Morphological, phylogenetic and biogeographic characterizations of three heterotrophic nanoflagellates isolated from coastal areas of Korea

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ABSTRACT

Heterotrophic nanoflagellates (HNFs, 2–20 µm in size) are important bacterivores in aquatic environments. We isolated and distinguished three HNF strains from Korean coastal samples: Ancyromonas kenti strain KM086, Cafeteria mylnikovii strain JS001 and Multimonas media strain JS004. Their 18S rDNA sequences aligned with previously known counterparts. However, under scanning electron microscopy, strain JS004 shows some features at odds with prior observations of M. media, including different putative extrusomes, an ‘unfrilled’ skirt margin, and a putative ‘tusk’. We also used the V4 region of 18S rDNA to study species distribution globally using Malaspina-2010 data from 122 surface stations and 13 various depth stations. Intriguingly, we found that A. kenti was most abundant in low-oxygen conditions of the bathypelagic zones (1000–4000 m deep) in the Pacific Ocean, suggesting a specialization for thriving in low-oxygen habitats. In contrast, we only found M. media represented at one surface site, and C. mylnikovii was not found at all. Thus, A. kenti may be one of the most important feeders on bacteria in the bathypelagic zones with low-oxygen concentrations, while the other two isolates appear to be rare species in marine systems.

Introduction

Heterotrophic nanoflagellates (HNFs, eukaryotes 2–20 µm in size) play critical roles as prokaryotic grazers, as prey to organisms of higher trophic levels and as nutrient remineralizers in freshwater, marine and extreme ecosystems (Sherr et al. 1989; Park et al. 2003; Fischer et al. 2006). They are diverse as well, with an estimated ∼36,400 species of marine protozoans (predominantly HNFs) (Mora et al. 2011). For the classification of HNFs, a combination of molecular phylogeny with morphological analysis is the predominant approach. For instance, members of the Cafeteria group are morphologically almost indistinguishable under light microscopy (Schoenle et al. 2022), so molecular phylogenetic analyses are essential for their identification. Furthermore, many undescribed species have been found in marine environment samples using metabarcoding technology (De Vargas et al. 2015; Schoenle et al. 2021). Recent circumglobal expeditions such as Malaspina-2010 and TARA-Oceans obtained DNA sequence data from the ocean, which have been used to document the biogeographical distribution of marine protists worldwide (Logares et al. 2020; Massana et al. 2021), albeit without morphological characterization. However, the local distribution and diversity of protists is often understudied. In particular, although a number of HNFs have been reported so far from Korean waters (Park et al. 2006; Park and Simpson 2010; Heiss et al. 2015; Lee and Park 2016; Jhin and Park 2019; Lax et al. 2019; Tikhonenkov et al. 2019; Jeong and Park 2021), our knowledge about the diversity of HNFs in the natural ecosystems of Korea remains poor.

In the present study, we document the presence of three marine HNFs in Korea: Ancyromonas kenti Glücksmann and Cavalier-Smith, 2013, strain KM086; Cafeteria mylnikovii Cavalier-Smith and Chao, 2006, strain JS001; and Multimonas media Cavalier-Smith, 2010, strain JS004. This account includes the first reported occurrences of A. kenti and C. mylnikovii in Korea. For all three strains, we describe their morphological features and present molecular phylogenetic analyses of their
18S rDNA. We also assessed their global distribution patterns using the large Malaspina-2010 metabarcoding dataset.

Materials and methods

Isolation and cultivation

Three HNFs, strains KM086, JS001 and JS004, were isolated from surface water/sediment interface samples collected from coastal areas (Gwangam beach and Jangsa beach) in Korea (Table I). Monoprotistan cultures were established by manual single-cell picking with a micropipette. In brief, isolated single cells were inoculated into 24-well culture plates (30024, SPL Life Sciences Co., Ltd., Pocheon, Republic of Korea) containing either LB or R2A broth (Korea) containing either Luria Bertani broth (LB; final concentration of 0.5%) or R2A broth (final concentration of 1%, MB-R2230, Kisan Bio Co., Ltd., Seoul, Republic of Korea) containing autoclaved seawater. Live flagellates were observed with phase contrast microscopy (Nikon ECLIPSE Ts2, Tokyo, Japan) after the inoculum was incubated at 25°C for seven days. The resulting monoprotistan cultures were transferred to T12.5 plug-cap cell culture flasks (70112, SPL Life Sciences Co., Ltd., Pocheon, Republic of Korea) or six-well culture plates (30006, SPL Life Sciences Co., Ltd., Pocheon, Republic of Korea) containing autoclaved seawater with R2A broth (final concentration of 1%) plus an autoclaved barley grain for enrichment. The three HNF strains were incubated at 25°C and subcultured every two weeks.

Microscopy

Live HNFs were mounted on glass slides and observed with differential interference microscopy and phase contrast microscopy using a Leica DM5500B microscope equipped with a DFC550 digital camera (Leica, Wetzlar, Germany). The sizes of the live cells were measured from digital images using the open-source ImageJ (ImageJ.net). Live HNFs were mounted on glass slides and observed with differential interference microscopy and phase contrast microscopy using a Leica DM5500B microscope equipped with a DFC550 digital camera (Leica, Wetzlar, Germany). The sizes of the live cells were measured from digital images using the open-source software ImageJ v2.9.0 (WS Rasband, US National Institutes of Health, Bethesda, MD, USA; https://imagej.net).

For scanning electron microscopy, 2-ml cultures were centrifuged at 3,000 × g for 5 min. The lower 900 µl of cultures were fixed by adding 100 µl of 25% v/v glutaraldehyde (electron microscopy grade, Sigma-Aldrich, St Louis, MO, United States), and incubating overnight at 4°C. Specimens were initially post-fixed with aqueous 4% (w/v) OsO4 (Crystalline, SPI Supplies, West Chester, PA, USA) vapour for 45 min at room temperature. After this, one to two drops per ml of culture of 4% (w/v) OsO4 were added to each of the samples, which were incubated for an additional 45 min in a 12-well culture plate (30012, SPL Life Sciences Co., Ltd., Pocheon, Republic of Korea) on a glass coverslip coated with 0.01% poly-L-lysine (P8920, Sigma-Aldrich, St Louis, MO, United States). After fixation, specimens were washed first with fresh media, then with double-distilled water. Subsequently, fixed cells were dehydrated using a graded ethanol series [30%–50%–70%–80%–90%–95% (×2)–100% (×3)] with 10 min for each change, and critical-point-dried with CO2 (Hitachi HCP-2, Hitachi Co., Ltd., Tokyo, Japan). Glass coverslips were attached to stubs and coated with platinum using an ion-sputter system. The specimens were subsequently examined under an SU8220 field emission scanning electron microscope (Hitachi, Tokyo, Japan).

DNA extraction and PCR amplification

DNA from the three HNF strains KM086, JS001 and JS004 was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. 18S ribosomal DNA sequences were obtained by PCR amplification using the primer set EukA [5’-AACCTGGGTATCGCCTGCAGTG-3’] / EukB [5’-TGATCCTTCTCAGTTCAACCTAC-3’] (Medlin et al. 1988). PCR employed Takara Taq™ DNA polymerase (Takara, Shuzo, Japan) in 20-µl reactions, cycled as follows: initial denaturation at 94°C for 5 min, 40 cycles of 45 s of denaturation at 94°C, 1 min of annealing at 55°C and 3 min of extension at 72°C, and a final extension for 20 min at 72°C. PCR products corresponding to the expected size were gel-

<table>
<thead>
<tr>
<th>Taxonomic name</th>
<th>Strain</th>
<th>Sampling site</th>
<th>Conditions at sampling site</th>
<th>Accession code</th>
</tr>
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<tbody>
<tr>
<td>Ancyromonas kenti</td>
<td>KM086</td>
<td>Gwangam Beach; Changwon; South Korea (35°06'08.01&quot;N, 128°30'00.09&quot;E)</td>
<td>Temperature: 14.1°C, Salinity: 34.0 PSU, Habitat: Surface water/sediment interface</td>
<td>OQ642318</td>
</tr>
<tr>
<td>Cafeteria mylnikovii</td>
<td>JS001</td>
<td>Jangsa Beach; Yeongdeok; South Korea (36°16'58.05&quot;N, 129°22'33.02&quot;E)</td>
<td>Temperature: 25.0°C, Salinity: 30.7 PSU, Habitat: Surface water/sediment interface</td>
<td>OQ642319</td>
</tr>
<tr>
<td>Multimonas media</td>
<td>JS004</td>
<td>Jangsa Beach; Yeongdeok; South Korea (36°16'58.05&quot;N, 129°22'33.02&quot;E)</td>
<td>Temperature: 25.0°C, Salinity: 30.7 PSU, Habitat: Surface water/sediment interface</td>
<td>OQ642320</td>
</tr>
</tbody>
</table>
isolated using gel electrophoresis and then purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the supplied protocols. Amplicons were directly sequenced by the Sanger sequencing method (Bioneer, Daejeon, Republic of Korea). Individual reads were assembled into contigs using the Vector NTI ContigExpress program version 6.0 (Invitrogen Corporation, Carlsbad, California, USA). The assembled 18S rDNA sequences from the three HNFs were deposited in GenBank under accession codes OQ642318–OQ642320.

Phylogenetic analysis

Three parallel 18S rDNA data matrices were constructed, one each for ancyromonads (KM086 and 10 previously published Anycromonas sequences, plus four representative Planomonas species as an outgroup), Cafeteria (JS001 and 31 previously published Cafeteria species, plus two Caecitellus strains as an outgroup) and apusomonads (JS004 and five previously published Multimonas sequences, and three sequences from Podomonas, two each from Catacumbia and Cavaliersmithia and one from Mylnikiovia, for an eight-taxon outgroup). Seed alignments originated from Glücksman et al. (2013) for strain KM086 (1,806 unambiguously aligned sites in this study), Schoenle et al. (2022) for strain JS001 (1,548 sites) and Heiss et al. (2015) for strain JS004 (1,704 sites). All three 18S rDNA sequence 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 (Nguyen et al. 2015) with the GTR + I + G model. Statistical support was assessed using ultrafast bootstrapping with 1,000 replicates. Bayesian analyses were performed using MrBayes v. 3.2.7 (Ronquist et al. 2012) with two independent runs, with four chains using a heating parameter of 0.1, running for 2 × 10⁶ generations and sampled every 10 generations. A burn-in of 5 × 10⁵ generations (25%) was used. The average standard deviation of split frequencies for the last 75% of generations was <0.01.

Global distribution patterns

The global distribution patterns of the three HNFs that we analysed were visualized using the MicroMap web application (ICM-CSIC, https://micromap.icm.csic.es/, Obiol et al. 2021). MicroMap creates global biogeography estimates of amplicon sequence variant (ASV) abundance from the Malaspina-2010 expedition database by assessing BLAST results of the V4 region in user-supplied 18S rDNA sequences (Logares et al. 2020 for 122 stations of surface water; Giner et al. 2020 for 13 stations of vertical profiles). The relative abundances of ASVs are reported for four water zones (surface, deep chlorophyll maximum, mesopelagic, and bathypelagic).

Results

Ancyromonas kenti

Observations. Cells of strain KM086 were bean-shaped, with a prominent leftward/ventral-oriented rostrum at their anterior ends (Figure 1). The average length and width of the live cell body was 4.0 ± 0.4 µm and 2.9 ± 0.4 µm, respectively (mean ± standard deviation; n = 30), with a length/width ratio of 1.38. The average lengths of the anterior and posterior flagellum were 1.8 ± 0.4 µm (n = 25) and 7.7 ± 1.0 µm (n = 30), respectively. The anterior flagellum was thinner than the posterior flagellum and was not easy to see under the light microscope (Figure 1A–E). The posterior flagellum had an acronematic tip (Figure 1B,E). Cells were usually found near, and generally attached to, the substrate. When the body or posterior flagellum was fixed to the substrate, the posterior flagellum or the body (respectively) shook randomly. When moving forward, the cell body oscillated quickly from side to side, while the posterior flagellum trailed posteriorly, during which the angle between the posterior flagellum and the body varied through 45–90 degrees. Scanning electron micrographs (SEMs) confirmed most light microscopic observations (Figure 1F–H), additionally showing that cells had a ventral groove (Figure 1G,H) and extrusomes on the ventral/left side of the rostrum (Figure 1B,D,H,I).

Molecular phylogeny. The partial 18S rDNA sequence of strain KM086 was 1,612 bp long, with a G + C content of 47.52%. The 18S rDNA sequence of strain KM086 was an exact match to that of Ancyromonas kenti strain 3b (NCBI accession code: GU001166; the 18S rDNA sequence with 1,784 bp long, with a G + C content: 49.35%). The maximum likelihood tree of strain KM086 was an exact match to that of Ancyromonas kenti strain 3b (NCBI accession code: GU001166; the 18S rDNA sequence with 1,784 bp long, with a G + C content: 49.35%). The maximum likelihood tree (Figure 2) likewise placed KM086 with strains of A. kenti, with acceptable ultrafast bootstrap support (95%) and a posterior probability of 1 (Figure 2).

Cafeteria mylnikovii

Observations. Living cells of strain JS001 were generally D-shaped, although they were more ovoid when swimming. The dorsal side was convex, and the ventral side flattened (Figure 3A,B,D–F). The average
Figure 1. Light and scanning electron micrographs of Ancyromonas kenti strain KM086. Anterior is to top of page in all images. (A–C) Differential interference contrast images. (D–E) Phase contrast images. All cells viewed from dorsal/left side. Note that anterior flagellum is visible in some (B, D) but not other (A, C, E) images under both contrast enhancement systems. (F–I) Scanning electron microscope images. Panels F–H show cell's ventral/right aspect; panel I is a dorsal/left view. Note groove (most clearly visible in F, G, I) and extrusomes (seen in typical aspect in G, more exposed and probably having discharged in H). Scale bars = 5 µm for panels A–E; 2 µm for panels F, G, I; 1 µm for panel H. AF: anterior flagellum, PF: posterior flagellum, arrowhead: rostrum, diamond: extrusomes.

Multimonas media str. JS004 (OQ642320)

- Multimonas media str. 7C (EU542597)
- Multimonas media str. 6B (EU542596)
- Multimonas media str. Millport (AY050181)
- Multimonas media str. KM030 (KP996852)
- Multimonas media str. MMROSKO2018 (OM966643)

Podomonas capensis (AV050182)
- Podomonas capensis str. SPRINTER (OM966641)
  - Podomonas magna str. CCAP1901/4 (OM966636)
  - Mylnikovia oxonienis str. CCAP1901/2 (OM966635)
  - Cavaliarsmithia sp. str. CCAP979/5 (AY050179)
  - Cavaliarsmithia chaoae str. FABANU (OM966645)

Catacumbia lutetiensis str. CAT (OM966640)
- Catacumbia lutetiensis str. ORSAYFEB19APU2 (OM966619)

Figure 2. Maximum likelihood phylogenetic tree inferred from 18S rDNA sequences of ancyromonads, including representatives of all cultured Ancyromonas strains as well as A. kenti strain KM086, and all available environmental sequences corresponding to A. kenti. Note that, although the sequences attributed to A. kenti strains 3b and edm11b do differ minimally, the two names actually refer to the same culture (see text for details). Ultrafast bootstrap support values (>95%) are shown at nodes. Solid circles indicate Bayesian posterior probability of 1.
length and width of the cell body was 4.0 ± 0.6 µm and 3.1 ± 0.5 µm, respectively, with a length/width ratio of ∼1.28 (n = 30). The average lengths of the anterior and posterior flagella were 4.8 ± 0.6 µm and 4.1 ± 0.7 µm, respectively (n = 30). There were three types of cell movements observed: (1) swimming, with the anterior flagellum directed forwards and the posterior flagellum trailing behind (Figures 3D); (2) rotating in place, with both flagella held in a parallel fashion (Figure 3C); and (3) attached to the substrate by the posterior flagellum, displaying jerking movements while the anterior flagellum beat for feeding (Figure 3A,B).

Cells prepared for scanning electron microscopy were somewhat rounder than those observed under light microscopy, but the basic D-shape was still apparent (Figures 3G,H). A ventral groove or pouch was evident, from which the posterior flagellum emerged (Figure 3H). The anterior flagellum had two rows of mastigomemmes (Figure 3G,H). Neither flagellum appeared to have an acroneme.

Molecular phylogeny. The partial 18S rDNA sequence of the strain JS001 was 1,541 bp long (G + C content: 48.54%). The 18S rDNA sequence of strain KM086 most closely matched Cafeteria mylnikovii strain A.M. (CCAP1900/2: NCBI accession number; DQ102392, 1,693 bp long, G + C content: 47.84%), with a high identity of 99.68% (1,536/1,541 bp). The maximum likelihood tree placed strain JS001 with C. mylnikovii (as represented by strain A.M.) with a high ultrafast bootstrap value (96%) and a posterior probability of 0.94 (Figure 4).

Multimonas media

Observations. Cells of strain JS004 had a generally fusiform but plastic body (Figure 5). The average length and width of the cell body was 7.0 ± 0.7 µm and 3.5 ± 0.4 µm, respectively (n = 30), with a length/width ratio of ∼2.0. The entire proboscis was 1.3 ± 0.3 µm long (n = 30) with an acronematous anterior flagellum emerging from the end of the proximal sleeve (Figure 5A,D,G,I). Thin pseudopodia of various lengths (3.7 ± 3.0, n = 19) usually were seen extending from the cell posterior. Food vacuoles were found throughout the cell (e.g. Figure 5B,C,H). The anterior flagellum rapidly flicked through a range of approximately 90° on the cell’s left side. The posterior...
flagellum was thin and ran within the ventral groove, and was thus infrequently observed under the light microscope. The cell displayed gliding movement and deformed while turning.

SEM images showed the proboscis and some long trailing pseudopodia (Figure 5K–M). The average lengths of the anterior and posterior flagella were 1.0 ± 0.2 µm (n = 30) and 5.23 ± 2.9 µm (n = 14), respectively. Other known features, such as a diagonal anterior crease, were also prominent (Figure 5L,M), along with irregularly shaped putative extrusomes on the dorsal surface of the cell. In some cases, these formed a dense coating (Figure 5L), although they appeared to be entirely absent on other cells (Figure 5M). Some extrusomes appeared to have ejected (Figure 5K,L). The edges of the skirt appeared slightly beaded but not otherwise elaborated, and some cells had a possible tusk (sensu Heiss et al. 2010) (Figure 5K).

**Molecular phylogeny.** The partial 18S rDNA sequence of strain JS004 was 1,656 bp long and had a G + C content of 46.25%. This sequence was an exact match to all overlapping bases in those of *Multimonas* strains 6B, 7c and MMRosko2018 (respective NCBI accession codes EU542596 [1,866 bp long, G + C content: 45.82%], EU542597 [1,843 bp long, G + C content: 45.81%] and OM966643.1 [1,877 bp long, G + C content: 45.87%]). The maximum likelihood tree accordingly placed strain JS004 with all known strains of *M. media* with a high ultrafast bootstrap value (100%) and a posterior probability of 1 (Figure 6).

**Global distribution patterns of the three HNFs**

Amplicon sequence variants (ASVs) matching *Ancyromonas kenti* showed the highest abundance from bathypelagic (1000–4000 m; relative abundance: 0.16%, the relative abundance was calculated against the number of reads per sample, Massana et al. 2021) and mesopelagic (200–1000 m; relative abundance: 0.13%) depths of a single site in the North Pacific Ocean (Figure 7A). Two other North Pacific sites also had high abundances (0.10% and 0.07%) from the bathypelagic depth, but much lower abundances from the mesopelagic (both <0.01%). A lower abundance (0.04%) was also found from the mesopelagic zone of one South Atlantic site. Otherwise, ASVs matching *A. kenti* were only detected with very low relative abundances (all <0.01%) from the bathypelagic zone of a different site in the South
Atlantic and from the mesopelagic zone of a single site in the Indian Ocean. No ASVs were found from any surface or deep chlorophyll maximum zones, nor from the North Atlantic or South Pacific Oceans (Figure 7A). No ASVs matching *Cafeteria mylnikovii* were found anywhere in the Malaspina-2010 expedition database. ASVs matching *Multimonas media* were identified in only one of 122 surface-depth (3 m) samples: this was from the South Pacific Ocean, with a relative abundance of 0.0046% (Figure 7B). No ASVs matching *M. media* were found in any subsurface samples.

**Emended taxonomy**

*Ancyromonas kenti* Glücksman and Cavalier-Smith, 2013  
Order: Ancyromonadida Cavalier-Smith, 1998  
Family: Ancyromonadidae Cavalier-Smith, 1993 sensu Cavalier-Smith in Glücksman et al., 2013  
Genus: *Ancyromonas* Saville Kent, 1882  
**Description.** As per Glücksman and Cavalier-Smith in Glücksman et al., 2013  
**Type strain.** CCAP 1953/1 (This is *Ancyromonas kenti* (fmr. *Planomonas* sp.) strain 3b; 18S sequence accessioned in GenBank under code GU001166)  
**Emended world distribution of strains.** England and Korea.

*Ancyromonas* *Cavalier-Smith and Chao, 2006*  
Class: Bicoeccea Cavalier-Smith, 1993  
Order: Bicosoecida Grasse, 1926 sensu Karpov in Karpov et al., 1998  
Family: Cafeteriaceae Moestrup, 1995

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*Figure 5.* Light and scanning electron micrographs of *Multimonas media* strain JS004. (A–F) Differential interference contrast images. (G–J) Phase contrast images. Anterior is to top of page, and all cells are viewed from the dorsal side, in all light micrographs. Note long, thin trailing pseudopodium, superficially resembling flagellum but clearly tapering in B, E, I, J. (K–M) Scanning electron microscope (SEM) images. Anterior is to top of page in K, L; to left of page in M. K: Ventral view of cell. Skirt has wrapped around venter of cell, such that skirt margins are overlapping or adjacent, and ventral surface of cell and posterior flagellum are not visible. Note smooth margin of skirt (in contrast to ‘frilled’ edge noted in previous observations of *M. media*). L, M: Dorsal view of cell. Note prominent ‘crease’ in both images and dense proliferation of putative extrusomes posterior to ‘crease’ in L. However, note also complete lack of putative extrusomes in M. Scale bars: 5 µm for A–J; 2 µm for K–M. AF: anterior flagellum, PF: posterior flagellum, Ps: pseudopodia, FV: food vacuole, Ex: putative extrusome, Cr: crease, T: putative tusk, SF: skirt flange, Pr: proboscis, double arrowhead: sleeve.
Genus: **Cafeteria** Fenchel and Patterson, 1988  
**Description.** As per Cavalier-Smith and Chao, 2006  
**Type strain.** CCAP 1902/2 (This is *Cafeteria mylnikovii* (fmr. *Pseudobodo tremulans*) strain A.M.; 18S sequences accessioned in GenBank under codes DQ102392 and MN334558)  
**Emended world distribution of strains.** Russia and Korea.

**Multimonas media** Cavalier-Smith and Chao, 2010  
**Order:** Apusomonadida Karpov and Mylnikov, 1989  
**Family:** Apusomonadidae Karpov and Mylnikov, 1989  
**Genus:** *Multimonas* Cavalier-Smith and Chao, 2010  
**Description.** As per Cavalier-Smith and Chao, 2010  
**Type strain.** CCAP 1901/3 (This is *Multimonas media* strain 6B; EU542596)  
**Emended world distribution of strains.** Canada, England, France and Korea.

**Discussion**

Members of *Ancyromonas kenti* strain KM086 are morphologically very similar to the type strain (edm11b/3b) of *A. kenti* in cell body size (3.5–4.1 × 2.5–3.5 µm), proportion (length/width ratio: ~1.27) and posterior flagellum length (5–11 µm; all measurements from Glücksmann et al. 2013). Expressed in ranges (calculated from the standard deviation), our measurements were 3.6–4.4, 2.5–3.3 and 6.7–8.7 µm for length, width and posterior flagellum length, respectively; our measured length was slightly greater, while the other measurements were within the previously reported ranges, explaining why our length/width ratio (~1.38) was slightly greater. It likewise appears that KM086 is identical to *A. kenti* strains 3b and edm11b from Cumbria in England based on their molecular phylogenetic analyses.

All previously cultured *A. kenti* strains were isolated from seawater and beach sediment (Glücksmann et al. 2013), similar to the environment in which KM086 was isolated. This indicates that *A. kenti* is likely an obligately marine species. Intriguingly, *A. kenti* was mainly detected in the bathypelagic zone of the Pacific Ocean rather than the mesopelagic and surface zones of the Pacific Ocean, or the bathypelagic zone of other oceans. This implies the presence of a distinct ecotype within the *A. kenti* group. Notably, *A. kenti* appears to thrive in low-oxygen concentrations of the bathypelagic zones. According to the Malaspina-2010 expedition database reported by Giner et al. (2020), as seen in Table S1, the oxygen concentrations of the bathypelagic zones in the Pacific Ocean (1.9–3.6 O₂ ml/l) were lower than those in the Atlantic (3.6–5.5 O₂ ml/l) and Indian Oceans (3.3–4.7 O₂ ml/l). Moreover, ancyromonads usually attach to surfaces (Scheckenbach et al. 2005; Glücksmann et al. 2013), suggesting that *A. kenti* may be a common grazer of particle-attached bacteria in the bathypelagic depths of the Pacific Ocean. Taken together, this suggests that *A. kenti* is a specialized organism in the ocean and well-adapted to be a bacterial grazer in low-oxygen habitats.

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**Figure 6.** Maximum likelihood phylogenetic tree inferred from 18S rDNA sequences of genus *Multimonas*, including *M. media* strain JS004, with various other apusomonad genera as outgroups. Ultrafast bootstrap support values (> 95%) are shown at nodes. Solid circles indicate Bayesian posterior probability of 1.
There are only minor morphological differences between species of the genus *Cafeteria* (Schoenle et al. 2020). As such, it is not surprising that our strain, JS001, was morphologically quite similar to *C. mylnikovii*, *C. loberiensis*, *C. burkhardae*, *C. roenbergensis*, *C. maldiviensis* and *C. baltica* (Fenchel and Patterson 1988; Cavalier-Smith and Chao 2006; Schoenle et al. 2020, 2022). Therefore, morphological classification using light microscopy should not be considered appropriate for *Cafeteria* species. Instead, it has been phylogenetic analysis that has significantly improved the classification of this genus (Cavalier-Smith 1998). Based on our phylogeny of its 18S rDNA sequence, strain JS001 is unambiguously a member of *C. mylnikovii*. In spite of this, we note some morphological differences with previous accounts. In particular, Schoenle et al. (2022) observed a furrow on the cell body of *C. mylnikovii* strain A.M., which was not confirmed in our strain. This difference might be due to the effects of long-term cultivation or sampling locality, which has been reported to affect the cell size in different strains, or of individuals within strains, in other organisms (Cerino et al. 2005; Petrova et al. 2020).

*Figure 7.* Global distribution pattern maps of two heterotrophic nanoflagellates, showing relative abundance of amplicon sequence variants (ASVs) of the V4 region of 18S rDNA, generated using the MicroMap web application (https://micromap.icm.csic.es/). (A) Two vertical depth profiles (mesopelagic and bathypelagic) of ASVs matching *Ancyromonas kenti*. (B) Relative abundance of ASVs matching *Multimonas media* at surface regions. Black circles indicate sampled sites with absence of the ASV.
Our study is only the second to report *Cafeteria mylnikovii*. The known worldwide distribution of *C. mylnikovii* was previously limited to the White Sea (Cavalier-Smith and Chao 2006); our finding shows its occurrence in Korean waters as well. Unfortunately, sampling site conditions were not published in the original account of *C. mylnikovii*. As such, there are insufficient data by which one might predict where else to find this species, but the fact that neither it nor sequences attributable to it have been detected elsewhere suggests that it is not common. However, *Cafeteria burkhardae* is known to be present in high abundance in marine environments and is globally distributed (Massana et al. 2021). We did not find evidence for any *Cafeteria* species with wide distributions in the Malspina-2010 dataset (data not shown). Based on our results, then, we suggest that *C. mylnikovii*, and probably other *Cafeteria* species, may have a smaller role as a bacterial grazer in the marine environment than *C. burkhardae*.

The 18S rRNA sequence of *Multimonas media* strain JS004 is 100% identical to *M. media* strains 6B (the type strain, isolated from England) and 7C. In addition, our phylogenetic tree grouped strain JS004 with previously cultured strains of *M. media* in a highly supported clade (BS: 100%, PP: 1). The body length of strain 6B is reported as ∼8.5 µm (Cavalier-Smith and Chao 2010), slightly longer than in our strain. Under the light microscope, other morphological features were similar between 6B and JS004. While scanning electron microscopy (SEM) of 6B has not been published, studies of one strain of *M. media* from France (MMRosko2018: Torruella et al. 2023), one from Korea (KM002: Heiss et al. 2015) and one from Canada (KM030: Heiss et al. 2015) have. These show additional features, in particular a diagonal crease at the anterior of the cell body, a ‘frilled’ skirt flange and small rounded putative extrusomes on the dorsal surface of the cell. While we observed a similar anterior crease in our SEM specimens, other features differed from those observed in previous studies. Our strain did not exhibit a ‘frilled’ skirt margin, this margin instead only appearing slightly thickened (compare Figure 5K with Heiss et al. 2015, figure 3A,B,D, and with Torruella et al. 2023, figure 3G). While putative extrusomes were seen on the dorsal side of the cell, their shapes were clearly different from previously observed examples, and their density much higher than that in previous observations (compare Figure 5L with Heiss et al. 2015, figure 3A,C, and with Torruella et al. 2023, figure 3G). The appearance of putative discharged extrusomes is roughly comparable across all three studies (Figure 5KL; Heiss et al. 2015, figure 3A, C,D; Torruella et al. 2023, figure 3G), but the differences between these are probably attributable to differences in preparation. Our SEM preparation also included well-preserved cells apparently lacking putative extrusomes entirely (Figure 5M); it is possible that some cells in the previous studies also lacked extrusomes (e.g. Heiss et al. 2015, figure 3B). In all three studies, the dorsal surface of the cell anterior to the ‘crease’ was completely devoid of extrusomes. Finally, a tusk (sensu Heiss et al. 2013) has never previously been identified in *Multimonas*, but a structure strongly resembling a short, thin tusk, such as has been seen in *Chelonemonas* (see Heiss et al. 2015, figure 3F,J), was observed in our strain. It is possible that the differences seen in SEM may be due to differences in preparation rather than to inherent differences between strains. Curiously, published transmission electron microscopy (TEM) of strain MMRosko2018 shows no obvious evidence for any extrusomes and does not suggest a ‘frilled’ skirt margin (Torruella et al. 2023). Additional TEM studies may be able to clarify the presence, internal detail and distribution of these unusual structures.

In contrast to the situations with *A. kenti* and *C. mylnikovii*, our results are compatible with the known global distribution of *M. media*. Previous *M. media* strains were also isolated from marine sediments in Canada, England and France (Cavalier-Smith and Chao 2010; Heiss et al. 2015; Torruella et al. 2023). Actually, our culture may be the second of this species from Korean waters (Heiss et al. 2015; Torruella et al. 2023). Heiss et al. (2015) reported that *M. koreensis* strain KM002, isolated from Korea, was indistinguishable from *M. media* strain KM030, isolated from Canada, based on light microscopic observations. However, the 18S rRNA gene sequence of KM002 was found to be chimeric, with the 5’ ∼1000 bases being identical to *M. media* strain MMRosko2018, and the 3’ ∼575 bases coming from an unidentified eukaryote, probably a fungus. No full sequence is currently available for this strain. Overall, it is likely that *M. koreensis* strain KM002 was in fact *M. media*. In global ocean surveys, *M. media* was confirmed in only one station in the South Pacific Ocean and furthermore showed only a very low relative abundance there. *Multimonas media* ASVs were found only at surface water depth, which is at odds with the understanding that apusomonads in general, and in this case *Multimonas* in particular, are mainly found gliding and attached to a surface. Torruella et al. (2023) reported that members of another apusomonad genus, *Podomonas*, mainly float in the water column. Our results lead us to entertain the possibility that, under some conditions at
least, *Multimonas* might also persist in the water column. There is a lack of environmental community analyses regarding Apusomonadida. In the light of our findings, we suggest that further ecological studies of apusomonads may produce significant results. Our study proposes *Multimonas media* as a reasonable model for this.

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