–50	4.4–9.9	Spherical to oval-shaped; one point collapsed ectocysts; no cyst pores	9–12	Sawyer et al. (1998)
	GO001	Monopodial limax; polypodial fan-shaped; eruptive pseudopodia; fine trailing uroidal filaments; no flagellates form	Limax: 16.3–32.2 Fan-shaped: 16.9–27.5	Limax: 6.7–17.6 Fan-shaped: 10.6–20.8		8.4–13.6	This study
Naegleria australiensis	PP 397	Eruptive, hyaline pseudopodia; amoebostome; flagellates with 2–8 flagella	14.5–29.5	4–19	Spherical-shaped; 3–8 distinct cyst pores with a thickend rim	8.5–19	De Jonckheere (1981)
	NR14-1	Monopodial limax; polypodial fan-shaped; eruptive pseudopodia; fine uroidal filaments or long and trailing filaments; moebostome; flagellates with two flagella	Limax: 16.0–33.4 Fan-shaped: 14.8–26.6	Limax: 6.9–14.3 Fan-shaped: 10.9–19.3	Spherical-shped with smooth outline; 1–4 cyst pores	8.6–14.5	This study

NR: Nakdong River; GO: Gonggeomji Reservoir; NA: not applicable.

scribed by Sawyer et al. (1977), is an amoeba with characteristic fine acanthopodia, an obtuse triangular shape, and without a hyaline anterior zone. It has an average length of 23.6 μm and width of 16.0 μm. The cysts of *A. hatchetti* have an average diameter of 13.1 μm, and possess a thick ectocyst and an endocyst with 3–4 vertices. The type species *A. polyphaga* Page-23 belong to morphological Group 2, and morphological characteristics of *A. hatchetti* and *A. polyphaga* are very similar to each other. *Acanthamoeba polyphaga* has a length range of 13–42 μm, with a broad hyaline cap at the anterior and filamentous projections (acanthopodia), and with neither eruptive movement nor uroidal filaments (Page 1967a; Table 2). The cysts of *A. polyphaga* have an average diameter of 13 μm, an ectocyst with folds and ripples, and an irregular polyhedral endocyst (Page 1967a; Table 2). As mentioned earlier, *A. hatchetti* strain 2HH is considered to be *A. polyphaga*; thus, we identified both strains NR5-2 and NR12-1 as members of *A. polyphaga* due to their strongly supported clustering with the type species *A. polyphaga* Page-23. However, strains NR5-2 and NR12-1 differ from previously known strains of *A. polyphaga*, which was not observed in obvious uroidal filaments (Page 1967a; Table 2). Thus, the presence or absence of uroidal filaments may not be a distinguishable key characteristic of *Acanthamoeba*.

Classification of *Tetramitus* and *Naegleria*

Strains GO001 and NR14-1 showed the presence of the eruptive pseudopodia that correspond to the morphology of a typical heterolobosean amoeba. Strain GO001 was morphologically identified as a member of *Tetramitus*, which is characterized by limax amoebae and cysts with smooth outlines and no cyst pores (Baumgartner et al. 2009; Brown and De Jonckheere 2004; Park 2017; Robinson et al. 2007). Strain NR14-1 exhibited morphological features consistent with typical *Naegleria* species: the presence of a flagellate stage with two flagella, pores in cysts, and an amoebostome in its amoeba stage (John and De Jonckheere 1985; Khwon and Park 2018; Page 1967b). The previous exclusively morphology-based approaches misassigned some species of *Tetramitus* and *Naegleria*; subsequent DNA analyses using 18S rRNA gene and ITS sequences (including the 5.8S rRNA gene) have given a more nuanced picture of *Tetramitus* and *Naegleria* species (De Jonckheere 2004; De Jonckheere and Brown 2005b). Our molecular phylogeny placed the strains GO001 and NR14-1 as members of the genera *Tetramitus* and *Naegleria*, respectively. In particular, strain GO001 was closely related to strains of *T. waccamawensis*, while strain NR14-1 exhibited a close relationship with *N. australiensis* strains.

Categorization within the genus *Tetramitus* distinguishes between amoeboflagellate and amoeba-only species. The known amoeboflagellates are *T. aberdonicus*, *T. angularis*, *T. dokdoensis*, *T. entericus*, *T. jugosus*, *T. lobospinosus*, *T. rostratus*, *T. thermacidophilus*, *T. thorntoni*, *T. vestfoldii*, and *T. waccamawensis* (Baumgartner et al. 2009; Brown and De Jonckheere 2004; De Jonckheere et al. 1997; Murtagh et al. 2002; Page 1988; Park 2017; Robinson et al. 2007; Sawyer et al. 1992, 1998). *Tetramitus anasazii*, *T. hohokami*, *T. horticolus*, *T. parangularis*, *T. pararusselli*, *T. ovis*, and *T. russelli* do not exhibit the flagellate form (De Jonckheere 2007; De Jonckheere and Brown 2005a; De Jonckheere et al. 2005; Read et al. 1983; Robinson et al. 2007). The morphological characteristics of our strain GO001 are similar to those of *T. waccamawensis* except for a small difference in size (Table 2). *Tetramitus waccamawensis* is a limax amoeba with an average length of 38 μm and width of 7.5 μm, and its cysts occasionally collapse at one point on the cyst wall, and they exhibit no cyst pores (Sawyer et al. 1998; Table 2). (*Tetramitus waccamawensis* was previously classified as *Learamoeba waccamawensis* due to subtle morphological differences from *T. rostratus*, but through additional molecular analysis, it was reclassified into the genus *Tetramitus*: De Jonckheere and Brown 2005b.) Although no flagellate form was observed in our strain, the absence of the flagellate form is actually unreliable for taxonomic discrimination within the Heterolobosea due to the difficulty of inducing transformation into the flagellate form (De Jonckheere and Brown 2005b). Therefore, based on its morphological and molecular phylogenetic characteristics, we classify the strain GO001 as *T. waccamawensis*, the first strain of this species reported in the Republic of Korea.

In general, the morphological characteristics of *Naegleria* species include a limax amoeba with eruptive pseudopodia and amoebostome, ovoid and rounded shaped flagellate with two flagella, and spherical cyst with a smooth outline and multiple cyst pores (De Jonckheere 1981; John and De Jonckheere 1985; Table 2). Similar to *Tetramitus*, some *Naegleria* species, such as *N. arctica*, *N. chilensis*, *N. indonesiensis*, *N. koreanum*, *N. neochilensis*, and *N. paradobsoni* also lack a flagellate form (De Jonckheere 2014; De Jonckheere et al. 2001; Khwon and Park 2018). Similarly to *Tetramitus*, the criterion of flagellate formation cannot be relied upon for *Naegleria* species differentiation, as the conditions for inducing flagellate transformation vary among different species under differing laboratory culture conditions (De Jonckheere et al. 2001). The morphology of different *Naegleria* species is very similar under the light microscope, and morphological data for *Naegleria* species are not well documented (De Jonckheere 2002, 2014; Page 1988). *Naegleria australiensis* is an amoeboflagellate with an amoeba-cyst-flagellate life cycle. The strain NR14-1 also has both biflagellate and amoeba life stages, the latter with an amoebostome. Based on 18S rDNA sequences and morphology, then, we identify the strain NR14-1 as the first reported *N. australiensis* strain from the Republic of Korea.

Putative risk and ecological importance of free-living amoebae

Remarkabely, some FLA are amphizoic (i.e., they can directly affect not only the environment but also humans and animals as opportunistic pathogens: Martinez and Visvesvara 1997; Smirnov and Brown 2004). Most *Acanthamoeba* species, including *A. castellanii*, *A. polyphaga*, *A. culbertsoni*, *A. hatchetti*, *A. rhysodes*, *A. lugdunensis*, *A. quina*, and *A. griffini*, are known to cause fatal diseases, such as granulomatous amoebic encephalitis (GAE) and AK in humans and animals (Khan 2006; Marciano-Cabral and Cabral 2003; Visvesvara et al. 2007). *Acanthamoeba polyphaga* and *A. castellanii* can each cause GAE and AK, and are commonly found in both natural and artificial environments, such as lakes, swimming pools, and tap water worldwide, through the use of the plate culture methods (Marciano-Cabral and Cabral 2003; Siddiqui and Khan 2012). Moreoever, *Acanthamoeba* spp. have been detected in various water samples using PCR/real-time PCR techniques (Lorenzo-Morales et al. 2015). The genus *Naegleria* is also reported to cause infections in humans and laboratory animals. *Naegleria fowleri*, popularly known as the "brain-eating amoeba," causes primary amoebic meningoencephalitis (PAM), from which approximately 95% of infected individuals die; whereas *N. italica* and *N. australiensis* are pathogenic protozoa in experimental animals (Carter 1970; De Jonckheere 2014; Visvesvara et al. 2007). Especially, *N. australiensis* has been abundantly detected in lakes, tap water, and wastewater treatment systems in North America, Europe, Asia, and Oceania (De Jonckheere 2014; Milanez et al. 2019; Sousa-Ramos et al. 2023). Our study is the first report of potentially pathogenic FLA, specifically two strains of *A. polyphaga* and one strain of *N. australiensis*, to have been discovered in the Nakdong River in the Republic of Korea. Thus, the largest Nakdong River can provide a home for potentially pathogenic protozoa, and the putative contamination risk of these pathogenic protozoa emerging from natural habitats is present to date.

Meanwhile, in aquatic and soil ecosystems, FLA contribute to microbial population predation and are in particular considered important feeders of surface-adherent bacteria (Rodríguez-Zaragoza 1994). In addition, some FLA play several important ecological roles by contributing to the cycling of nutrients such as nitrogen and carbon in the ecosystem, through decomposing organic matter such as biofilms, and various chemical substances, by serving as a food source for organisms in higher trophic levels such as nematodes, and promoting plant growth (Rodríguez-Zaragoza 1994; Rosenberg et al. 2009; Smirnov 2009). Most observations have been made in the Nakdong River, and there is still a significant decifiency in fundamental knowledge of FLA in the Republic of Korea. Further studies are continuously needed to understand the diversity, distribution, and habitat of potentially pathogenic FLA in natural ecosystems of the Republic of Korea, regardless of their ecological importance.

Conclusions

Free-living amoebae play important ecological roles as bacterial predators and prey at higher trophic levels. In this study, four FLA isolated from freshwater environments in the Republic of Korea were identified as *A. polyphaga*, *T. waccamawensis*, *and N. australiensis*, based on morphological and molecular phylogenetic analyses. These amoebae were previously unrecorded in the Republic of Korea, and *A. polyphaga* and *N. australiensis* are known to be potentially pathogenic to animals including humans. Further studies are needed to confirm the pathogenicity of *A. polyphaga* and *N. australiensis* as well as to further explore the distribution pattern and diversity of freshwater pathogenic FLA in the Republic of Korea.

Supplementary Information

Supplementary information accompanies this paper at https://doi.org/10.5141/jee.23.030.

Taxonomic Summary.

Abbreviations

FLA: Free-living amoebae
GO: Gonggeomji Reservoir
NR: Nakdong River
PAS: Page's amoeba saline
UFBS: Ultrafast bootstrap support
PP: Posterior probability
GAE: Granulomatous amoebic encephalitis
PCR: Polymerase chain reaction
AK: *Acanthamoeba* Keratitis
PAM: Primary amoebic meningoencephalitis

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Authors' contributions

JSP sampled and supervised the study. HBL performed the majority of experiments. HBL and JSP drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated and analyzed in this study are deposited in the related repositories. The names of the repositories and accession numbers are included in the article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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