

## ORIGINAL ARTICLE

# *Vannella salarenaria* sp. nov. (Amoebozoa, Vannellida) and its implications for the distribution of amoebae

Alexander Kudryavtsev<sup>1\*</sup>, Ekaterina Volkova<sup>1</sup>, and Alexey Smirnov<sup>2</sup>

<sup>1</sup> Laboratory of Cellular and Molecular Protistology, Zoological Institute, Russian Academy of Sciences, 199034 St. Petersburg, Russia

<sup>2</sup> Department of Invertebrate Zoology, Faculty of Biology, St. Petersburg University, 199034 St. Petersburg, Russia

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## Summary

*Vannella salarenaria* sp. nov. was isolated from the upper layer of sand at the dry bottom of Paralimniou lake (Cyprus). This species can be cultured under the salinity up to 40‰, like a typical marine amoeba. However, it also tolerates freshwater conditions and demonstrates a contractile vacuole in this case. Based on the morphological and SSU rRNA data, this species is most closely related to the previously sequenced marine strain *Vannella* sp. ED40Ana, isolated from the Ebro River delta on the Spanish coast of the Mediterranean Sea in 2000th. We present here archived morphological data on this strain. Among named species, the strain CY18.4SO.2 is most similar morphologically to *V. flabellata*, described by F.C. Page in 1974 as *Platyamoeba*. However, it differs from this species in the presence of pentagonal glycostyles in the cell coat. This is the first case of isolation of an amoeba species which can be cultured under marine conditions from the terrestrial habitat. It suggests that amoebae, showing a wide range of salinity tolerance, may populate terrestrial sites in the proximity of the sea.

**Key words:** Amoebozoa, biogeography, cytochrome c oxidase, molecular phylogeny, morphology, small-subunit ribosomal RNA, *Vannella*

## Introduction

The genus *Vannella* Bovee, 1965 (Amoebozoa, Vannellida) comprises 38 species isolated from marine, brackish, freshwater, and terrestrial habitats worldwide (Smirnov et al., 2007; Kudryavtsev et al., 2021). Marine and brackish water biotopes harbor at least 28 named species and 54 unnamed isolates that

were observed in all niches available for amoebae (e.g., Schaeffer, 1926; Sawyer, 1975; Dyková et al., 2005; Dyková and Kostka, 2013; English et al., 2019; Kudryavtsev, 2022). Yet, distribution patterns and biogeography of vannellid amoebae are poorly studied. Most marine and brackish water *Vannella* species were isolated only once, and never re-isolated since their initial description, thus no data

on their geographic distribution are available. There are few cases when a vannellid species was reported from geographically distant locations, but in all these studies they were identified based on morphology (Davis et al., 1978; Rogerson and Hauer, 2002; Patsyuk, 2016; Patsyuk and Onyshchuk, 2019).

There are a number of unnamed strains assigned to the genus *Vannella*, but their identity has never been established (Dyková et al., 2005; Dyková and Kostka, 2013; English et al., 2019). Strains originating from different geographic locations and different biotopes in the phylogenetic tree form clusters of closely related sequences. Potentially, they can be assigned to the same species (Kudryavtsev, 2022). Simultaneously, some morphologically different *Vannella* species and unnamed strains show extremely low genetic distances (e.g., *Vannella arabica* and *V. bursella*; Smirnov et al., 2007; Nasonova et al., 2010). Therefore, to clarify the species concept in *Vannella* and to better understand geographic distribution patterns in these amoebae, comparative studies of different strains from remote biotopes are needed.

In this paper, we compare a pair of closely related *Vannella* strains isolated from remote contrasting habitats in the Mediterranean region. One of these strains was isolated from the sand of the dry bed of Paralimniou Lake (Famagusta District, Cyprus). Another one – from the Ebro River delta on the Spanish Mediterranean coast. The SSU rRNA gene of the latter strain was sequenced under the strain designation *Vannella* sp. ED40 (Smirnov et al., 2007). No morphological data on this strain were published at that time; therefore, we publish its archived light micrographs. We discuss the implications of these findings for the geographic distribution patterns of vannellid amoebae.

## Material and methods

### ISOLATION OF AMOEBAE, CULTIVATION AND MICROSCOPIC STUDY

Amoebae of the strain CY18.4SO.2 were isolated from a sample of sand collected in a dry bed of Paralimniou Lake (Famagusta District, Cyprus) in May 2018 (35.03859N, 33.97197E). The sand sample was aseptically inoculated on the surface of SMY agar plates (15 g NN agar, 0.02 g yeast extract, 0.02 g malt extract per 1 l water) prepared with artificial seawater diluted to 25‰. Amoebae were purified and cloned using agar migration technique

(Page, 1983). Cloning was repeated twice. Amoebae were further cultured in plastic 50-ml tissue culture flasks in artificial filter-sterilized seawater diluted to 20‰ with the addition of two autoclaved wheat grains per flask. For investigations, culture was temporarily established in 60-mm plastic Petri dishes. For light microscopic studies, amoebae were transferred on the coverslips using glass capillary pipettes. Wet mounts were sealed with petroleum jelly and examined using a Leica DM2500 upright microscope equipped with differential interference contrast (DIC). Amoebae in culture were investigated using DMI 3000 and DM IL inverted microscopes (Leica) equipped with phase contrast and integrated modulation contrast (IMC). Amoebae were measured on micrographs and video records using FIJI software (Schindelin et al., 2012).

Data on *Vannella* sp. ED40Ana strain are compiled from A. Smirnov's images captured in 2002, before molecular study of this amoeba. Methods of isolation, cultivation and morphological studies of this strain were the same as described for *Vannella ebro* (Smirnov, 2001); molecular methods are described in Smirnov et al. (2007).

For transmission electron microscopic (TEM) study amoebae were fixed at +4 °C in culture using a 2.5% (v/v) solution of glutaraldehyde for 40 min followed by postfixation in a 1% (w/v) solution of osmium tetroxide for 1 h. Both fixatives were prepared in 0.05M sodium cacodylate buffer supplemented with artificial seawater diluted to 25‰ (all concentrations final). Cells were washed with the same buffer (3×5 min) between fixation steps. Before dehydration, amoebae were collected from the Petri dish bottom using a cell scraper, concentrated with gentle centrifugation, and the buffer was gradually diluted to distilled water followed by embedding in 1.5% (v/w) agar. Pieces of agar (1 mm<sup>3</sup>) containing cells were dehydrated in a graded ethanol series (30 to 90% in 20% steps followed by 100%), infiltrated through 100% acetone and embedded in Embed812 epoxy resin. Ultrathin sections were cut from polymerized blocks using Leica UC ultramicrotome, double-stained with solutions of uranyl acetate and Reynolds' lead citrate, and observed using FEI Morgagni 268 transmission electron microscope operated at 80 kV.

### THE SALINITY TOLERANCE TEST

To perform salinity tolerance test, amoebae in a flask where the locomotive forms were prevailing were washed several times with the 20‰-seawater.

Afterwards, the flask was shaken vigorously to produce a cell suspension, and its contents were inoculated into 60-mm Petri dishes (0.25 ml of cell suspension per dish) with artificial seawater (salinities of 0.3, 10, 40, and 100‰) and two wheat grains per dish. Petri dishes with 20‰ seawater were inoculated identically as a control. Dishes were sealed with Parafilm and incubated at +18 °C. Three dishes were inoculated for each salinity. Cultures were observed using Leica DM IL inverted microscope equipped with Integrated Modulation Contrast (IMC) one hour after inoculation, on the next day after inoculation, and during one month after inoculation once in 3–4 days.

#### MOLECULAR PHYLOGENETIC STUDY

Total DNA was isolated from the purified culture after two rounds of cloning using guanidine isothiocyanate method (Maniatis et al., 1982). Genes encoding for small subunit rRNA and *Cox1* were amplified using polymerase chain reaction (PCR) with the primer pairs RibA + RibB (Medlin et al., 1988) and LCO1490 + HCO2198 (Folmer et al., 1994), respectively. SSU rRNA amplicon was cloned, and four molecular clones were sequenced in both directions; *Cox1* amplicon was sequenced directly using PCR primers. PCR product purification, cloning, and sequencing were done as described by Kudryavtsev et al. (2022). The sequences obtained were checked against the NCBI/GenBank database using BLAST (Altschul et al., 1990) and aligned with our curated databases of SSU rRNA and *Cox1* genes of Amoebozoa. Alignment was performed using MAFFT software (Kato and Standley, 2013) with parameters set for accurate alignment (–localpair –maxiterate 1000). Poorly aligned positions from the SSU rRNA gene alignment were trimmed using trimAl (Capella-Gutiérrez et al., 2009) set for automatic trimming for the optimal set of positions for maximum likelihood analysis. The final alignment of the SSU rRNA gene used for tree reconstruction consisted of 67 sequences of *Vannella* spp. and deeply-branching lineages of Vannellida as outgroups and 1899 nucleotide positions. The alignment of *Cox1* gene consisted of 20 sequences of Vannellida with 663 nucleotide positions.

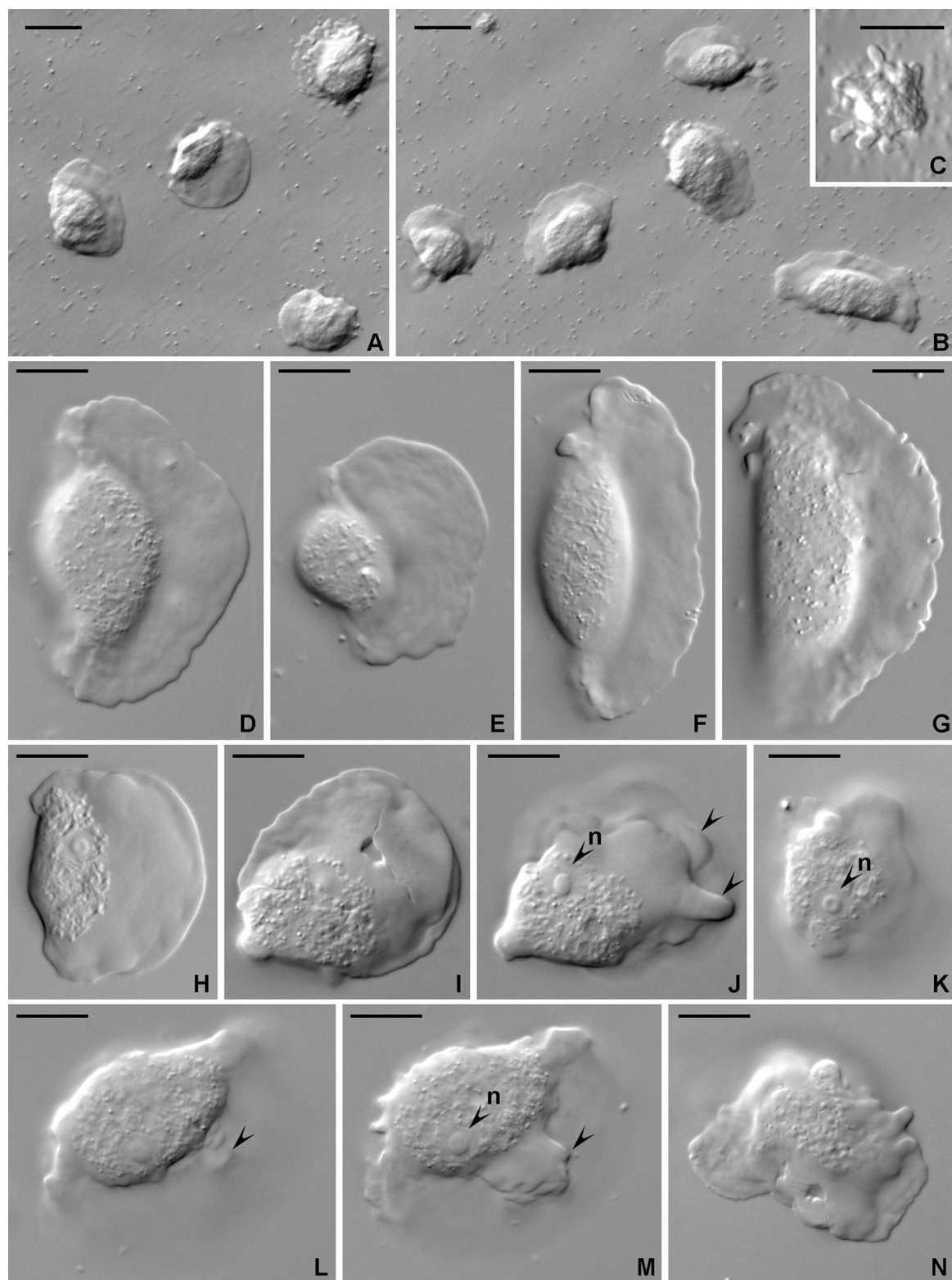
Phylogenetic analyses were performed using the maximum likelihood algorithm with IQ-TREE v.2.2.0.3 (Nguyen et al., 2015) and RAxML v.8.2.10 (Stamatakis, 2014), and Bayesian analysis

using MrBayes v.3.2.7 (Ronquist et al., 2012). IQ-TREE used automatic substitution model selection with ModelFinder (Kalyaanamoorthy et al., 2017) and non-parametric bootstrap with 1000 pseudoreplicates. The models selected were GTR+F+I+R4 for SSU rRNA gene dataset and K3Pu+F+G4 for *Cox1* gene dataset. RAxML used GTRGAMMA substitution model and 100 independent tree searches with random starting trees followed by bootstrapping with 1000 pseudoreplicates and mapping of bootstrap values on the best tree. Bayesian MCMC analysis was performed using the GTR + G + I substitution model (eight rate categories), sampling was performed for 20,000,000 generations with a random starting tree and a burn-in of 0.25 of the samples. All analyses were performed using a computational cluster of the Zoological Institute of the Russian Academy of Sciences. The sequence similarity was evaluated using BioEdit (Hall, 1999) on the alignments with terminal gaps and PCR primer sites removed.

## Results

#### MORPHOLOGY AND SALINITY TOLERANCE OF *VANNELLA* SP. CY18.4SO.2

During locomotion, amoebae were 23–34 µm long by 23–50 µm broad (average 27.5 and 35.1 µm, respectively), length:breadth ratio was 0.5–1.2 (average 0.8); n=44. The locomotive forms of this strain were identical both on the plastic surface in culture (Fig. 1, A, B) and on the glass surface in wet mounts (Fig. 1, D-H; Supplementary Video S1). The most typical locomotive forms were fan-shaped, with the broad anterior hyaline area, which occupied up to two-thirds of the cell length (Fig. 1, D-G). Some cells were rounded or oval during locomotion. Temporarily, the posterior end of the cell could become slightly extended (Fig. 1, H, I). The hyaline area surrounded the posterior granuloplasm anteriorly and laterally. Frontal margin of anterior hyaline area was usually slightly wrinkled and produced shallow waves. Small endocytic vesicles could be produced on the margin of hyaloplasm and transported posteriorly. The lateral areas of the hyaloplasm contracted and often became extensively wrinkled while the cell was advancing (Fig. 1, D, F, G). Numerous wrinkles and hyaline waves were produced on the dorsal surface of anterior hyaline area. They usually extended



**Fig. 1.** Light micrographs of *Vannella salarenaria* sp. nov. A–C – Amoebae in liquid medium in culture, IMC. A, B – Locomotive forms; C – floating form. D–I – Locomotive forms on glass slides in temporary mounts, DIC. J, L, M – Wrinkles and hyaline waves (*arrowheads*) on the dorsal surface during locomotion, DIC; K – amoeba showing nucleus with the central lacune in the nucleolus, DIC; N – stationary amoeba, DIC. *Abbreviations:* n = nucleus. Scale bars: 20  $\mu$ m in A–C; 10  $\mu$ m in other figures.

towards the anterior margin of the cell starting from the granuloplasm (Fig. 1, J, L, M). The posterior granuloplasm was usually hemispherical or spindle-shaped when viewed from above. Sometimes during fast locomotion, the anterior hyaline area rapidly contracted and became strongly wrinkled. Amoeba temporarily ceased locomotion and produced a new hyaline area, which continued to expand in a new direction of movement. The locomotive rate on the glass surface was 32–52  $\mu\text{m}/\text{min}$  that roughly corresponded to 1–2 cell lengths per minute.

Floating forms were not observed in cultures, and were temporarily present only after the amoebae were detached from the substratum by vigorous shaking. Floating amoebae contracted and became irregularly wrinkled with numerous blunt-ended, bent pseudopodia that never exceeded the diameter of the central cytoplasmic mass (Fig. 1, C). Stationary amoebae on the substratum had a flattened, irregular shape with numerous folds and wrinkles (Fig. 1, N). Amoebae showed a single nucleus that was irregularly rounded or ovoid, with a central spherical nucleolus (Fig. 1, J, K, M). Sometimes the nucleolus contained a central lacune (Fig. 1, K). The diameter of the nucleus was 4.3–6.8  $\mu\text{m}$  (average 5.6  $\mu\text{m}$ ), of the nucleolus 2.2–3.9  $\mu\text{m}$  (average 3.1  $\mu\text{m}$ );  $n=40$ . The granuloplasm contained numerous spherical and ovoid granules about 1  $\mu\text{m}$  in diameter and several rounded food vacuoles filled with bacteria. We never observed encystment.

Transmission electron microscopy demonstrated a cell coat consisting of a basal amorphous layer 5–8 nm thick and pentameric tower-shaped glycostyles (Fig. 2, A, B). The length of the glycostyles was 100–220 nm (average 150 nm;  $n=38$ ), lower part of a glycostyle (from plasma membrane to central, narrowest part) comprised 37–72% of its length (average 60%;  $n=38$ ). Simple filaments 186–250 nm long were occasionally seen between the glycostyles (Fig. 2, A). The nucleus in sections was irregularly shaped with shallow depressions of its envelope (Fig. 2, C) or spindle-shaped (Fig. 2, D) with the rounded electron-dense nucleolus. Mitochondria were rounded, with electron-dense matrix and branching, tubular cristae (Fig. 2, C–E). Dictyosomes scattered over the cytoplasm had a shape of stacks of 5–6 flattened cisternae (Fig. 2, F).

After inoculation into seawater medium diluted to different concentrations, amoebae showed different responses. Normal activity, adhesion and locomotion were seen at 10, 20, and 40‰

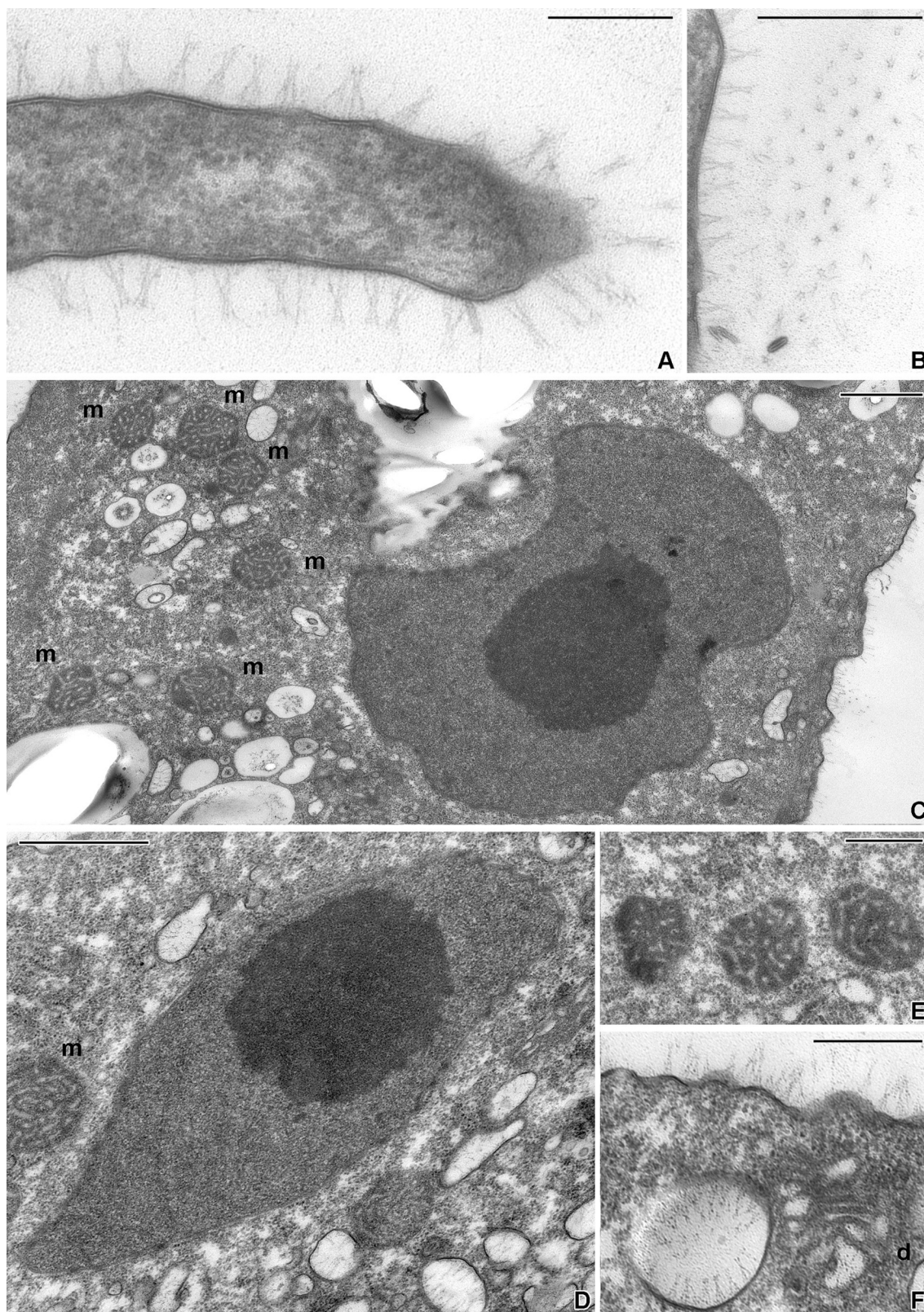
immediately after inoculation. The increment in cell density was evident under these salinities the next day after inoculation. After inoculation into 0.3‰ medium, amoebae were immobile, either adhered to the substratum or inflated. Five days after inoculation, most of the cells in this salinity remained immobile, while seven days after inoculation locomotion was evident, and the density of the culture gradually became comparable to that observed at 10–40‰. The locomotive rate of amoebae under this salinity was lower than in 10–40‰ medium. Each cell in culture possessed a contractile vacuole with a maximal diameter during diastole 9–12  $\mu\text{m}$  (Fig. 3, A–D), and a period of contraction 4–5 min at +22 °C. At the beginning of a diastole stage, the formation of the contractile vacuole started from 2–3 smaller vacuoles (Fig. 3, B) that later fused and the resulting vacuole increased in diameter until contraction. Amoebae in 100‰ artificial seawater contracted immediately after inoculation, and neither adhered to the substratum nor multiplied during the experiment.

#### MORPHOLOGY OF *VANNELLA* SP. ED40ANA

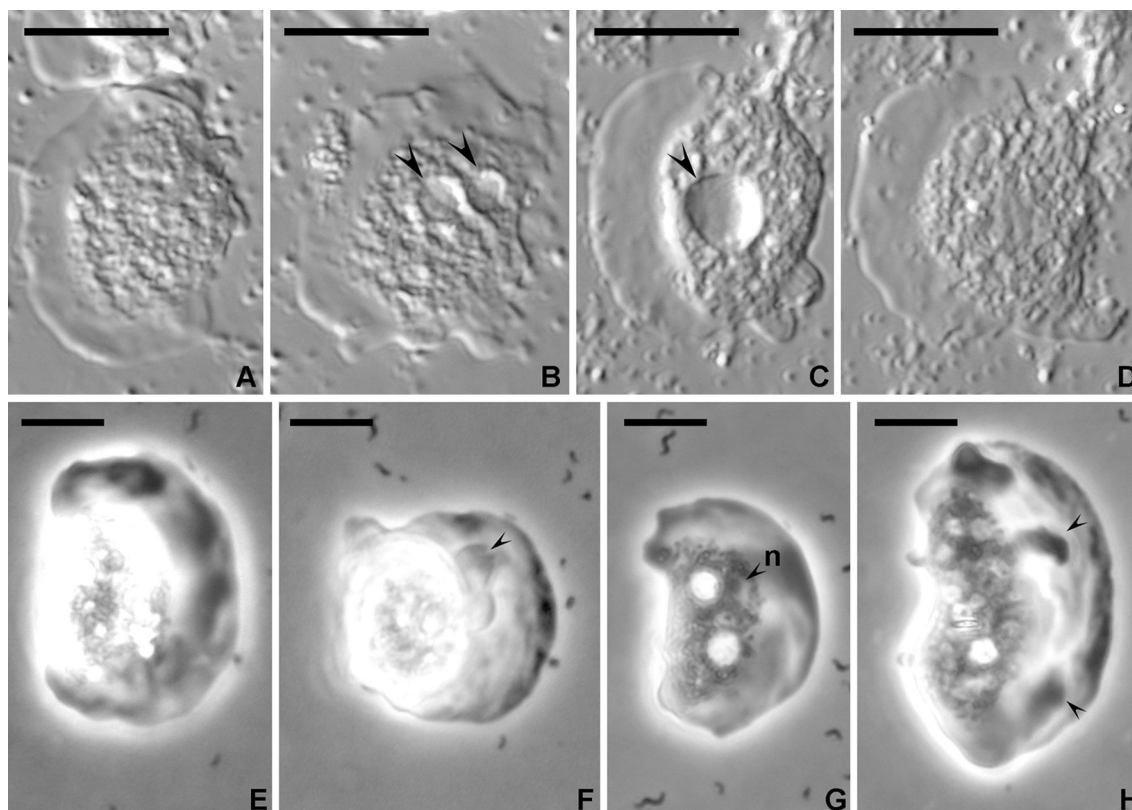
Judging from the available micrographs, the size of the locomotive forms of these amoebae was 15–25  $\mu\text{m}$  long by 20–40  $\mu\text{m}$  broad, and length:breadth ratio varied between 0.7 and 1.1. During locomotion, amoebae of the strain *Vannella* sp. ED40Ana were usually oval or fan-shaped with the broad area of the hyaloplasm that surrounded the posterior granuloplasm anteriorly and laterally (Fig. 3, E–H). Breadth was usually the greatest dimension of the locomotive form, and hyaloplasm occupied about a half of the cell length. Amoebae produced numerous wrinkles in the lateral parts of the hyaloplasm, and on the dorsal surface of the anterior part (Fig. 3, F, H). The nucleus was vesicular, with a large central nucleolus (Fig. 3, G).

#### MOLECULAR PHYLOGENETIC ANALYSES

**Small-subunit ribosomal RNA gene.** Fully sequenced fragments of the SSU rRNA gene of CY18.4SO.2 strain were 1936–1940 bp long (excluding primers). Differences between overlapping, completely sequenced parts of the molecular clones of the same amplicon were in 1.40–4.20% of the 1836 nucleotide positions compared. The variable positions were scattered over the sequence length, but most of them were concentrated in the variable



**Fig. 2.** Transmission electron micrographs of *Vannella salarenaria* sp. nov. A, B – Sections of the cell coat. C, D – Different shapes of nucleus in the sections. E – Mitochondria. F – Dictyosome. *Abbreviations:* d – dictyosome, m – mitochondria. Scale bars: 0.25  $\mu\text{m}$  in A, 1  $\mu\text{m}$  in C and D, 0.5  $\mu\text{m}$  in other figures.



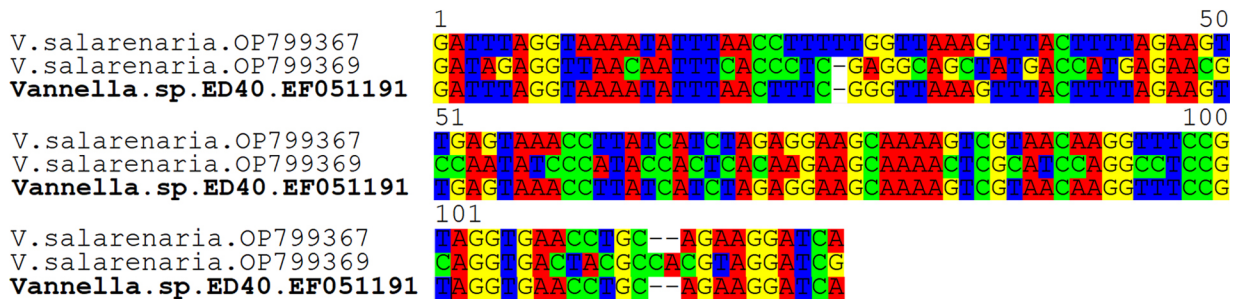
**Fig. 3.** A–D – Light micrographs of *Vannella salarenaria* n. sp. in culture in seawater diluted 100 $\times$  (salinity 0.3‰) showing stages of contractile vacuole activity (*arrowheads*), IMC; E–H – locomotive forms of *Vannella* sp. ED40Ana in culture on plastic surface (phase contrast); *arrowheads* indicate cytoplasmic wrinkles. *Abbreviations:* n = nucleus. Scale bars: A–D – 20  $\mu$ m, E–H – 10  $\mu$ m.

region V9. The variability pattern in this area suggested that the sequenced clones demonstrated two distinct sequence variants, each represented by two molecular clones (Fig. 4). Among the known sequences, SSU rRNA gene of *Vannella* sp. ED40Ana (EF051191) obtained by Smirnov et al. (2007) was the most similar to those of our strain. This sequence differed from obtained sequences in 2.50–4.90% of the nucleotide positions. Twenty-four unique substitutions (1.3% of all positions compared) were detected between this sequence and the molecular clones of CY18.4SO.2 scattered over the entire sequence length.

Molecular phylogenetic analysis based on the SSU rRNA gene (Fig. 5) demonstrated a robust clade comprising strains CY18.4SO.2 and ED40Ana that occupied a relatively isolated position among *Vannella* with the only relative being an unidentified amoeba *Vannella* sp. CHOR (Dyková and Kostka, 2013). However, the support for this clade was relatively low (Bayesian posterior probability 0.96, bootstrap support 52 and 67 with IQ-TREE and

RAxML, respectively). The entire clade was sister to the one comprising *V. robusta*, *V. australis*, *V. arabica*, *V. bursella*, *V. ebro*, and *V. samoroda*. It occupied a position in the crown part of *Vannella* tree among other species isolated from marine/brackish biotopes or marine hosts. The tree topology and support values were mostly congruent between the three programs used for tree reconstruction (Bayesian reconstruction using MrBayes, maximum likelihood using IQ-TREE and RAxML). The only clade that had different topology in the Bayesian tree was the one comprising *V. septentrionalis* and *V. contorta* (Fig. 5).

**Cytochrome c oxidase subunit 1 gene.** The sequenced fragment of Cox1 gene was 656 bp long and had a G + C content of 30.1%. The sequence shared all typical features of *Vannella*, in particular, it had a putative site of insertional editing in position 182 (Nassonova et al., 2010; Kudryavtsev, 2022). Additionally, it had three more putative sites of insertional editing located in positions 52, 515, and 603. No traces of within-amplicon sequence



**Fig. 4.** The last 125 nucleotide positions of the alignment of two molecular clones (OP799367 and OP799369) of *Vannella salarenaria* sp. nov. and *Vannella* sp. ED40Ana (EF051191) for reference showing intra-amplicon variation in the region V9 of *V. salarenaria*.

variation were seen in the phoregrams. In the maximum likelihood phylogenetic tree based on nucleotide sequences (Fig. 6), the new sequence branched as a sister clade to *V. robusta* and *V. australis*, but this position was not supported. Although the taxonomic sampling of the Cox1 gene tree is much smaller than in the SSU rRNA tree, the general structure of the tree was similar. There are deep-branching freshwater lineages of *V. croatica* and *V. danica* + *persistens* + *simplex* ('SPD' clade; Nasonova et al., 2010), and derived lineage comprising all presently sequenced marine and brackish water species.

## Discussion

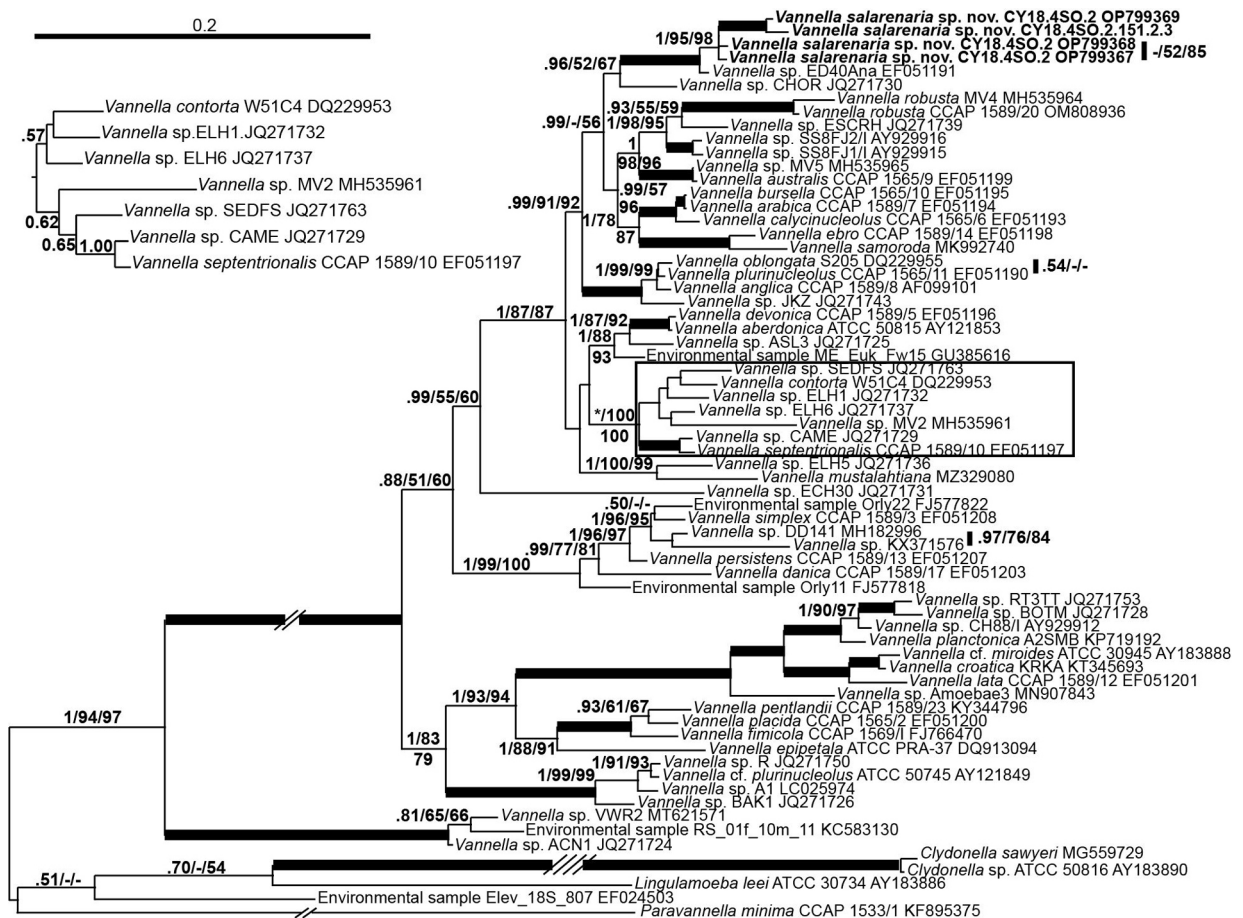
### IDENTIFICATION OF THE NEW STRAIN

Based on the morphological and molecular phylogenetic data, strain CY18.4SO.2 can be reliably identified as a member of the genus *Vannella* sensu Smirnov et al. (2007). Gene sequence data on these amoebae exclude their identification as one of the already sequenced species. Therefore, a set of candidate species among known marine/brackish water ones is limited to *Vannella flabellata* and *V. mainensis*, showing the similar size range (Page, 1971, 1974). *Vannella mainensis* differs from our strain in locomotive form. It usually has a rounded shape with the hemispherical posterior edge and has the ability to produce extended pseudopodia when floating (Page, 1971). In contrast with our species, the cell coat of *V. mainensis* does not contain pentagonal glycostyles (Page, 1980). *Vannella flabellata* is very similar in size and locomotive form to our strain (Page, 1974), but Page did not observe pentagonal glycostyles in this species. Instead,

*Vannella flabellata* has amorphous stratified glyco-calyx 15–20 nm thick with the compact inner layer and a less dense outer layer (Page and Blakey, 1979). The current hypothesis in the taxonomy of vannellids is that the structure of glyco-calyx (pentagonal glycostyles or absence thereof) is species-specific. Although there are cases of closely related species with and without pentagonal glycostyles (e.g., *V. arabica* and *V. bursella*; Smirnov et al., 2007), there is no evidence for the presence of both types of the glyco-calyx in the same strain or switch between the glyco-calyx types in the life cycle. Therefore, under the current hypothesis, different structures of glyco-calyx warrant different species. Given that no molecular data are available for *V. flabellata*, we cannot assign strain CY18.4SO.2 to this species. Unidentified *Vannella* strain ED40Ana (SSU rRNA gene accession number EF051191) is most closely related to this species, its sequence divergence from this species is comparable to the divergence between molecular clones of the same amplicon of CY18.4SO.2 strain. Unfortunately, a limited amount of morphological data available for this strain does not permit us to perform the full comparison of this organism with our isolate.

There is an unnamed *Vannella* strain, closely related to the one described here and also isolated from the Mediterranean. It is *Vannella* sp. strain CHOR (Dyková and Kostka, 2013). Based on the published micrographs, the size and the shape of its locomotive form, as well as nuclear structure, it is similar to our strain. However, the cell coat of this isolate was designated as "amorphous". In available electron micrograph it shows tiny filaments about 30 nm long without any evident structure (Fig. 4 in Dyková and Kostka, 2013, p. 245). There are no pentagonal glycostyles in the strain CHOR. This strain considerably differs from our isolate in SSU





**Fig. 5.** Maximum-likelihood (IQ-TREE) phylogenetic tree of *Vannella* spp. rooted with selected Vannellida based on the SSU rRNA gene sequences (1899 nucleotide positions). New sequences are in bold. Numbers at nodes indicate Bayesian posterior probability/bootstraps support with IQ-TREE/bootstraps support with RAxML (if above 0.5/50/50). Thick branches = 1/100/100. Slashed branches are shortened to a half (//) or a quarter (///) of their lengths. An alternative topology revealed in Bayesian analysis for the framed clade is shown to the left. Scale bar = 0.2 substitutions/site.

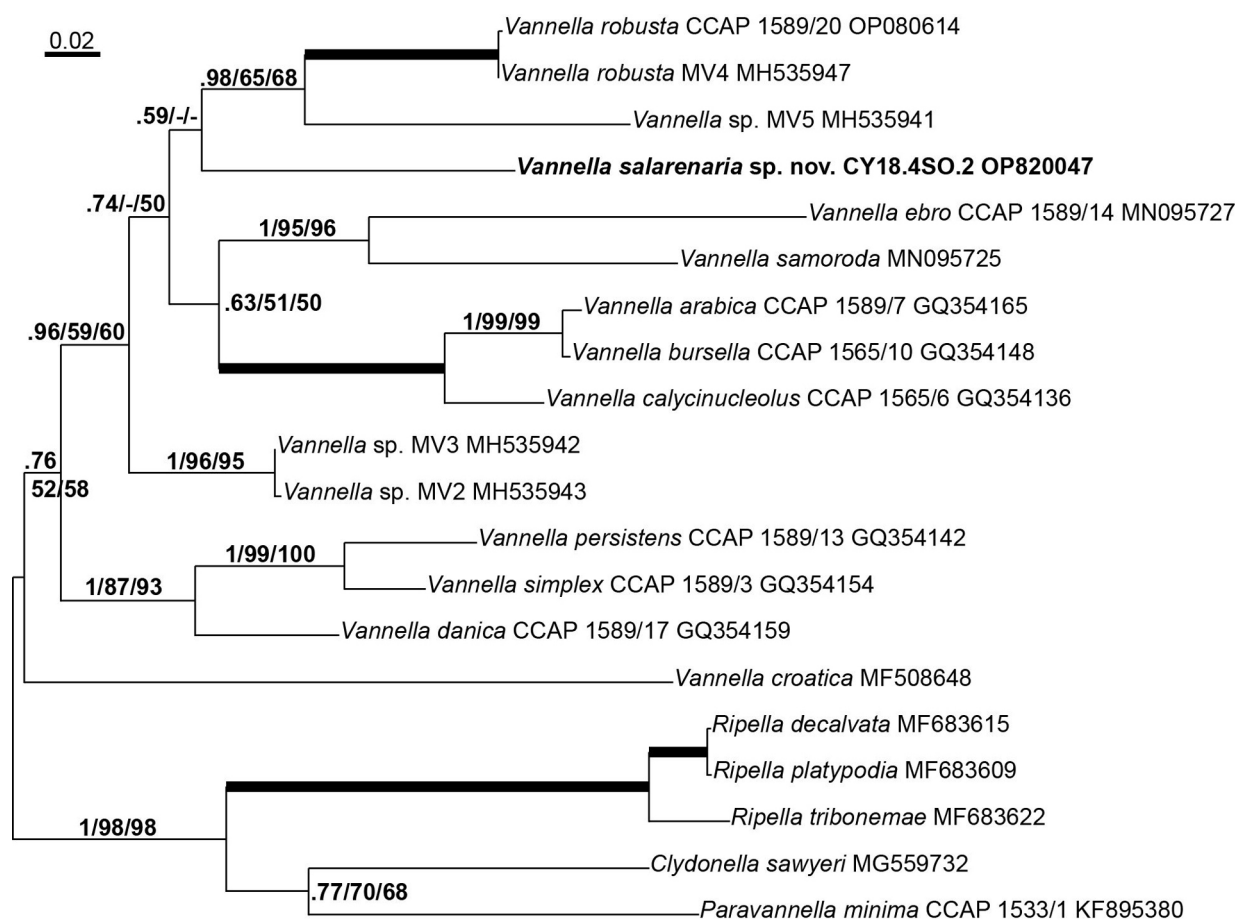
rRNA gene sequence, in particular, in the V4 and V9 regions of the SSU rRNA gene. So, it cannot be co-specific with our isolate. Based on the above analysis, we conclude that our strain should be described as a new species.

**SALINITY TOLERANCE AND IMPLICATIONS FOR GEOGRAPHIC DISTRIBUTION**

Strain *Vannella* sp. CY18.4SO.2 shows growth and reproduction in the range of salinities between 0.3‰ and 40‰. This is congruent with previous data on the salinity tolerance in *Vannella*. In particular, Page (1974) demonstrated reproduction of *V. flabellata* in the freshwater medium based on amoeba saline solution (Page, 1967). This species,

like our strain, also formed contractile vacuole in the freshwater medium. In our study, amoebae did not survive under the salinity value of 100‰. The studied strain was isolated from the dry bottom of Paralimniou Lake that had properties of a dry soil habitat at the moment of sampling. At the same time, this lake is filled with slightly brackish water during wet seasons of the year and is located close to the sea shore (direct distance to the nearest shore site is about 4 km). These facts suggest that this biotope, in addition to freshwater and soil species, may harbor species of brackish water and marine origin that can also temporarily thrive in fresh water.

The closest relatives of the studied strain have been isolated from marine biotopes. Strain ED40Ana originates from the Ebro River delta on



**Fig. 6.** Maximum-likelihood (IQ-TREE) phylogenetic tree of *Vannella* spp. rooted with selected Vannellida based on the Cox1 gene sequences (663 nucleotide positions). New sequence is in bold. Numbers at nodes indicate Bayesian posterior probability/bootstraps support with IQ-TREE/bootstraps support with RAXML (if above 0.5/50/50). Thick branches = 1/100/100. Scale bar = 0.02 substitutions/site.

the Spanish coast of the Mediterranean Sea, salinity 70–80‰ at the moment of sampling. The salinity level in this habitat could significantly oscillate (see Smirnov, 2001). The strain CHOR was isolated from a decomposing crab on the beach in the Adriatic Sea (Savudrija, Croatia; Dyková and Kostka, 2013). This suggests that the strain CY18.4SO.2 might also have a marine origin. Biotopes, like the one sampled in this study, may be considered a transitional “meeting point” between marine and freshwater/soil microbial faunas, harboring both types of species as a component of cryptic amoebae diversity. Either marine/brackish-water or freshwater/soil community gets active, depending on the current environmental conditions. Some organisms, like the studied CY18.4SO.2 strain, elaborated a wide range of salinity tolerance and probably can be active under almost any salinity level.

## Taxonomic summary

*VANNELLA SALARENARIA* SP. NOV., STRAIN CY18.4SO.2

**Diagnosis.** Length of the locomotive form between 20 and 40  $\mu\text{m}$ , breadth between 20 and 50  $\mu\text{m}$ , length:breadth ratio varies between 0.5–1.2, but does not exceed 1.0 on average. Fan-shaped to oval during locomotion, broad anterior hyaline area with arc-shaped margin occupies up to two-thirds of the cell length; posterior, spherical or spindle-like granuloplasm surrounded by the hyaline area anteriorly and laterally; numerous wrinkles on the dorsal surface and lateral parts of the hyaloplasm. Floating form compact, with wrinkled surface and short pseudopodia. Spherical or ovoid vesicular nucleus with the central nucleolus; nucleolar lacune about 1  $\mu\text{m}$  in diameter in some cells; nuc-

leus between 4 and 7 µm in diameter, nucleolus between 2 and 4 µm. Cell coat contains pentagonal glycostyles and sparse simple filaments.

**Zoobank LSID:** urn:lsid:zoobank.org:act:6334F8C2-4C58-4F71-9ABE-DE67B5B05BF3

**Type material:** holotype consists of a culture (accession No ZIN.2022.04), epoxy resin block with embedded cells for TEM (accession No F172), and purified DNA sample (accession No A621) stored with the Culture Collection of Heterotrophic Protists at the Zoological Institute of the Russian Academy of Sciences.

**Type locality:** sand at the dry bottom of Paralimniou Lake (Famagusta District, Cyprus; 35.03859N, 33.97197E), grows in brackish water conditions.

**Reference sequence data:** OP799367-OP799369 (SSU rRNA gene) OP820047 (Cox1 gene).

**Etymology:** *salarenaria*, L. “sal” – “salt”, “arenaria” – “of sand”, indicates isolation from the salty sand of the bottom of dry lake.

**Differences from congeners.** Clearly differs from all sequenced species of *Vannella* in the SSU rRNA and/or Cox1 gene sequences. Among species without molecular data available, most similar to *Vannella flabellata* (Page, 1974), but differs from this species by the presence of the pentagonal glycostyles in the cell coat.

## Acknowledgements

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## References

Altschul S.F., Gish W., Miller W., Myers E.W. and Lipman D.J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215 (3): 403–410. [https://doi.org/10.1016/S0022-2836\(05\)80360-2](https://doi.org/10.1016/S0022-2836(05)80360-2)

Capella-Gutiérrez S., Silla-Martínez J.M. and Gabaldón T. 2009. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics.* 25 (15): 1972–1973. <https://doi.org/10.1093/bioinformatics/btp348>

Davis P.G., Caron D.A. and Sieburth J. McN. 1978. Oceanic amoebae from the North Atlantic:

culture, distribution, and taxonomy. *Trans. Amer. Micros. Soc.* 97 (1): 73–88. <https://doi.org/10.2307/3225685>

Dyková I., Boháčová L., Fiala I., Macháčková B. et al. 2005. Amoebae of the genera *Vannella* Bovee, 1965 and *Platyamoeba* isolated from fish and their phylogeny inferred from SSU rRNA gene and ITS sequences. *Eur. J. Protistol.* 41 (3): 219–230. <https://doi.org/10.1016/j.ejop.2005.05.004>

Dyková I. and Kostka M. 2013. Illustrated guide to culture collection of free-living amoebae. Academia, Praha.

English C.J., Tyml T., Botwright N.A., Barnes A.C., Wynne J.W., Lima P.C. et al. 2019. A diversity of amoebae colonise the gills of farmed Atlantic salmon (*Salmo salar*) with amoebic gill disease (AGD). *Eur. J. Protistol.* 67: 27–45. <https://doi.org/10.1016/j.ejop.2018.10.003>

Folmer O., Hoeh W.R., Black M.B. and Vrijenhoek R.C. 1994. Conserved primers for PCR amplification of mitochondrial DNA from different invertebrate phyla. *Mol. Mar. Biol. Biotechnol.* 3 (5): 294–299.

Hall T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41: 95–98.

Kalyaanamoorthy S., Minh B.Q., Wong T.K.F., von Haeseler A. and Jermin L.S. 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. *Nat. Methods.* 14: 587–589. <https://doi.org/10.1038/nmeth.4285>

Katoh K. and Standley D.M. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30 (4): 772–780. <https://doi.org/10.1093/molbev/mst010>

Kudryavtsev A. 2022. From Argentine Abyssal Plain to farmed turbot in Spain: A ubiquitous amoeba species *Vannella robusta* sp. nov. (Amoebozoa, Vannellida). *Eur. J. Protistol.* 85: 125912. <https://doi.org/10.1016/j.ejop.2022.125912>

Kudryavtsev A., Parshukov A., Kondakova E. and Volkova E. 2022. *Vannella mustalahtiana* sp. nov. (Amoebozoa, Vannellida) and rainbow trout nodular gill disease (NGD) in Russia. *Dis. Aquat. Org.* 148: 29–41. <https://doi.org/10.3354/dao03641>

Kudryavtsev A., Volkova E. and Plotnikov A. 2019. *Vannella samoroda* n. sp. (Amoebozoa) – first member of the genus from a continental saline habitat placed in a molecular tree. *Eur. J. Protistol.* 71: 125634. <https://doi.org/10.1016/j.ejop.2019.125634>

- Kudryavtsev A., Volkova E. and Voytinsky F. 2021. A checklist of Amoebozoa species from marine and brackish-water biotopes with notes on taxonomy, species concept and distribution patterns. *Protistology*. 15 (4): 220–273. <https://doi.org/10.21685/1680-0826-2021-15-4-3>
- Maniatis T., Fritsch E.F. and Sambrook J. 1982. *Molecular cloning, a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Medlin L., Elwood H.J., Stickel S. and Sogin M.L. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA coding regions. *Gene*. 71 (2): 491–499. [https://doi.org/10.1016/0378-1119\(88\)90066-2](https://doi.org/10.1016/0378-1119(88)90066-2)
- Nassonova E., Smirnov A., Fahrni J. and Pawlowski J. 2010. Barcoding amoebae: comparison of SSU, ITS and COI genes as tools for molecular identification of naked lobose amoebae. *Protist*. 161 (1): 102–115. <https://doi.org/10.1016/j.protis.2009.07.003>
- Nguyen L.T., Schmidt H.A., Von Haeseler A. and Minh B.Q. 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol. Biol. Evol.* 32 (1): 268–274. <https://doi.org/10.1093/molbev/msu300>
- Page F.C. 1967. Taxonomic criteria for limax amoebae, with descriptions of 3 new species of *Hartmannella* and 3 of *Vahlkampfia*. *J. Protozool.* 14 (3): 499–521. <https://doi.org/10.1111/j.1550-7408.1967.tb02036.x>
- Page F.C. 1971. A comparative study of five fresh-water and marine species of Thecamoebidae. *Trans. Amer. Micros. Soc.* 90 (2): 157–173. <https://doi.org/10.2307/3225022>
- Page F.C. 1974. Some marine *Platyamoeba* of East Anglia. *J. Mar. Biol. Assoc. U. K.* 54 (3): 651–664. <https://doi.org/10.1017/S0025315400022827>
- Page F.C. 1980. Fine structure of some marine strains of *Platyamoeba* (Gymnamoebia, Thecamoebidae). *Protistologica*. 16 (4): 605–612.
- Page F.C. 1983. *Marine gymnamoebae*. Institute of Terrestrial Ecology, Cambridge.
- Page F.C. and Blakey S.M. 1979. Cell surface structure as a taxonomic character in the Thecamoebidae (Protozoa: Gymnamoebia). *Zool. J. Linn. Soc.* 66 (2): 113–135. <https://doi.org/10.1111/j.1096-3642.1979.tb01905.x>
- Patsyuk M.K. 2016. New finds of naked amoebae (Protista) in water reservoirs of Ukraine. *Vestn. Zool.* 50 (4): 291–300.
- Patsyuk M.K. and Onyshchuk I.P. 2019. Diversity and distribution of naked amoebae in water bodies of Sumy region (Ukraine). *Vestn. Zool.* 53 (3): 177–186.
- Rogerson A. and Hauer G. 2002. Naked amoebae (Protozoa) of the Salton Sea, California. *Hydrobiologia*. 473: 161–177. [https://doi.org/10.1007/978-94-017-3459-2\\_12](https://doi.org/10.1007/978-94-017-3459-2_12)
- Ronquist F., Teslenko M., van der Mark P., Ayres D.L. et al. 2012. MRBAYES 3.2: Efficient Bayesian phylogenetic inference and model selection across a large model space. *Syst. Biol.* 61 (3): 539–542. <https://doi.org/10.1093/sysbio/sys029>
- Sawyer T.K. 1975. Marine amoebae from surface waters of Chincoteague Bay, Virginia: one new genus and eleven new species within the families Thecamoebidae and Hyalodiscidae. *Trans. Amer. Micros. Soc.* 94: 305–323. <https://doi.org/10.2307/3225496>
- Schaeffer A.A. 1926. *Taxonomy of the amoebas: with descriptions of thirty-nine new marine and freshwater species*. Carnegie Inst. Washington, Washington.
- Schindelin J., Arganda-Carreras I., Frise E., Kaynig V. et al. 2012. Fiji: an open-source platform for biological-image analysis. *Nat. Methods*. 9 (7): 676–682. <https://doi.org/10.1038/nmeth.2019>
- Smirnov A.V. 2001. *Vannella ebro* n. sp. (Lobosea, Gymnamoebia), isolated from cyanobacterial mats in Spain. *Eur. J. Protistol.* 37 (2): 147–153. <https://doi.org/10.1078/0932-4739-00811>
- Smirnov A.V., Nassonova E., Holzmann M. and Pawlowski J. 2002. Morphological, ecological and molecular studies of *Vannella simplex* Wohlfarth-Bottermann 1960 (Lobosea, Gymnamoebia), with a new diagnosis of this species. *Protist*. 153 (4): 367–377. <https://doi.org/10.1078/14344610260450109>
- Smirnov A.V., Nassonova E.S., Chao E. and Cavalier-Smith T. 2007. Phylogeny, evolution, and taxonomy of vannellid amoebae. *Protist*. 158 (3): 295–324. <https://doi.org/10.1016/j.protis.2007.04.004>

## Supplementary material

**Video S1.** Locomotion of *Vannella salarenaria* Kudryavtsev et al., 2022 strain ZIN.2022.04 on the glass surface in a temporary preparation, salinity 20‰, DIC.