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Protistology

ORIGINAL ARTICLE

Morphology and phylogeny of *Dermamoeba fibula* n. sp., (Amoebozoa, Discosea) – the new species of the genus *Dermamoeba* isolated from leaf litter

Yelisei S. Mesentsev[1,*], Arseniy A. Shklyar[1], Oksana G. Kamyshatskaya[1,2], Alina A. Surkova[1], Elena S. Nassonova[2], and Alexey V. Smirnov[1]

[1] *Department of Invertebrate Zoology, Faculty of Biology, St. Petersburg State University, 7/9 199034 St. Petersburg, Russia*
[2] *Laboratory of Cytology of Unicellular Organisms, Institute of Cytology RAS, 194064 St. Petersburg, Russia*

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Summary

Amoebae of the genus *Dermamoeba* can frequently be isolated from terrestrial and freshwater habitats. They have oval or lingulate outlines and a smooth dorsal surface. The thick cell coat of these organisms is a complex, multilayered glycocalyx. Nowadays, three species within the genus *Dermamoeba* are recognized, with molecular data available for just one of them. In this paper, we describe one more species, *D. fibula* n. sp., isolated from a sample of leaf litter. Light microscopy and ultrastructure, as well as the 18S rRNA gene sequence show clear differences between our isolate and any known species. In a conserved region, the obtained sequence contains a long insertion, which corresponds to a S516 group I intron.

Key words: Amoebozoa, *Dermamoeba*, Dermamoebida, phylogeny, systematics

Introduction

Lobose amoebae of the genus *Dermamoeba* Page and Blakey, 1979 are flattened, elongated cells of lingulate morphotype (Smirnov and Goodkov, 1999; Smirnov and Brown, 2004), characterized by smooth outlines and the absence of regular folds and ridges on the dorsal surface of the cell (Page, 1977; Page and Blakey, 1979; Pussard et al., 1979; Smirnov et al., 2011a). These amoebae have rounded anterior and posterior ends, and do not form discrete pseudopodia during locomotion. The first organism, now classified in this genus, was described in the mid-19th century (Greeff, 1866) as "*Amoeba granifera*". Further Page (1976) transferred it to the genus *Thecamoeba*. The genus *Dermamoeba* was established in 1979, after the electron-microscopic investigations of thecamoebids, and the main character of this genus was the presence of a thick multilayered glycocalyx (Page and Blakey, 1979). In the morphological system of naked amoebae, the genus *Dermamoeba* belonged to the family Thecamoebidae (Page, 1987).

According to the results of molecular phylogenetic analysis, the genus *Dermamoeba* was found to be related to *Mayorella* rather than to *Thecamoeba*

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***Corresponding author**: Yelisei S. Mesentsev. Department of Invertebrate Zoology, Faculty of Biology, St. Petersburg State University, 7/9 Universitetskaya Emb., 199034 St. Petersburg, Russia; e.mezentsev@spbu.ru

(Fahrni et al., 2003; Smirnov et al., 2005). A separate family, Dermamoebidae, unifying the genera *Mayorella, Dermamoeba*, and *Paradermamoeba* was created (Smirnov et al., 2011b). In the modern system, the genus *Dermamoeba* belongs to the class Discosea, subclass Flabellinia, order Dermamoebida, family Dermamoebidae (Smirnov et al., 2011b). The same position it occupies in the system by Adl et al. (2019), except for the absence of ranks in the latter case.

Three species belonging to the genus *Dermamoeba* are described at present. These are *D. granifera* (Greeff, 1866) Page et Blakey, 1979, *D. minor* (Pusard, Alabouvette et Pons, 1979) Page, 1988, and *D. algensis* Smirnov, Bedjagina et Goodkov, 2011. The molecular data are available only for the latter species (Smirnov et al., 2011a). This single species on the phylogenetic trees forms a sister clade to the genus *Paradermamoeba*. Numerous references to findings of various *Dermamoeba*-like organisms in faunistic investigations, as well as our own observations – suggest the widespread distribution of them in freshwater (e.g., Anderson, 2013; Surkova et al., 2022) and terrestrial habitats (e.g., Mrva, 2005; Robinson et al., 2002). This makes the small number of known *Dermamoeba* species suspicious, and there is a high probability that the genus actually contains more species.

During our studies of lobose amoebae from terrestrial habitats, we isolated a culture of amoebae identified as a representative of the genus *Dermamoeba*. The combination of morphological characteristics of this strain did not allow us to identify it as any known species of this genus. We performed light microscopic and ultrastructural studies, obtained the sequence of the 18S rRNA gene of this strain, and described it here as a new species, *Dermamoeba fibula* n. sp.

Material and methods

Isolation and cultivation

The described strain, initially named "Da161", was isolated from a sample of leaf litter collected near Kremiotis waterfall, Kritou Terra, Cyprus (34°57'45.1"N 32°26'00.8"E). To isolate cells, tiny (2–4 mm across) pieces of collected substratum were placed in sterile 60 mm Petri dishes filled with wMY agar (Spiegel et al., 1995). To make a clonal culture, tiny fragments of agar containing the single amoeba cell were cut off and transferred to a fresh dish filled with the same medium. The culture was subcultured once a month with the same method by transferring a piece of agar with cells. Amoebae fed on accompanying bacteria and fungi.

Light microscopy

Live cells were studied, measured, and photographed on the object slides using Leica DM2500 upright microscope equipped with DIC and phase contrast optics and a DS-Fi3 camera (Nikon, USA). To increase the focal depth, we applied z-stacking as described by Mesentsev et al. (2020).

Electron microscopy

For electron microscopy, individual cells of the strain Da161 were collected by tapered-tip Pasteur pipette and placed in a glass embryo dish. Fixation was carried out with the mixture of 2.5% glutaraldehyde and 1.6% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 90 min at +4 °C. After fixation, the cells were washed in the same buffer 3 times at room temperature (rt) for 5 min and postfixed with 1% osmium tetroxide (final concentration) in 0.1 M phosphate buffer for an hour at +4 °C. Further amoebae were washed in the same buffer for 3×10 min (rt), and embedded in 2% low-melting agarose (Amresco).

Small pieces of agarose (about 1 mm^3) containing amoebae were dehydrated in a graded series of ethanol followed by 100% acetone. The agarose pieces were further embedded in Epon-812 resin according to the manufacturer's instructions. Ultrathin sections were obtained using a Leica Ultracut 7 ultratome and double-stained with 2% uranyl acetate aqueous solution and Reynolds' lead citrate. The samples were viewed using a JEOL JEM-1400 (JEOL, Ltd., Tokyo, Japan) electron microscope at 80 kV.

DNA extraction and sequencing

To isolate DNA, individual amoeba cells were transferred with a tapered-tip Pasteur pipette into the fresh Petri dish with wMY agar and filled with Millipore-filtered (0.2 μm pores) PJ solution. Cells were left to starve under these conditions for three days; every day, cells were transferred to a fresh dish. After three days of starvation, cells were washed twice in Millipore-filtered PJ solution and transferred into 200 μl PCR tubes with 1–2 μl of the medium. DNA was extracted using the Arcturus

PicoPure DNA Extraction Kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions; 12 μl of extraction buffer was added to each tube. We performed the Multiple Displacement Amplification (MDA) of the DNA from the sample showing good trace quality in Sanger sequencing, using the REPLI-g Single Cell DNA Amplification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

For PCR amplification of the 18S rRNA gene, we used forward RibA (5'>ACCTGGTTGATC CTGCCAGT<3') primer, which is a second half of the original "Primer A" (Medlin et al., 1988) and reverse RibB (5'>TGATCCTTCTGCAGGT TCACCTAC<3') primer (Pawlowski, 2000). The thermal cycle parameters were initial denaturation (10 min at 95 °C) followed by 39 cycles of 30 s at 94 °C, 60 s at 58 °C and 120 s at 72 °C, followed by 10 min at 72 °C for the final extension. Amplicons were purified in 1.5% agarose gel using Cleanup mini Purification Kit (Eurogene, Moscow, Russia). All amplicons were sequenced directly using ABI-PRISM Big Dye Terminator Cycle Sequencing Kit with the RibA, s6F, s12.2, s12.2R, s14, s20R, and RibB primers for 18S rRNA gene (Medlin et al., 1988; Pawlowski, 2000; Adl et al., 2014). The system by Petrov et al. (2014) was used as a reference to identify regions and helices in the sequence of the 18S rRNA gene.

Phylogenetic analysis

The obtained sequence was mounted in the alignment containing all named culture-derived 18S rRNA gene sequences of Dermamoebida and a number of discosean sequences used to form a proper set of outgroups. Sequences were automatically aligned using the Muscle algorithm as implemented in SeaView 4.0 (Gouy et al., 2010); the alignment was further refined manually. The phylogenetic analysis was performed using the maximum likelihood method as implemented in RaxML program (Stamatakis, 2014) with GTR + γ model; 1560 sites were selected for the analysis, 1000 bootstrap pseudoreplicates were used. Bayesian analysis of the same dataset was performed using MrBayes 3.2.6, GTR model with gamma correction for intersite rate variation (8 categories), and the covarion model (Ronquist and Huelsenbeck, 2003). Trees were run as two separate chains (default heating parameters) for 10 million generations, by which time they had reached converging (final average standard deviation of the split frequencies was less than 0.01). The quality of chains was estimated using built-in MrBayes tools and additionally – using the software Tracer 1.6 (Rambaut et al., 2014); based on the estimates by Tracer, the first 25 % of generations were discarded as burn-in. RaxMl and MrBayes programs were run at Cipres V.3.3 website (Miller et al., 2010).

The obtained sequence was deposited with GenBank under the number OR671173 (*Dermamoeba fibula* strain Da161, length 3004 bp).

Results

Light microscopy

Usually, amoebae showed a good ability to adhere to the glass surface of the object slide, but sometimes cells did not adhere to the substrate for a long time. Locomotive amoebae of the strain Da161 on glass were flattened (Fig. 1, A–P). They moved as a whole, and did not form distinct pseudopodia or subpseudopodia (Fig. 1, A–K). During active locomotion, amoebae were elongated in the direction of movement and had smooth outlines. The overall shape of the cell varied from oval to elongate or lanceolate (Fig. 1, A–I). The length of locomotive cells was 36–71 μm (mean 54.8 μm, n = 54). The breadth was 24–45 μm (mean 34.7 μm, n = 54). The length to breadth ratio (L/B) was 1.3–2.3 (mean 1.6). The anterior end of the cell was narrowed smoothly, rounded at a tip (Fig. 1, A, B, E, and I). The posterior end was also rounded and often had a waved surface (Fig. 1, A–I). However, when cells changed the direction of movement or started movement from stationary form, amoebae often demonstrated a posterior end covered with thick, curved wrinkles (Fig. 1, P and Q). Sometimes, cells formed narrow, thin lateral lobes (Fig. 1, A, B, and E). When the cell suddenly changed direction, a new frontal hyaline zone appeared on the lateral side, and the cell branched for a short time (Fig. 1, N and O). As the moving cell gradually turned, slowly changing the direction of movement, the anterior hyaline region smoothly moved to the lateral side of the cell. Because of this, such amoebas had curved outlines (Fig. 1, K). Slowly moving amoebae sometimes had a slightly bifurcated frontal region with parallel growing hyaline areas (Fig. 1, G and H). In non-directed movement, amoebae often had irregularly triangular outlines (Fig. 1, L and M). The dorsal surface of the locomotive cell was usually smooth.

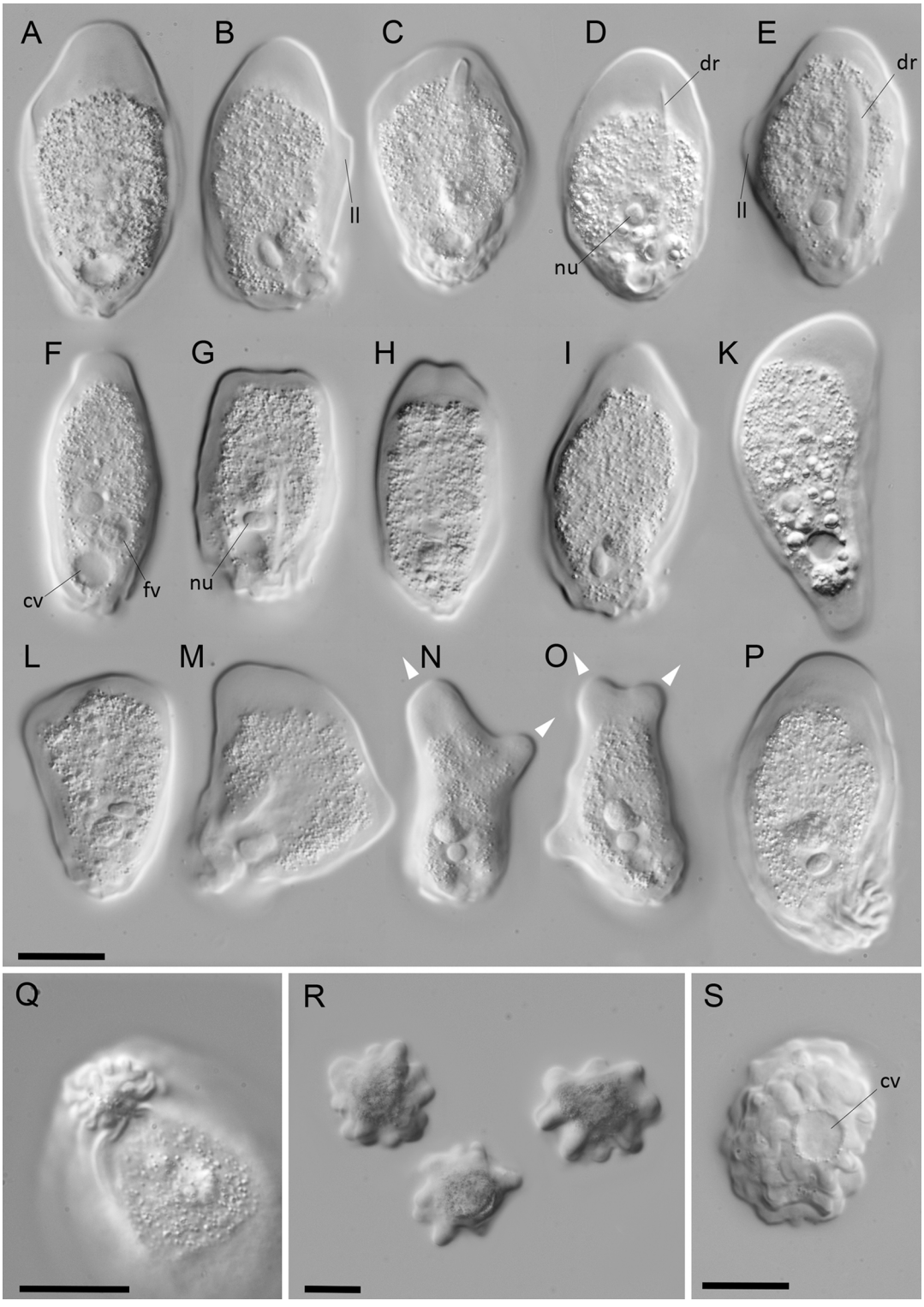

Fig. 1. Light microscopy of *Dermamoeba fibula* n. sp. strain Da161, DIC, z-stacking. A–I – Locomotive forms; K – cell slightly changes the direction of movement; L and M – slowly moving locomotive forms; N and O – cells suddenly change its direction of movement; P – cell after start locomotion; Q – wrinkled posterior end; R – floating forms; S – stationary form. *Abbreviations*: cv – contractile vacuole; dr – dorsal ridge; fv – food vacuole; ll – lateral lobe; nu – nucleolus; white arrowhead shows the direction of pseudopodia growth. Scale bars: 20 μm.

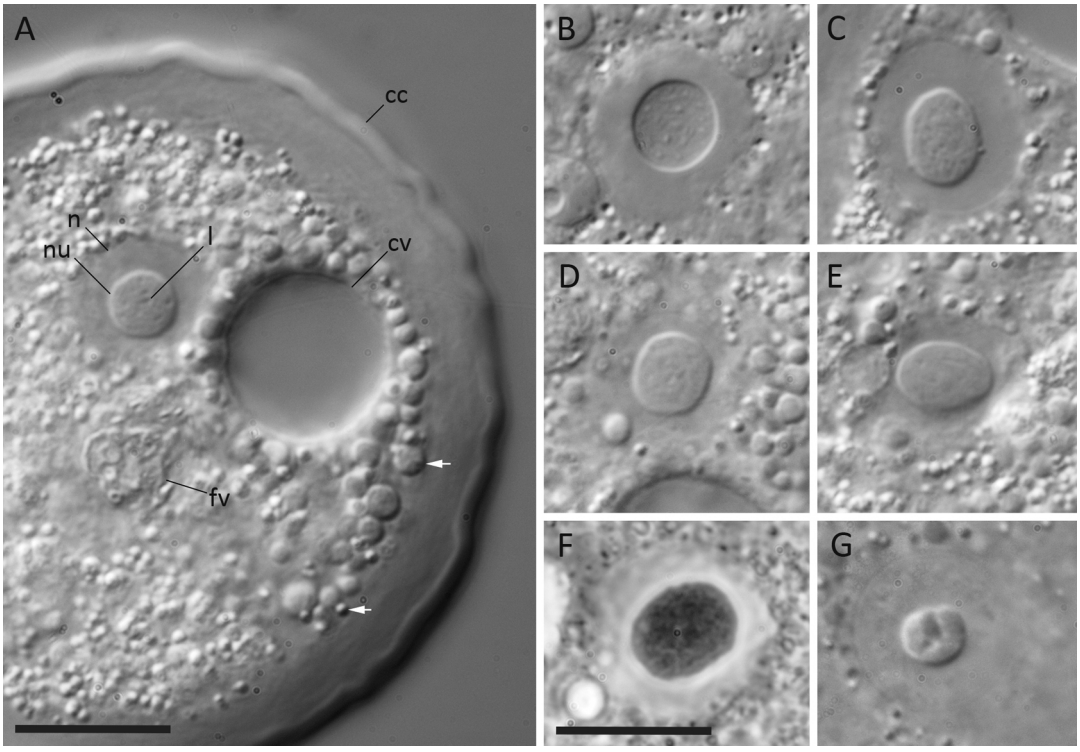

Fig. 2. Light microscopy of cytoplasm of *Dermamoeba fibula* n. sp. strain Da161, DIC. A – Higher magnification of the cell showing granuloplasm, nucleus, and cytoplasmic inclusions; B–F – nuclei of *D. fibula*; G – unusual nucleus of *D. fibula*. *Abbreviations*: cc – cell coat; cv – contractile vacuole; fv – food vacuole; l – lacuna; n – nucleus; nu – nucleolus; white arrow – spherical bodies in cytoplasm. Scale bar: 10 μm.

However, sometimes amoebae formed pronounced dorsal folds during locomotion (Fig. 1, C–E). The number of folds did not exceed three; most often, only one ridge was observed. The ridges began at the posterior end of the cell and generally continued to the middle part of the amoeba, but the largest ridges sometimes extended to the frontal hyaline region (Fig. 1, C–E). The stationary form had a shape close to a hemisphere (Fig. 1, S); the surface of the stationary form was wrinkled. The contractile vacuole as well as the nucleus of a stationary cell often was located near the apex of the hemisphere. In a liquid medium, amoebae often started floating (Fig. 1, R). The floating form was compact, rounded, with smooth short outgrowth consisting of the hyaloplasm.

The anterior part of the moving cell consisted of the hyaloplasm, which occupied up to 1/5 of the total length of the cell. The hyaloplasm extended along the sides of the cell, forming a lateral hyaline edge. Often this edge reached the posterior end of the cell (Fig. 1, A–K, P). The single nucleus in a locomotive cell was usually located at the posterior half (Fig. 1, B, D–G, I, N–P). Nucleus had a spherical or slightly elongated shape (Fig. 2, A–G), its length (measurement in maximal dimension) varied from 6.5 to 11 μm (mean 8.6 μm, n = 35), the breadth was 5–9 μm (mean 7 μm). The outlines of the nucleolus varied from spherical (Fig. 2, A, B, and D) to slightly oval (Fig. 2, C, E, F). The size of the nucleolus in maximal dimension was 4.3–6.5 μm (mean 5.3 μm). Nucleolus always contained numerous lacunae (Fig. 2, A–G). In one cell, we observed a nucleus containing unusually small nucleolus consisting of several closely apposed parts (Fig. 2, G). Except the nucleus, the granuloplasm contained spherical granules of different sizes. The largest ones reached 2 μm in maximal dimension, those were food vacuoles and a contractile vacuole, which was usually located near the posterior end of the cell. The surface of the amoeba was covered with a relatively thick layer of the cell coat, well visible under the light microscope (Fig. 2, A). In our cultures, amoebae of the strain Da161 did not form cysts.

Transmission electron microscopy

The plasma membrane of the cell was covered with a multilayered glycocalyx approximately 430 nm in thickness (Fig. 3, A–B). The main part of the glycocalyx was a layer composed of electron-dense spindle-shaped structures located parallel to the plasma membrane. The space between this layer and the plasma membrane was filled with fibrillar structures located perpendicular to the plasma membrane. Additionally, in this space we have seen small spherical or teardrop-shaped protrusions of the plasma membrane measuring about 50 nm in height (Fig. 3, B). The number of these protrusions varied in different parts of the cell. Under the main layer of the glycocalyx, there were separate clusters of thread-like membrane protrusions (Fig. 3, C), which were noticeably thinner and longer than the spherical protrusions. Above the main layer of the membrane, there was a spotted layer of lumpy material. The upper layer of the glycocalyx consisted of a more homogeneous fibrillar material. Above the top layer, there were individual light clumps of amorphous material. Bundles of thin filaments resembling actin microfilaments in appearance were seen in various areas of the cell, primarily at the cell periphery. Dictyosomes of the Golgi complex were not numerous and were represented by the stacks of 6–8 cisterns (Fig. 3, D). Many mitochondria were spherical or ovoid in our sections with cristae of the tubular type and electron-dense matrix (Fig. 3, E). In some mitochondria, all cristae were located predominantly parallel to each other. The cytoplasm lacked morphologically distinguishable MTOCs and obvious microtubules. In addition, there were numerous structures in the cytoplasm, rounded or elongated in outline (Fig. 3, A, D, E, G, and H). These structures had an electron-dense, clear boundary, underlain by a loose material. In the central part of these structures, there was a dark area, fenced off from the outer contour by a brighter space (Fig. 3, H). Vacuoles containing one or several bacteria were frequently seen in the cytoplasm (Fig. 3, F and G). These vacuoles were often surrounded by a layer of small spherical bodies resembling the endoplasmic reticulum with ribosomes.

In our sections, the amoeba nuclei usually had irregularly rounded or oblong outlines (Fig. 4, A and B). The karyoplasm was not homogeneous, containing areas of varying electron density and numerous small patches of dense granular material. The inner nuclear lamina was not found. Inside the nucleus, there was a rounded body of complex structure, consisting of electron-dense material. It was located centrally or somewhat eccentrically. This body can be interpreted as a nucleolus visible at the light microscopic level (Fig. 2, A–E and Fig. 4, C). In our sections, it had irregularly oval outlines. Its internal structure was quite complex and heterogeneous. It consisted of areas of granular material surrounded by clusters of more electron-dense material. Those areas were separated by a complex network of channels filled with karyoplasm. In some areas, the channels were noticeably widened and formed gaps.

Molecular phylogenetic analysis

The 18S rRNA gene sequence of the strain Da161, 3004 bp long, was obtained. This length corresponded to a full-length molecule from the 1st to 45th helices (Petrov et al., 2014). The main structural difference in the 18S rRNA gene between the strain Da161 and the closest relative, the species *D. algensis*, was observed in the 21st helix, which roughly corresponded to a V4 region. In addition, the strain Da161 possessed a unique insertion in the conservative region, located in the helix 18, positions 571–1511 bp. Direct comparison of the Da161 strain sequence excluding the insertion with the *D. algensis* sequence showed a level of the sequence identity of 95.44%.

Phylogenetic analysis based on 18S rRNA gene sequences indicated that the closest relative of the strain Da161 is the species *D. algensis*. These two species form a fully supported clade (Fig. 5). The sister group to them is the clade formed by two species of the genus *Paradermamoeba*. More distant clade contained *Coronamoeba* and *Mycamoeba*; next was the clade consisting of *Mayorella* sequences. Other groups in the tree were traditional for Discosea phylogeny (e.g., Smirnov et al., 2020; Kudryavtsev et al., 2022).

Discussion

Strain "Da161" represents a new species of *Dermamoeba*

The amoebae of the strain Da161 do not form a distinct pseudopodia or subpseudopodia; the outlines of locomotive cells are smooth and elon-

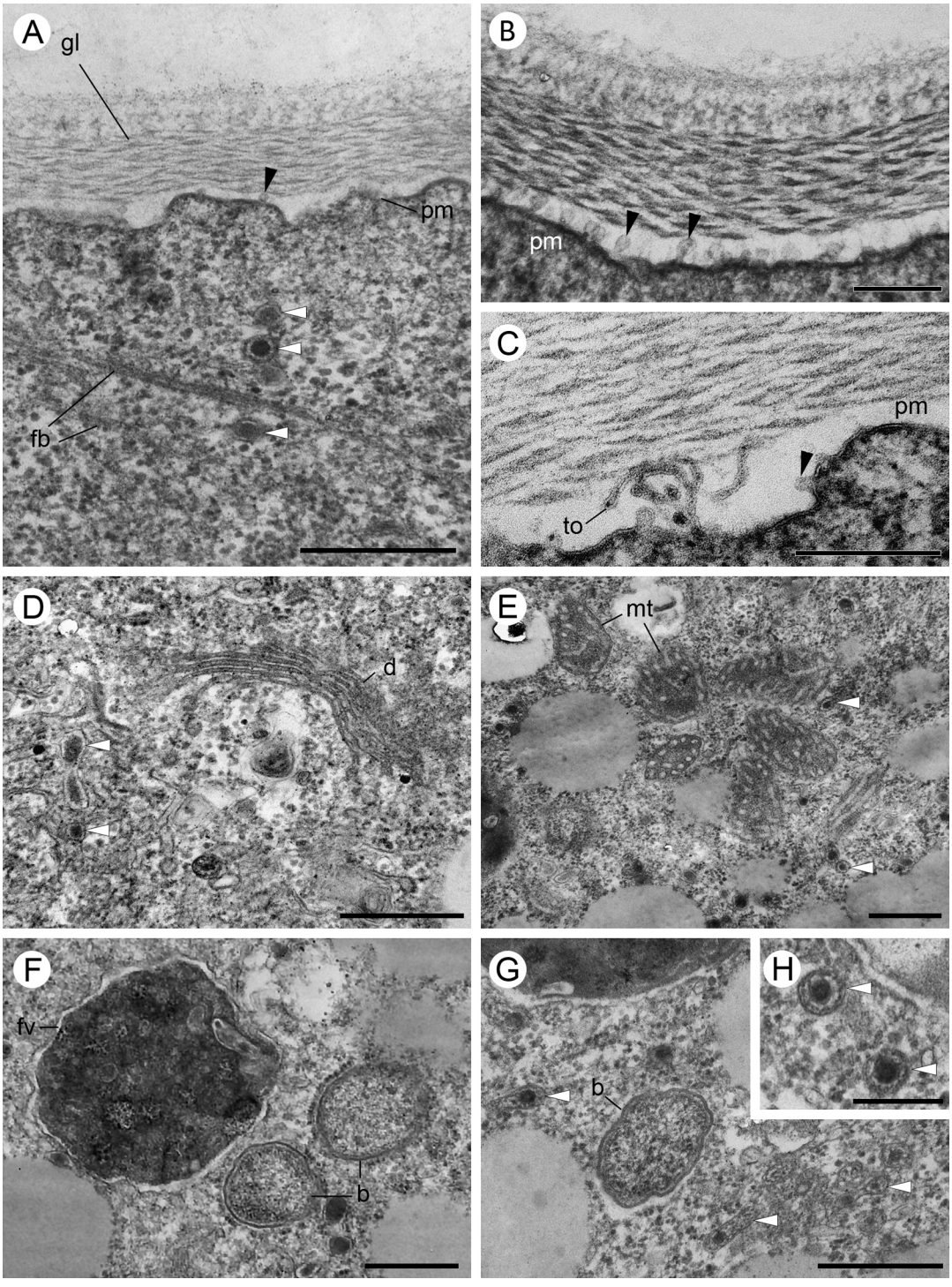

Fig. 3. General ultrastructure of *Dermamoeba fibula* n. sp. strain Da161, TEM. A – General view of the peripheral cytoplasm area and cell coat; B – higher magnification of the cell coat; C – the main layer of the glycocalyx and the membrane outgrowths under it; D – area of cytoplasm containing dictyosomes of the Golgi complex; E – the fragment of the cell with mitochondria; F and G – part of amoeba cell containing food vacuole and single bacteria; H – higher magnification of the spherical cytoplasmic bodies. *Abbreviations*: b – bacteria; d – dictyosomes of the Golgi complex; fb – bundles of microfilament; gl – glycocalyx; mt – mitochondria; pm – plasma membrane; to – thread-like membrane protrusions; black arrowhead – spherical membrane protrusion; white arrowhead – spherical cytoplasmic body. Scale bars: A, D–G – 500 nm; B, C, and H – 200 nm.

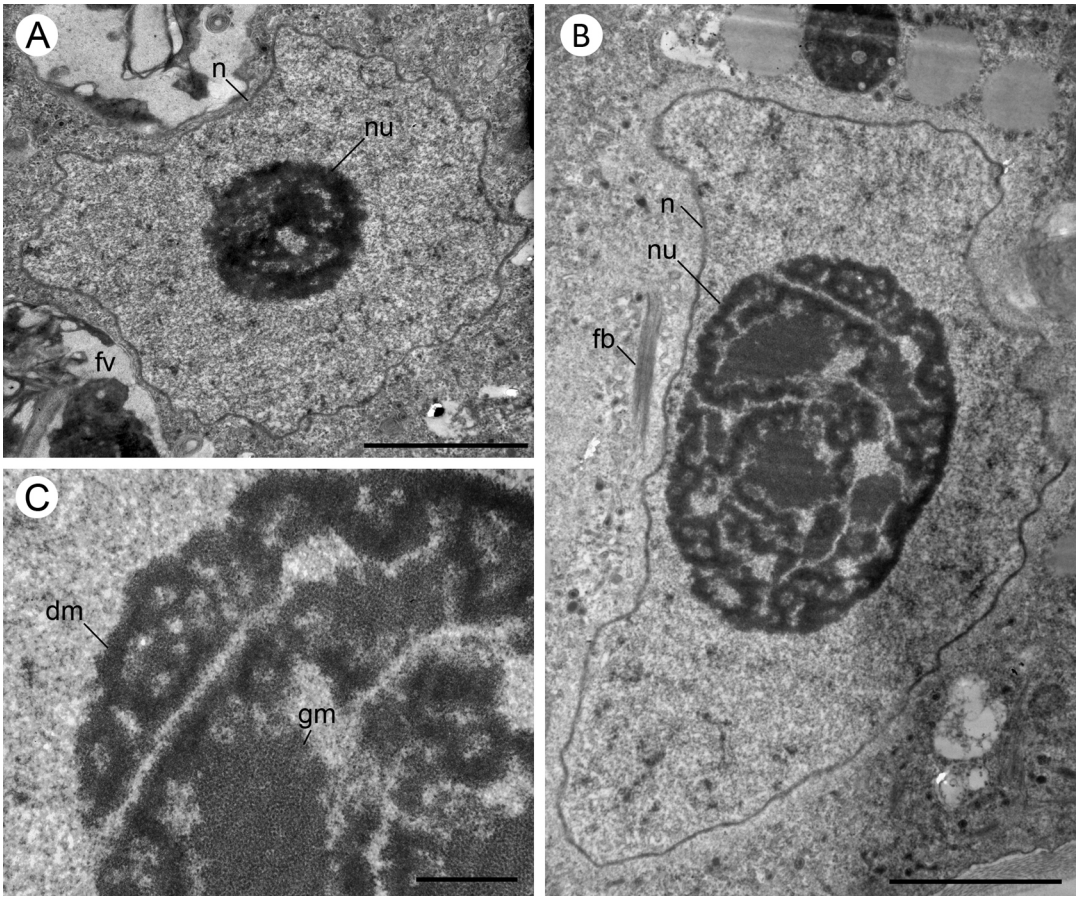

Fig. 4. Ultrastructure of nucleus of *Dermamoeba fibula* n. sp. strain Da161, TEM. A and B – Cross-section through the nucleus of the amoeba; C – higher magnification of the nucleolus. *Abbreviations*: dm – dense material, fb – microfilamentous bundles; fv – food vacuole; gm – granular material; n – nucleus; nu – nucleolus. Scale bars: A and B – 2 μm, C – 500 nm.

gated in the direction of locomotion. The dorsal surface of the cell is usually smooth; sometimes a cell forms inconstant longitudinal ridges. Based on these morphological features, these amoebae certainly belong to lingulate morphotype (Smirnov and Goodkov, 1999; Smirnov and Brown, 2004). The morphotype and presence of the thick multilayer glycocalyx revealed by TEM studies allow us to identify the strain Da161 as a representative of the genus *Dermamoeba*.

Among the three known species of the genus *Dermamoeba*, the species *D. minor* is much smaller than the present isolate (max. length is 50 μm, with an average of 41 μm). The nucleus of *D. granifera* contains two closely apposed endosomes (Page and Blakey, 1979; Page, 1988), which is not a characteristic feature of the present isolate. Moreover, Page (1988) mentioned that the cytoplasm of *D. granifera* was always filled with numerous yellowish or brownish spheres, probably – lipid globules. There is nothing like this in the cytoplasm of the strain Da161. Amoebae belonging to the species *D. algensis* are usually larger than the amoebae of the strain Da161 (*D. algensis*: 50–100 μm, mean 75 μm in length, vs Da161: 36–71 μm, mean 54.8 μm). In contrast, the nuclei in Da161 strain are much larger than the nuclei of *D. algensis* (Da161: 6.5–11 μm, mean 8.6 μm, vs *D. algensis*: 2.9–5.7 μm, mean 4.7 μm).

Molecular data and phylogenetic analysis revealed the relationship between strain Da161 and *D. algensis*. These amoebae have differences in the 18S rRNA gene sequence, sufficient to recognize strain Da161 as a separate species. In addition to differences in homologous regions, the 18S rRNA gene of strain Da161 has a long insertion of 941 bp,

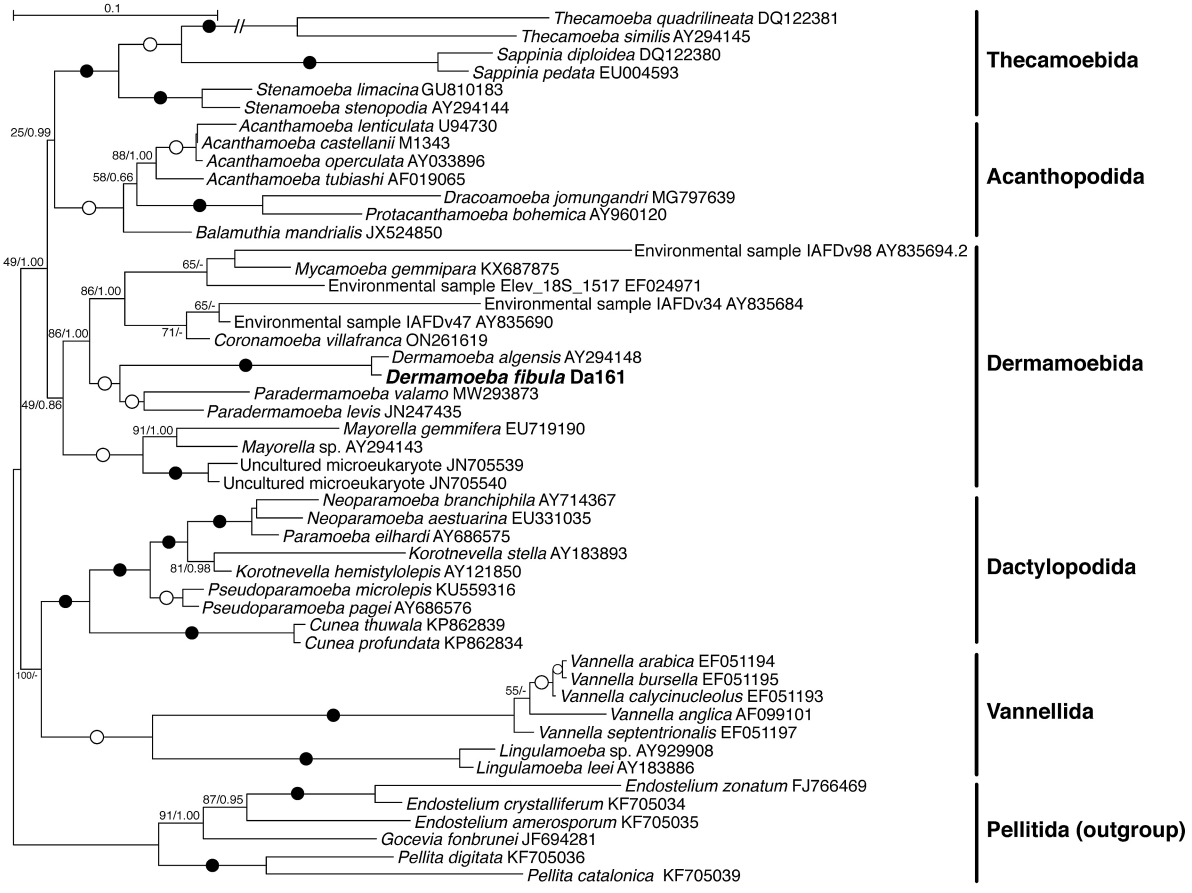

Fig. 5. Molecular phylogenetic tree based on 18S rRNA gene sequences of Dermamoebida and a number of discosean sequences used to form a proper set of outgroups. 1560 sites used in the analysis. Node supports indicated as BS/PP values; black circles mark fully supported nodes (100/1.0 support); white circles mark highly supported nodes (BS > 95 and PP > 0.95).

which is not known in any other Dermamoebida species. This insertion is located in a helix 18 conservative region. It is corresponding to a S516 group I intron, which was described for fungi and protists, including myxomycetes (Amoebozoa) (Haugen et al., 2003).

Dermamoeba fibula n. sp.

Diagnosis: Amoebae of lingulate morphotype; sometimes form thin lateral lobes; may form from one to three temporary longitudinal ridges on the dorsal surface. Length in locomotion 36–71 μm; breadth 24–45 μm; length/breadth ratio 1.3–2.3. Single vesicular nucleus, rounded or slightly elongated, 6.5–11 μm in maximal dimension with the single central nucleolus 4.3–6.5 μm in diameter.

Type material: The type slide (Heidenhain's iron hematoxylin-stained permanent preparation) is deposited with the collection of slides of the Laboratory of Unicellular organisms, Institute of Cytology RAS, under the No 1071. The sequence of the 18S rRNA gene of this strain is deposited with GenBank under the accession number OR671173.

Type location: leaf litter near Kremiotis waterfall, Kritou Terra, Cyprus (34°57'45.1"N 32°26'00.8"E).

Etymology: "*fibula*" is a type of brooch clothes fasteners. An amoeba with one longitudinal ridge is similar to the penannular brooch.

Differences from closely related species: *Dermamoeba fibula* has a smaller locomotive form compared to *D. algensis*. In addition, *Dermamoeba fibula* has larger nuclei. There are also differences in the 18S rRNA gene sequence.

ZooBank registration: 6449C878-9A50-43C1-9484-3C637EE4E2D2

Present work: C7A69ED4-A7C1-4E78-B49B-75427C5A8519

Ultrastructure of *Dermamoeba fibula*. At the ultrastructural level, amoebae of the strain Da161 have a number of tiny differences from other species. Information about the ultrastructure of the species *D. granifera* is limited only to images of the cell coat, which is similar to the glycocalyx of the studied strain (Page and Blakey, 1979). *Dermamoeba algensis* has clusters of electron-dense oval structures in the space between the main layer of the glycocalyx and the plasma membrane (Smirnov et al., 2011a). In *D. fibula*, only spherical or thread-like protrusions of the membrane were found in this space. The size, shape and density of these protrusions never closely resembled clusters of dark oval structures, so we cannot suggest their homology. In the cytoplasm, amoebae of the species *D. fibula* have numerous rounded bodies with a dark central zone. No such structures were described in *D. algensis*. However, in the published images, similar bodies can be found at the site of food vacuole formation (Smirnov et al., 2011a, Fig. 23). It can be assumed that such bodies are unique to *Dermamoeba*, since nothing similar was found even in cells of the closest relative of *Dermamoeba* – the genus *Paradermamoeba* (Smirnov and Goodkov, 2004; Kamyshatskaya and Smirnov, 2016). In contrast, the mysterious trichocyst-like bodies, shared by all studied *Paradermamoeba* species, were not seen in any *Dermamoeba* species (as well as in no other amoebae species in general). These bodies are likely unique inclusions in the cells of *Paradermamoeba*.

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