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Molecular phylogeny of *Paradermamoeba valamo* (Amoebozoa, Discosea, Dermamoebida)

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Summary

A remarkable amoeba species – *Paradermamoeba valamo* Smirnov et Goodkov,1993 was re-isolated from the bottom sediment in Izmailovo Park pond (Moscow, Russia). For the first time, we obtained modern DIC images of this species. High-quality light-microscopy allowed us to resolve some subcellular structures, including trichocyst-like bodies, and show congruence of the present optical images with the results of earlier electron-microscopic studies. We applied single-cell PCR to obtain full sequence of the 18S rRNA gene of this species. The analysis of genetic distances, as well as the result of phylogenetic analysis, shows that *P. valamo* is a valid species, related to *P. levis* but showing clear morphological and molecular differences.

Key words: Amoebozoa, Discosea, morphology, phylogeny, SSU gene, light microscopy

Introduction

An amoebae genus *Paradermamoeba* was established by Smirnov and Goodkov (1993) to accommodate *P. valamo* – a remarkable species of flattened, lanceolate amoebae possessing thick cell coat consisting of spiral glycostyles. One year later, another species of this genus, *P. levis* was described (Smirnov and Goodkov, 1994). This organism, being similar in appearance to *P. valamo*, was smaller in size and had almost twice thinner cell coat, hence of the same structure. At the time of description, both these species were found to be difficult objects for TEM studies, so only the images of the cell coat were published in both above-cited paper, also this was sufficient for morphological species distinction. The comprehensive ultrastructure of *P. valamo* and *P. levis* was published ten years later

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(Smirnov and Goodkov, 2004). This publication highlighted differences in the ultrastructure of the nucleus between these two species and confirmed their status of individual species. The same paper showed that while *P. levis* was found to be widely distributed in Europe and Asia, the only confirmed finding of *P. valamo* remained the original finding at Valamo Island (North-West Russia). Mrva (2005; 2006) listed *P. valamo* (as well as *P. levis*) among species recovered in Slovakia (from mosses of the Malé Karpaty and rainwater pool in Bratislava, respectively), but the taxonomic identification was based only on the light-microscopic morphology. The SSU sequence of *P. levis* was obtained by Smirnov et al. (2011) and the phylogenetic analysis performed in this study resulted in the establishment of the order Dermamoebida, unifying the genera *Mayorella, Paradermamoeba* and *Dermamoeba*. High-quality light microscopic images of *P. levis* and further data on its ultrastructure became available from Kamyshatskaya et al. (2016), while the re-isolation of *P. valamo* was found to be a problem. This species appeared in the area of our attention several times, but we were never successful in culturing it. In the year 2020, we isolated *P. valamo* from a sample, collected in Izmailovo Park pond (Moscow), and managed to obtain SSU sequence of this species. The results of phylogenetic analysis and the first high-quality DIC images of this species are reported in the present paper.

Material and methods

The strain of *Paradermamoeba valamo* designated as strain MSK1, which is the subject of the present paper was isolated from the sample, containing material from the top 5 cm layer of the sandy bottom sediments of a freshwater pond in Izmailovo park, Moscow, Russia (55°46'46.8"N, 37°46'09.2"E). Amoebae were found in the dish, inoculated with the sampled sediments diluted 1:1000 with 0.25% WG infusion (see Geisen et al., 2014 for protocol), made on PJ medium (Prescott and James, 1955). After two weeks of incubation, the initial culture contained about a hundred of *P. valamo* cells. Species identification was performed basing on the unique locomotive morphology, nuclear structure and size data on this species.

All attempts to maintain or to clone culture failed, so observations and study were done in initial enrichment culture. All cells in this culture clearly belong to the same species (further molecular studies confirmed this). Live cells in culture were observed using Leica M205C dissection microscope equipped with Rottermann contrast and Leica DMI3000 inverted microscope equipped with phase-contrast and IMC (Integrated Modulation Contrast) optics. Cells were studied, measured and photographed on the glass object slides using Leica DM2500 microscope equipped with DIC optics on PL-fluotar and plan-apochromatic lenses. Amoebae were photographed and video-recorded using DS-Fi3 Nikon camera powered by NisElements – AR software (Nikon).

For DNA isolation, individual cells were transferred in sterile 40 mm Petri dishes containing a layer of NN agar (see Page, 1988) covered with Millipore-filtered (0.22 μm) PJ medium. Cells were left to starve for three days, every day they were transferred to the fresh dish with sterile medium. To isolate DNA, individual amoeba cells were collected manually with the tapered-end Pasteur pipette, washed in two subsequent changes of the same medium and placed with 1–2 μl of the medium in a 200 μl PCR tube. DNA was extracted using the Arcturus PicoPure DNA Extraction Kit (Thermo Fischer Scientific, USA). The extraction mixture was prepared according to the manufacturer's instructions; 12 μl of the mixture was added to the tube containing the single cell.

The SSU sequence was obtained in two ways. First, PCR amplification was performed using RibA (forward, 5'> ac ctg gtt gat cct dcc agt <3') and reverse S12.2R (5'> gac tac gac ggt atc tra tc <3') primers (Pawlowski, 2000). PCR products were sequenced directly, without purification, using the Big Dye Terminator Cycle sequencing kit and an ABI PRISM automatic sequencer using the same primers. In total, 12 different cells were treated and sequenced, one sequence was found to belong to a contaminant, while all other were almost identical. The consensus sequence was assembled using CodoneCode software (https://www.codoncode.com) on the base of six sequences showing the best trace quality.

The second way included the whole genome amplification followed by NGS sequencing. We performed the Multiple Displacement Amplification (MDA) of the DNA from the sample showing good trace quality in Sanger sequencing (cell #2), using the REPLI-g Single Cell DNA Amplification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The resulting MDA

products were sequenced, using Illumina HiSeq 2500 system at the core facility center "Development of Molecular and Cell Technologies" of the SPSU Research Park according to the manufacturer's protocols; as a result, 20M paired reads with the length about 150 bp were obtained. Quality control check of raw sequence data was performed, using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). SPAdes assembler was used for de novo genome assembly (Bankevich et al. 2012). The contig containing the SSU rRNA gene fragment was identified, using BLAST (Altschul et al. 1990) and the sequence fragment of this isolate obtained by Sanger sequencing as a query.

The alignment constructed for this study was based on one used by Glotova et al. (2018) for *Mayorella* analysis. It contained all named culture-derived 18S rRNA gene sequences of Dermamoebida, two full-length sequences belonging to unnamed strains of *Mayorella* and a number of discosean sequences used to form a proper set of outgroups. Other available *Mayorella* sequences were not used because of their short length. Sequences were aligned using the Muscle algorithm as implemented in SeaView 4.0 (Gouy et al., 2010); the alignment was further polished manually. The phylogenetic analysis was performed using maximum likelihood method as implemented in PhyML program (Guindon and Gascuel, 2003) with GTR (eight rate categories) + γ + I model; 1651 sites were selected for the analysis. The initial selection was made using the g-blocks algorithm as implemented in SeaView 4.0, further the set was manually polished. The number of invariant sites, alpha parameter, and tree topology was optimized by PhyML. The program generated 25 random starting trees; the best tree was further optimized. To test the stability of branching, 1000 bootstrap pseudoreplicates were used. Bayesian analysis of the same dataset was performed using MrBayes 3.2.7, 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 ceased 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. MrBayes was run at Cipres V.3.3 website (Miller et al., 2010). Sequence identity level was calculated using an online tool "Ident and Sim" provided at http://www.bioinformatics.org/sms2/ident_sim.html website.

The obtained sequence was deposited with the GenBank under the number MW293873 (*Paradermamoeba valamo* strain MSK1, length 2096 bp).

Results and discussion

Light-microscopic observations made in culture, as well as those made on the surface of the object slide show almost identical cell morphology and locomotive behaviour. Amoebae were flattened and oblong in locomotion. They demonstrated lanceolate morphotype (Smirnov and Goodkov, 1999; Smirnov and Brown, 2004) and show clear lateral flatness in moving cells, characteristic to this species (Smirnov and Goodkov, 1993, 2004) (Fig. 1, A-E). Rapidly moving cells could be more flattened and often adopted oblong, lingulate shape without prominent lateral flatness (Fig. 1, F). When the cell moved more slowly and appeared to be "uncertain" with the direction of locomotion, it could temporarily form short prominent folds and wrinkles (Fig. 1, G-H). These structures were never stable and could be observed for a short time, often counted by seconds. When the cell moved slower, without the single, clear direction of movement it could form short triangular pseudopodia (Fig. 1, I-J). Amoebae like this resembled small representatives of the genus *Mayorella* and could be even missed with them, especially in mixed culture. However, much thicker cell coat of *Paradermamoeba* helped to distinguish these two genera.

We have not observed a differentiated floating form in our strain; few amoebae cells detached from the substrate only for a short time, and soon settled down and continue the movement. This might be a physiological thing because to get proper locomotive forms we worked with actively growing culture while floating forms are usually observed in older cultures, which are on the stationary phase.

The cell coat of *P. valamo* was well visible with DIC microscope as a thick line bordering the cell (Fig. 1, K). The nucleus was of the vesicular type and had a central nucleolus showing non-homogenous structure (Fig. 1, L). In the best DIC images, it was possible to see the "cords" of nucleolar material and eccentrically located homogeneous area looking as a lacuna inside the nucleolus. The observed

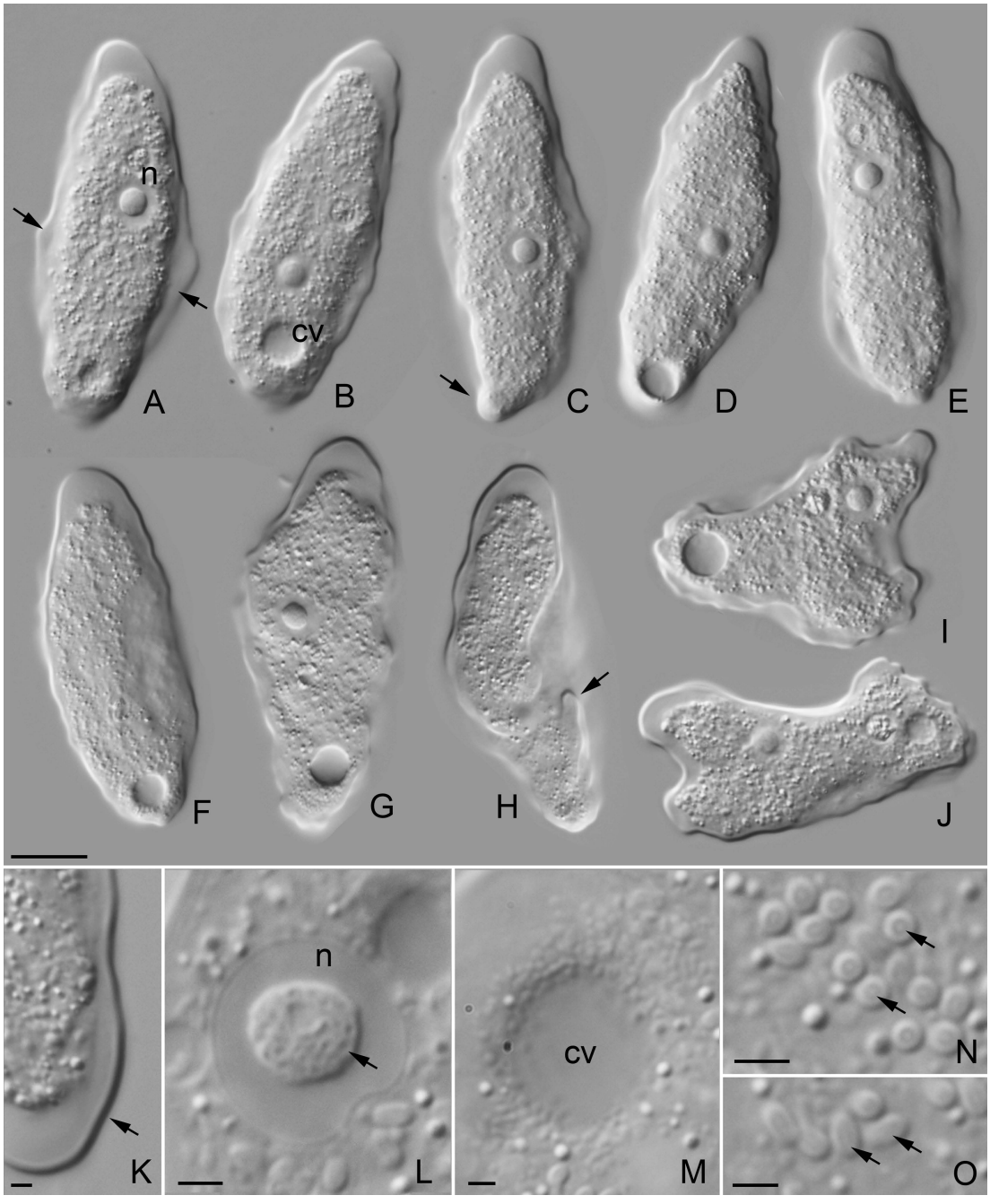

Fig. 1. Light-microscopic morphology of *Paradermamoeba valamo* strain MSK1. A-E – The most typical lanceolate locomotive form of this species with pronounced lateral flatness (arrowed in A); the nucleus (n) is usually located in the central part of the cell; the contractile vacuole (cv) is situated in the posterior part of the cell (B) or right in the uroid (D); uroidal structure of bulbous type is arrowed in C; F – flattened, elongate locomotive cell without pronounced lateral flatness; G-H –irregularly moving cell, showing temporary lateral folds and short ridges (arrowed in H); I-J – cells in non-directed locomotion, showing short triangular pseudopodia (amoebae like this may even be missed with *Mayorella*, especially in mixed culture); K – thick cell coat, forming a well-visible line in DIC images; L – nucleus (n) with central nucleolus (arrowed), consisting of tightly pack cords of nucleolar material (an eccentrically located lacuna is visible in the nucleolus); M – contractile vacuole (cv), surrounded by a vesicular spongiome; N-O – "trichocyst-like" bodies in transversal and longitudinal sections (the central axial core is arrowed in both images). Scale bars: A-J – 10 μm, K-O – 1 μm.

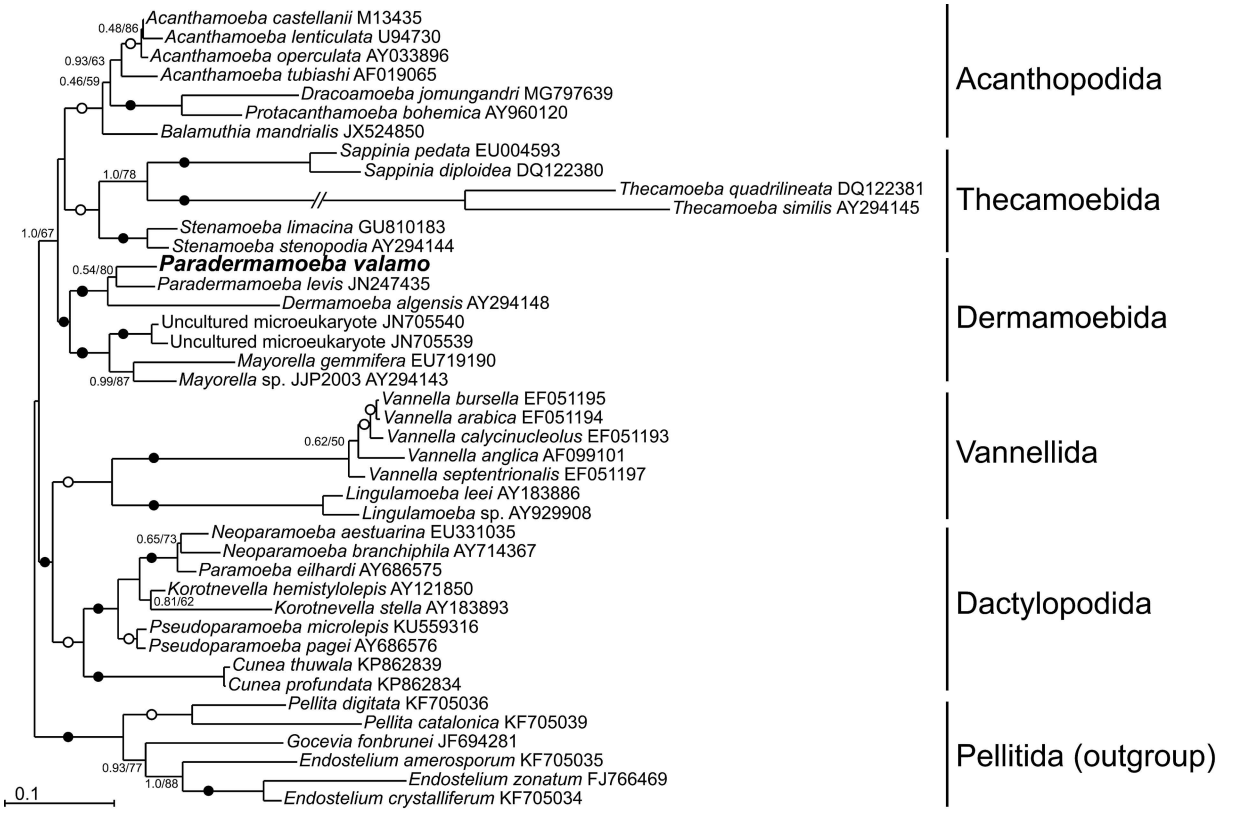

Fig. 2. Phylogenetic tree based on the 18S rRNA gene, showing the position of *Paradermamoeba valamo*. 1651 sites are used for the analysis; GTR + γ + I was used for ML analysis and GTR + γ with covarion – for Bayesian analysis. Labelling of nodes: PP/ML support. Black-filled circles are used to label fully supported nodes (1.0/100 support); white-filled circles are used to recognize highly supported nodes (PP > 0.95 and BS > 95).

structure is congruent with the TEM data obtained by Smirnov and Goodkov (2004); it is possible to suggest that the "lacuna" observed in DIC images (which could look as elevation depending on the side of the interference spectrum selected with the upper DIC prism) correspond to the central body of the nucleolar material visible with TEM in this species (op. cit.). No trace of the nuclear lamina was seen in DIC optics, this is congruent with TEM data as well.

The contractile vacuole was surrounded with numerous small bodies (0.3-0.75 μm across), rounded or oblong, which probably represented mitochondria, located around the vacuole.

DIC microscopy allowed us to observe mysterious trichocyst-like bodies mentioned in all studied strains of *P. valamo* and *P. levis* (Smirnov and Goodkov, 1993, 1994, 2004; Kamyshatskaya et al., 2016). These bodies were oblong in longitudinal section (0.75-1 μm in length) and circular in cross-section, ca 0.5 μm across. The central core penetrating the body was distinguishable in the best DIC photographs (Fig. 1, N-O). This observation confirms the structure of these bodies, earlier revealed by TEM and further show that the presence of these structures is characteristic for the entire genus *Paradermamoeba*. They are still not known in any other organism outside this genus.

Molecular phylogeny groups the present sequence with that of *P. levis* within fully supported Dermamoebida (Fig. 2). The nearest neighbour to the genus *Paradermamoeba* in our tree was the genus *Dermamoeba*, outgroup to this assemblage was formed by sequences of amoebae belonging to the genus *Mayorella*. All other groupings in the tree reflected usual molecular relationships between amoebae genera within the Discosea clade (e.g. Kudryavtsev et al., 2014; Cavalier-Smith et al., 2016; Udalov et al., 2017, 2020). The artificial grouping of Acanthopodida with Thecamoebida

was caused with the usage of Pellitida as an outgroup clade.

Before the present sequence was obtained, a little doubt on the validity of *P. valamo* as an individual species anyway remained. The major difference between these two species is the size, the thickness of the cell coat and the structure of the nucleus. Each of these characters is believed to be reliable, and in combination, they form a rather clear pattern of differences. However, there are amoebae species, showing a wide size range (Page, 1983, 1988; see for example size data in Page, 1985). For example, the smallest known specimens of *Flabellula baltica* are about three times smaller than the largest ones (Smirnov, 1999; Smirnov et al., 2017). The cell coat in *P. valamo* is twice thicker than in *P. levis*, but the overall structure of the cell coat is almost identical. Differences in the organization of the nucleolus are significant, but there are cases when the details of the nuclear structure in amoebae vary depending on the stage of the cell cycle or the fixation procedure used (e.g. Demin et al., 2016; Mesentsev et al., 2020). However, the present study dispels the last doubts. The level of identity between sequences of *P. valamo* and *P. levis* on the compared fragment shared by both sequences (1785 bp) was only 78.61% which is a rather low value. For comparison, the SSU sequence identity level among species of the genus *Stygamoeba* varies in the range from 83 to 85% (Lotonin and Smirnov, 2020). The identity between sequences of *Thecamoeba quadrilineata* and *T. cosmophorea* is lower and reaches only 70% (Mesentsev and Smirnov, 2019). However, the level of sequence identity between morphologically different *Vannella* species may be as high as 0.97 or even 0.99, and anyway, these species have clear morphological differences (Smirnov et al., 2016). So, the observed value is in the range, common for a reliable, well-differing amoebae species.

The present study indicates that *Paradermamoeba valamo* is a valid species, showing a clear difference from *P. levis* at the morphological level and well distant from it at the molecular level. The sequence of its SSU gene will facilitate reliable identification of this species at the organismal level as well as its recovery among the results of environmental DNA sequencing. This, in turn, should improve our knowledge on the geographic distribution of this amoeba species. DIC observation made with the top-quality optical system with the resolution close to the theoretical limit for conventional light microscopy shows perfect congruence with earlier LM and TEM data on this species.

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