Protistology 18 (1): 40–51 (2024) | doi:10.21685/1680-0826-2024-18-1-4

Protistology

ORIGINAL ARTICLE

New data on *Leptomyxa neglecta* (Amoebozoa, Tubulinea, Leptomyxida)

Nikita S. Kulishkin* and Alexey V. Smirnov

Department of Invertebrate Zoology, Faculty of Biology, St. Petersburg University, 199034 St. Petersburg, Russia

| Submitted February 13, 2024 | Accepted March 23, 2024 |

Summary

The species *Leptomyxa neglecta* (Smirnov, Nassonova, Fahrni and Pawlowski, 2009) Smirnov et al., 2017 (Amoebozoa, Tubulinea, Leptomyxida) was described from the bottom sediment of the Lake Leshevoe (Valamo Island, Northwestern Russia), based on the material collected in the year 1998. The description, published in 2009, was illustrated with phase-contrast microphotographs made by film camera. In 2019, we re-isolated this species from the Lake Nikonovskoe (Valamo Island), located in one kilometer from the type habitat. The amoebae of the studied strain called LVal were identified as *L. neglecta* by the congruence of basic morphological characters and complete sequence identity of the 18S rRNA gene sequence. We obtained high-quality DIC and IMC images of this species. New data on the possible shapes of the floating cell, morphology and behavior of flattened amoeba and on the structure of the nucleolus are presented. Our study shows that *L. neglecta* is capable of locomotion in a flabellate form and fusion of pseudopodia, but without the formation of fenestrated, reticulate cells.

Key words: Amoebozoa, Tubulinea, Leptomyxida, *Leptomyxa neglecta*

Introduction

The genus *Rhizamoeba* was established by Page (1972) for the marine limax amoeba *R. polyura*, possessing a posterior bunch of adhesive uroidal filaments. Later, he described one more species, *R. saxonica*, which had a similar morphology when moved in a monopodial form (Page, 1974). Initially, Page (1972) proposed close relationships of the genus *Rhizamoeba* with the family Hartmannelidae, based on the shared locomotive form. He placed the genus *Rhizamoeba* into the family Hartmannelidae of the order Amoebida (Page, 1976a). However, Pussard and Pons (1976a) suggested closer similarity of *Rhizamoeba* with the representatives of the order Leptomyxida, based on their shared ability to expand into flattened sheets and propensity to produce adhesive uroidal filaments. They proposed to consider the genus *Rhizamoeba* as a member of the family Leptomyxidae. Page (1976b) expressed a similar idea, and transferred more species to the genus *Rhizamoeba*, namely – a species *Leptomyxa flabellata* described by Goodey (1915) and the species described as "*Ripidomyxa australiensis*" by Chakraborty and Pussard (1985), thereby invalidating the genus *Ripidomyxa* (Page, 1988). As a result,

https://doi.org/10.21685/1680-0826-2024-18-1-4

***Corresponding author**: Nikita S. Kulishkin. Department of Invertebrate Zoology, Faculty of Biology, St. Petersburg University, Universitetskaya Emb. 7/9, 199034 St. Petersburg, Russia; **st069336@student.spbu.ru**

all amoebae species with adhesive uroid that tend to move in the monopodial shape and do not form a fenestrated plasmodium were assigned to the genus *Rhizamoeba*.

The monophyly of Leptomyxida was confirmed by molecular studies (Amaral Zettler et al., 2000; Bolivar et al., 2001; Fahrni et al., 2003; Smirnov et al., 2008; Tekle et al., 2008). Further revisions of the Amoebozoa system, based on morphological and molecular data (Cavalier-Smith et al., 2004; Smirnov et al., 2005), have led to the placement of the order Leptomyxida into the class Tubulinea (Smirnov et al., 2005, 2011) and in the Tubulinea lineage in unranked taxonomic schemes by Adl et al. (2005, 2012, 2018). Phylogenetic analysis performed by Smirnov et al. (2017) showed that sequences of *Leptomyxa* and *Rhizamoeba* species do not form isolated clusters. Instead, they are mixed in the tree. Smirnov et al. (2017) suggested that the tendency to adopt a limax-like locomotive form is not crucial for distinguishing these genera. Thus, most of *Rhizamoeba* species were transferred to the genus *Leptomyxa*. The genus *Rhizamoeba* was retained for three species only: *R. polyura*, *R. saxonica* and R. *matisi* (Smirnov et al., 2017).

During the studies of amoebae fauna of Lake Leshevoe (Valamo Island, Lake Ladoga, Northwestern Russia), carried out from 1992 to 2007, A. Smirnov found a monopodial amoeba with adhesive uroidal structures, tending to adopt a characteristic "comet-like" shape and form long pointed pseudopodia while resting. These amoebae were discovered and first documented with line drawings and stained preparations in 1992–1993. They were listed among other amoebae isolated from the bottom sediment of Lake Leshevoe at Valamo Island by Smirnov and Goodkov (1996) as "*Rhizamoeba* sp". In 1998 and 1999, it was re-isolated from the same site and documented with phase-contrast microphotographs made with film camera, and the ultrastructure of this organism was studied. Later, in 2007, it was re-isolated from this location once again, and the 18S rRNA gene sequence of this organism was obtained. Based on the accumulated data, Smirnov et al. (2009) described it as a species *R. neglecta*. This species was later transferred to the genus *Leptomyxa* by Smirnov et al. (2017) based on the phylogenetic analysis.

In the current study, we isolated a strain of leptomyxid amoeba named LVal from a freshwater lake Nikonovskoe at Valamo Island. This lake is located in one kilometer from the lake Leshevoe – the type habitat of *L. neglecta* (Smirnov et al., 2009). Representatives of this strain demonstrate clear morphological similarities with *L. neglecta*. The pairwise comparison of the 18S rRNA gene sequences revealed their complete identity (100%). Based on the obtained data, we identified amoebae of the LVal strain as *L. neglecta*. Here we provide modern light-microscopic images of this species along with a more detailed morphological description and improved data on cell morphology and the structure of its nucleolus.

Material and methods

Sampling and cultivation

Strain LVal was isolated from a sample of bottom sediment collected from Lake Nikonovskoe, Valamo Island, Lake Ladoga, Northwestern Russia (61°36'90.6"N, 30°90'27"E) in June 2019. A small amount of sand and detritus was placed in sterile 60mm Petri dishes filled with WG infusion made in PJ solution (Prescott and James, 1955; Geisen et al., 2014). To establish clonal cultures, single cells were collected using a tapered-tip Pasteur pipette, washed two times in sterile PJ solution and placed in a new 60mm Petri dish filled with fresh WG medium. Amoebae of the LVal strain fed on accompanying bacteria.

Light microscopy

Trophozoites and cysts were studied, photographed and measured on polylysine-coated glass slides using a Leica DM2500 upright microscope equipped with DIC optics and a DS-Fi-3 camera (Nikon, USA). Cultures of cells in 60mm plastic Petri dishes were observed and studied using a Leica DMI3000 inverted microscope with IMC (Integrated Modulation Contrast, Leica) optics and a Leica DFC295 camera powered by Leica Application Suite software (Leica Microsystems).

To make permanent stained preparations, amoebae were collected with a tapered-tip Pasteur pipette, placed on non-coated glass slides and left to adhere. Cells were fixed using Bouin solution and stained with Heidenhain's iron hematoxylin as described by Page (1988). Fixed and stained amoebae on slides were dehydrated in ethanol series followed by isopropanol and xylene and embedded in DPX mounting medium (Sigma-Aldrich, USA).

DNA isolation and amplification

Prior to the extraction of DNA, we washed cells with PJ solution, filtered through a Millipore filter with a pore diameter of 0.2 μm. Total DNA was extracted using the Arcturus PicoPure DNA Extraction Kit (Thermo Fischer Scientific, USA) following the manufacturer's instruction; 10 μl of extraction buffer was added to the tube. The 18S rRNA gene was amplified by PCR using RibA, RibB and s20r primers (Table 1). The thermal cycle parameters were: initial denaturation (five min at 95 °C) followed by 40 cycles of 60 s at 94 °C, 60 s at 60 °C, 150 s at 72 °C, followed by five min at 72 °C for the final elongation. Amplicons were sequenced directly using the ABI-PRISM Big Dye Terminator Cycle Sequencing Kit with the primers listed in Table 1. The consensus sequence was assembled using ChromasPro software (Technelysium) based on sequences with the best trace quality.

Alignment and phylogenetic analysis

The obtained sequence was aligned with the 18S rRNA gene sequences of leptomyxids based on Kulishkin et al. (2023) alignment. Sequences were first aligned automatically using the Muscle 4.0 algorithm (Edgar, 2004) built into SeaView 4.0 (Gouy et al., 2010). The resulting alignment was refined manually. The phylogenetic analysis was performed using the maximum likelihood method implemented in the RAxML program (Stamatakis, 2014) with the GTR + gamma correction model. 1809 sites for the analysis were manually selected; the number of invariant sites, alpha parameter and tree topology were optimized by RAxML; 1000 bootstrap pseudoreplicates were used. The Bayesian analysis was performed using MrBayes 3.2.6 (Ronquist et al., 2012), the GTR model with gamma correction for intersite rate variation (8 rate categories), and the covarion model. Trees were run as two separate chains (default heating parameters) for ten million generations. By that time, convergence had effectively ceased (final average standard deviation of split frequencies was less than 0.01). The first 25% of generations were discarded as burn-in. RAxML and MrBayes programs were run at the Cipres V 3.3 website (Miller et al., 2010). The pairwise comparison of sequences was performed using the "Ident and Sim" tool (Stothard, 2000; https://www.bioinformatics.org/sms2/ident_sim.html). The obtained 18S rRNA gene sequence of *Leptomyxa neglecta* strain LVal was deposited with GenBank under the accession number PP230162.

Table 1. List of amplification and sequencing primers (after Medlin et al., 1988; Pawlowski et al., 1996; Pawlowski, 2000; Fahrni et al., 2003; Adl et al., 2014). RibA, RibB and s20r primers were used for amplification. All listed primers were used for sequencing.

Name	Sequence (5'-3')	Orientation
RibA	ACCTGGTTGATCCTGCCAGT	Forward
RibB	TGATCCTTCTGCAGGTTCACCTAC	Reverse
s20r	GACGGGCGGTGTGTACAA	Reverse
A10S1	CTCAAAGATTAAGCCATGC	Forward
s6f	CNGCGGTAATTCCAGCTC	Forward
s12.2	GATCAGATACCGTCGTAGTC	Forward
s12.2r	GACTACGACGGTATCTGATC	Reverse
s14r	AAGTTTCAGCCTTGCGACCA	Reverse

Results

Light microscopy of *Leptomyxa neglecta* strain LVal

Cells of the LVal strain never acquired monopodial shape on a regular glass slides. Adhered cells became flattened and slowly crawled over the glass, forming pseudopodia with rounded ends in different directions. To study locomotion of the LVal strain we applied polylysine-coated glass slides. On these slides, the cells showed the same variety of shapes as in culture, as well as monopodial locomotive forms actively moving across the slide.

Locomotive cells on polylysine-coated glass slides were monopodial, subcylindrical, with a pronounced hyaline area occupying up to 1/8–1/6 of the cell length (Fig. 1, A-L). Such cells were slightly clavate (Fig. 1, A-B), sometimes elongated or narrowed (Fig. 1, C). Occasionally, cells stopped and formed a new leading pseudopodium at an angle of about 45° to the initial axis of movement. The previous one was retracted into the main cell mass. Thus, such cells appeared tortuous (Fig. 1, D-E). Typically, the formation of a new pseudopodium was preceded by a hyaloplasm eruption, spreading back along one side of the cell in the area of the hyaline cap (Fig. 1, F). At the posterior end, the cells had a naked swollen area (Fig. 1, A-D, F) or a bulbous uroid bearing adhesive filaments (Fig. 1, G). Sometimes, cells formed single adhesive filaments on the lateral surface of the posterior third of the cell

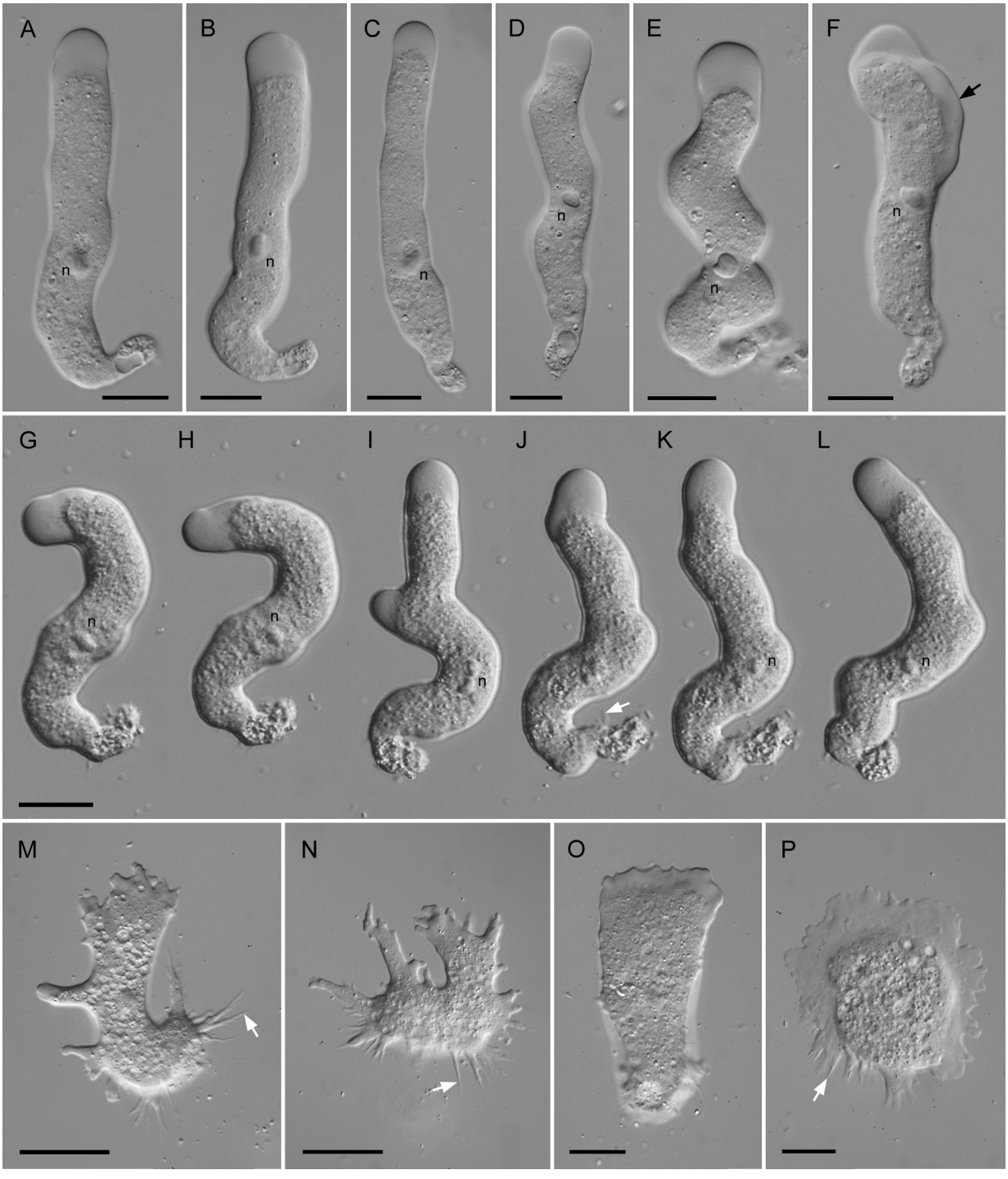

Fig. 1. Light micrographs of moving *Leptomyxa neglecta* strain LVal (DIC). A-C – Monopodial locomotive cell without pronounced uroid; D-E – tortuous monopodial cell; F – the cell showing the eruption for the cytoplasm opposite to the main direction of movement (*arrowed*); G-L – a sequence of shapes of the locomotive cell; M-N – slowly moving, irregular-shaped, flattened cells; O – trapezoidal slowly moving amoeba; P – flabellate slowly moving cell with uneven hyaloplasm border. *Abbreviations*: n – nucleus; white *arrow* – adhesive uroidal filaments. Scale bars: A-L, O-P – 20 μm; M-N – 40 μm.

(Fig. 1, J). The length of the monopodial amoebae ranged from 104 to 147 μm (mean 125 μm, n = 11), and the breadth (in maximal dimension) from 16 to 27 μm (mean 19 μm, n = 11). The L/B ratio was 4-8.

Slow-moving cells were flattened, expanded and branched (Fig. 1, M-N). From the branches, these amoebae formed short rounded or trapezoidal hyaline pseudopodia, elongated in the direction of movement. During the movement, the hyaloplasm often filled the space between them, forming the single anterior hyaline area. Such cells possessed numerous long adhesive filaments on the posterior end. Sometimes, the entire cell had a form of an elongated trapezoid (Fig. 1, O). The base of such trapezoids was a thin hyaline area.

Sometimes we observed flabellate cells (Fig. 1, P). The anterior hyaloplasm of such cells was uneven. It occupied about 1/4 of the total cell length and extended along the lateral sides. In the posterior part, the cell formed adhesive filaments from the lateral region of the hyaloplasm. These amoebae immediately started to shrink when the cells were exposed to the light of the microscope lamp and gradually adopted a branched shape. We also found two closely spaced flabellate cells with a smoother peripheral hyaline zone (Fig. 2, A). Very soon, the hyaline area started to shrink with a formation of numerous adhesive filaments (Fig. 2, B-D). As a result, the semicircular hyaline area was divided into two hyaline lobes that subsequently transformed into the pseudopodia of various shapes (Fig. 2, E).

Once we observed the fusion of two pseudopodia (Fig. 2, F-H). Initially, the cell formed two short, closely spaced pseudopodia, oriented in the same direction (Fig. 2, F). Later, these pseudopodia extended forward and, as a result, acquired an expanded contact area (Fig. 2, G). Further, the space between these two pseudopodia was filled with the hyaloplasm followed by the granuloplasm, and the anterior end of the cells transformed into the hyaline lobe (Fig. 2, H).

On non-coated and polylysine-coated glass slides, the settled cells immediately started to form hyaloplasm eruptions around the perimeter of the cell. For this reason, stationary amoebae were observed and photographed in cultures, on the plastic surface of the Petri dish. Such cells were branched, flattened and had numerous long conical pseudopodia and adhesive filaments along the cell periphery (Fig. 2, I-M). Larger cells were elongated, with few lateral pseudopodia (Fig. 2, I-J). Smaller cells were more compact and had a comet-like (Fig. 2, K) or irregular (Fig. 2, L-M) shape. The length of the elongated conical pseudopodia in smaller cells usually was almost equal to the size of the cell body. The size of resting cells measured 65–334 μm (mean 140 μm, n = 33) in the maximal dimension and 47–198 μm (mean 108 μm, n = 33) in the perpendicular direction.

The floating form was of a radial type, with multiple tapering pseudopodia (Fig. 2, N-O). Almost half of each pseudopodium consisted of the granuloplasm. These pseudopodia were approximately 0.5–1.5 times as long as the diameter of the central part of the floating cell.

Living cells were predominantly mono- or bi-nucleate, but sometimes we observed cells containing up to six nuclei (Fig. 3, F). Nuclei were rounded or ovoid in shape, but usually the nuclear membrane seemed slightly wrinkled (Fig. 3, G-K). The nucleoli were rounded (Fig. 3, G), ovoid (Fig. 3, H) or irregular in shape (Fig. 3, I-K). Sometimes the nucleoli contained small lacunae (Fig. 3, H-I). The maximal dimension of the nucleus varied from 6.9 to 17.5 μm (mean 11 μm, n = 18), and the nucleolus measured from 3.6 to 10.8 μm (mean 6.6 μm, n = 18). Cells had several contractile vacuoles that asynchronously fused into one or two larger vacuoles that later contracted. The cytoplasm also contained digestive vacuoles filled with bacterial cells and numerous small opaque granules. There were no crystals visible.

Cysts were not numerous and were observed only in old cultures (six months of cultivation or more). We simultaneously encountered spherical single- (Fig. 3, M) and double-walled (Fig. 3, N) cysts. In single-walled cysts, the wall thickness was approximately 1–1.5 μm. In double-walled cysts, the outer wall was represented by a slightly wrinkled thin layer, receding from the internal wall of the cyst at a distance of 2–4 μm. The inner cyst wall was thicker (about 0.5 μm) and appeared to be smooth. The nuclei of encysted cells were crumpled and contained nucleoli consisting of 10–12 closely apposed rounded granules of different sizes (Fig. 3, L). The diameter of cysts varied from 28 to 38 μm (mean 31.5 μm, n = 7).

Light microscopy of stained preparations

Smirnov et al. (2009) declared a permanent hematoxylin-stained preparation obtained from a strain isolated in 1993 to be the holotype of "*Rhizamoeba neglecta*". In 1998, he studied and pho-

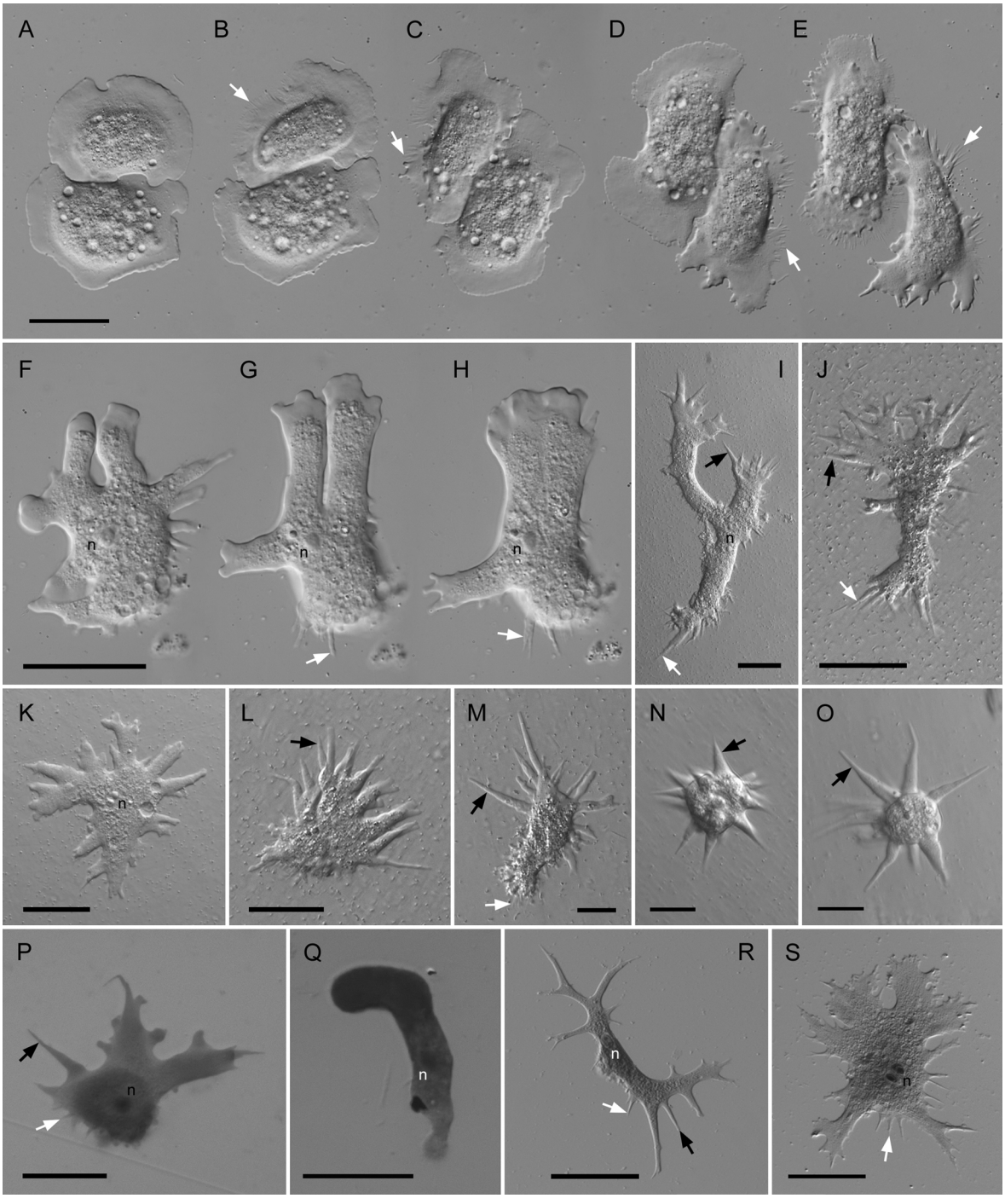

Fig. 2. Light micrographs of *Leptomyxa neglecta* strain LVal (A-O). A-E – Movement of fan-shaped cells with smooth hyaloplasm border, the sequence of images shows occasional contact of two cells; F-H – fusion of two pseudopodia and subsequent formation of the single frontal hyaline border; I-M – flattened, expanded (I-J) and more compact (K-M) cells observed in cultures on plastic surface; N-O – radial floating amoebae with multiple tapering pseudopodia. P-S – Permanent stained preparations of *Leptomyxa neglecta* made by A. Smirnov in 1993 (P-Q) and N. Kulishkin in 2023 (R-S); P – flattened and branched type representative of *L. neglecta* with conical pseudopodia; Q – small monopodial cell with bulbous uroid; R-S – flattened expanded amoebae of different shapes. A-H, R-S: DIC; I-O: IMC; P-Q: BF. *Abbreviations*: n – nucleus; white *arrow* – adhesive uroidal filaments; black *arrow* – conical pseudopodium. Scale bars: A-K, R-S – 50 μm; L-M, P, Q – 20 μm; N-O – 10 μm.

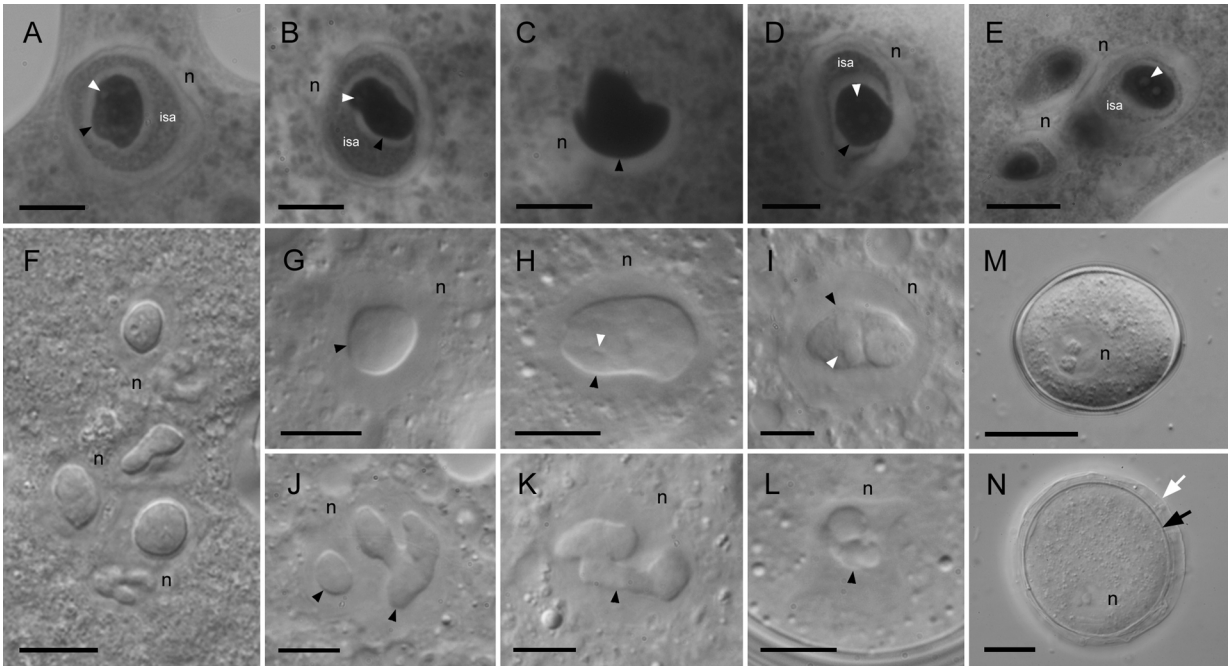

Fig. 3. Light micrographs of the nuclei of *Leptomyxa neglecta* strain LVal in the permanent stained preparations made in 2023 during the present study, bright field (A-E). A-D – Diversity of shapes of the nuclear membranes and nucleoli; E – trinucleated cell with comet-shaped intranuclear stained area. F-L – Light microscopy of the nuclei of living *Leptomyxa neglecta* strain LVal, DIC; F – cell granuloplasm containing six nuclei with nucleoli of different shapes; G-K – variety of nucleoli shapes. L-N – Light microscopy of cysts of *Leptomyxa neglecta* strain LVal, DIC; L – the nucleus of the cyst under a higher magnification; M – single-walled cyst; N – double-walled cyst. *Abbreviations*: n – nucleus; black *arrowhead* – nucleolus; white *arrowhead* – nucleolar lacuna; ISA – intranuclear stained area; black *arrow* – inner cyst wall; white *arrow* – outer cyst wall. Scale bars: A-E, G-L – 5 μm; F, N – 10 μm, M – 20 μm.

tographed another strain, isolated form the same location, and these images were the base for Smirnov et al. (2009) description of *R. neglecta*. The sequence published by Smirnov et al. (2009) was obtained from the third strain, isolated in the year 2007. All these strains originated from the same location and were recognized as co-specific (op. cit.). In the holotype preparation from 1993 there are two amoebae cells measuring about 40-45 μm in the maximal dimension (Fig. 2, P-Q). One of them (announced as a holotype and circled in preparation) is flattened, branched and has a conical pseudopodium (Fig. 2, P). The other one is monopodial, with a pronounced bulbous uroid (Fig. 2, Q). Cells in the permanent hematoxylin-stained preparations of the LVal strain made in 2023 were larger, predominantly elongated and branched (Fig. 2, R-S). Stationary and slowly moving amoebae had long, tapered pseudopodia and adhesive filaments.

The nuclei in preparations of the LVal strain were rounded or ovoid (Fig. 3, A-E). Besides the nuclear membrane and the nucleolus, central part of the karyoplasm inside the nucleus was more densely stained than its peripheral part. We call it an "intranuclear stained area" (ISA). The nucleoli had numerous rounded lacunae (up to 0.5 μm in maximal dimension) (Fig. 3, A-B, D-E). The shape of the nucleolus was ovoid (Fig. 3, A, E), slightly elongated (Fig. 3, B), rounded (Fig. 3, D) or irregular (Fig. 3, C). Usually, the nucleolus was located inside the ISA (Fig. 3, A), but sometimes it could be displaced (Fig. 3, B, D). Occasionally, the ISA had comet-like shape (Fig. 3, E). Usually, ISA was receded from the nucleolus by approximately 1 μm space. In permanent stained preparations, the maximal size of the nucleus varied from 10 to 18 μm (mean 11 μm, n = 18), the maximal size of the ISA varied from 8 to 12 μm (mean 10 μm, n = 18), the nucleolus measured from 4 to 7.9 μm (mean 5.7 μm, n = 18).

Molecular phylogenetic analysis

There are two sequences of *Leptomyxa neglecta*, available in GeneBank; both are labelled as "*Rhizamoeba neglecta*". The sequence FJ844435 was obtained by Smirnov et al. (2009). This is the partial sequence, 780 bp in length. Longer sequence KT945251 (1677 bp in length) was obtained by Smirnov et al. (2017) from the same DNA sample as the initial one. These sequences were identical in shared fragment. In the course of the present study, we obtained a partial 18S rRNA gene sequence of the strain LVal (1697 bp). Pairwise comparison of this sequence with the sequence named "*Rhizamoeba neglecta* KT945251" showed complete identity of a 1590 bp fragment, shared by both sequences. Subsequently, in the tree of 18S rRNA gene sequences of the order Leptomyxida the obtained sequence grouped with zero distance with this sequence (Fig. 4). Overall, genetic distances in the resulting tree were very low. Basal clades were supported with high values of bootstrap (BS) and posterior probability (PP). BS support degraded within the derived clades, while PP remained higher.

Discussion

The comparison of *Leptomyxa neglecta* (Smirnov et al., 2009) and *Leptomyxa neglecta* strain LVal

Amoebae of the strain LVal are very similar to the organisms described by Smirnov et al. (2009) in size and morphology. The locomotive forms of both are monopodial, clavate and have a pronounced hyaline cap. We estimate the size of the cap to be 1/8-1/6 of the cell length, but Smirnov et al. (2009) stated that it could occupy up to 1/3 of the total cell length. It may depend on the conditions of the observation. We have rarely seen amoebae with a bulbous uroid, in contrast to observations of Smirnov et al. (2009), but long trailing filaments are characteristic of both isolates (Smirnov et al., 2009, p. 252). The dimensions of the original *L. neglecta* were: length – 70-140 μm, breadth – 10-36 μm (Smirnov et al., 2009, p. 254). Our isolate is slightly longer, but narrower: length – 104-147 μm, breadth – 16-27 μm. Their L/B ratios are very similar: 5-8 in Smirnov et al. (2009) vs 4-8 in the current work.

Stationary cells of the original *L. neglecta* and strain LVal produce long conical pseudopodia. While not moving, both isolates are flattened, branched and irregular in shape. The trophozoite shown in Fig. 8 in Smirnov et al. (2009) can be compared with the one we call "comet-shaped" or "irregular" (Fig. 2, K-L). On the other hand, all resting cells studied by Smirnov et al. (2009) were relatively small (no more than 100 μm), and now we have found amoebae measuring up to 334 μm across.

Smirnov et al. (2009) did not provide a photomicrograph of the floating form of the original *L. neglecta*. The paper contains a scheme showing a radial cell with numerous (12 on the drawing) tapered pseudopodia. The length of these pseudopodia was often nearly equal to the diameter of the central cytoplasmic mass (Smirnov et al., 2009, p. 254). In the present study, we found similar floating cells with conical pseudopodia whose length varied from 0.5 to 1.5 compared to the diameter of the central area (Fig. 2, N-O).

The paper by Smirnov et al. (2009) contains a drawn scheme showing three types of nucleolar organization: rounded nucleus with irregularly ovoid nucleolus, elongated nucleus with elongated nucleolus and the nucleus with the nucleolus, consisting of several apposed fragments (op. cit., Fig. 20, B-D). In general, our observation is consistent with this scheme (Fig. 3, G-I), but we also observed nucleoli of irregularly elongated shape (Fig. 3, J-K). We also noticed that the nuclear membrane was slightly crumbled (cells were not compressed by the cover glass) and provided data on the presence of lacunae inside the nucleoli. Uneven border of the nuclear membrane and nucleolar lacunae can be also seen in Smirnov et al., 2009, Fig. 12-14. It is likely that the darker stained area (intranuclear stained area, or ISA) between the nuclear membrane and the nucleolus corresponds to the denser region of karyoplasm adjacent to the nucleolus in the TEM photos provided by Smirnov et al. (2009, Fig. 11-12). In those photographs, the nucleolus is located in the center of a denser area (op. cit., Fig. 12) or is displaced (op. cit., Fig. 11), as in Fig. 3, A and Fig. 3, D-E, respectively, in the current work. The maximal dimension of the nucleus is similar to that reported by Smirnov et al. (2009). Both original *L. neglecta* and amoebae of the LVal strain exhibit a high level of variability of the nucleolar material. The present observations show that *L. neglecta* can contain up to six nuclei with nucleoli of various shapes.

Based on the morphological similarity and the complete sequence identity, we conclude that the present isolate belongs to the species *L. neglecta* (Smirnov, Nassonova, Fahrni and Pawlowski, 2009) Smirnov et al., 2017.

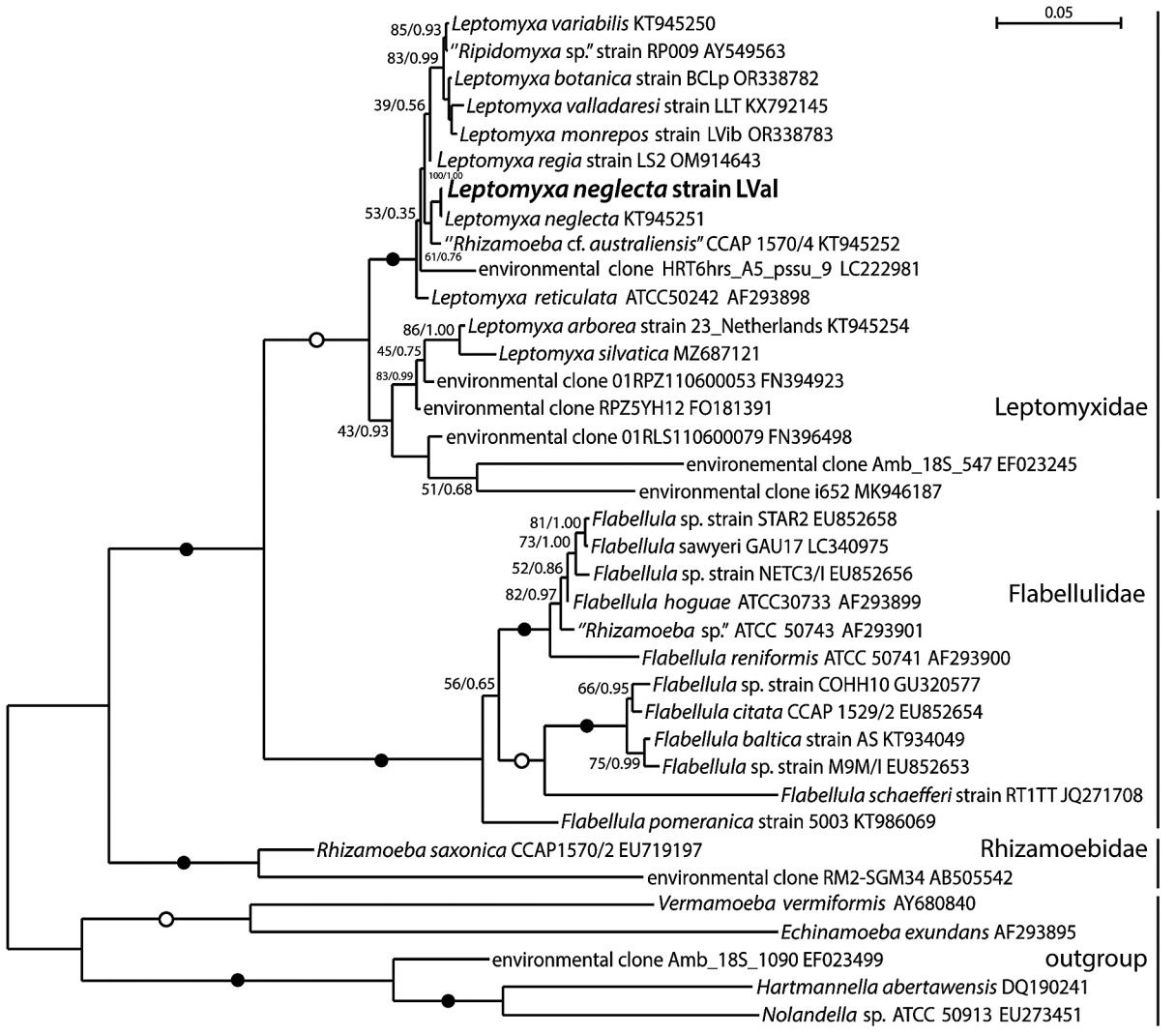

Fig. 4. Phylogenetic tree based on 18S rRNA gene, showing position of *Leptomyxa neglecta* strain LVal (GenBank: PP230162). The 1809 sites were selected for the analysis; GTR + gamma correction was used for the ML analysis and GTR + gamma correction with covarion for the Bayesian analysis. Labeling of nodes: bootstrap value/posterior probability. Black dots indicate fully supported nodes (100/1.00 support); circles indicate highly supported nodes (both BS > 95 and PP > 0.95). Supports lower 50/0.5 are not shown.

New data on the morphology of *Leptomyxa neglecta*

Smirnov et al. (2009) did not provide photographs of the movement of flattened cells and provided only one sentence describing this process: "When such cells started to move, the space between two neighbouring pseudopodia... was often filled by the eruption of the hyaloplasm, which softened the outlines of the cell" (op. cit., p. 254). This is consistent with our description and corresponds to Fig. 1, M-N. Photos of moving trapezoid or flabellate cells with smooth and uneven hyaloplasm border complement the data on the locomotion of flattened *L. neglecta*. It is important to note that the polylysine coating of the glass slide may have an impact on the shape of the flattened amoebae.

The ability of pseudopodia to anastomose to form fenestrae *sensu* Goodey (1915) was recently demonstrated by reticulate leptomyxid species, namely – *L. arborea* and *L. silvatica* (Smirnov et al., 2017; Glotova et al., 2021). Recently, Kulishkin et al. (2023) showed that one representative of the "mostly compact" clade – *L. reticulata* strain ATCC50242 – could do the same. In Fig. 2, F-G we show that *L. neglecta* is also able to fuse its

pseudopodia, but remains compact rather than reticulate. This makes a fenestration a much weaker taxonomic criterion.

No cysts were yet reported for *L. neglecta*, because "the cultures were never stable enough to ensure a complete observation of the entire life cycle of a cell" (Smirnov et al., 2009, p. 254). In our cultures, amoebae encyst rarely but successfully. Sometimes, double-walled cysts were observed (Fig. 3, N), as in *L. regia* (Kulishkin et al., 2022) or *L. reticulata* (Goodey, 1915; Pussard and Pons, 1976b).

***Leptomyxa neglecta* (Smirnov, Nassonova, Fahrni, Pawlowski, 2009) emend.**

Diagnosis. Length in locomotion 70–147 μm; breadth 10–36 mm; the cell is often clavate; adhesive uroidal filaments or bulbous uroid. Frontal hyaline cap occupies up to 1/3 of the total length of locomotive cell. Uninucleate or multinucleate cells. Nucleus 6–18 μm in length, oblong, flattened, rounded or of an irregular shape, sometimes with several fragments of the nucleolar material. The nucleolus sometimes contains lacunae, visible in LM, and a system of fine channels, visible in TEM. Cysts single- or double-walled, 28–38 μm in diameter. Freshwater.

Type location. Lake Leshevoe, Valamo Island (Lake Ladoga, Northwestern Russia). Also known from Lake Nikonovskoe, Valamo Island (Lake Ladoga, Northwestern Russia).

Type material. Holotype – the type slide mentioned by Smirnov et al. (2009), prepared in 1993 and deposited with the collection of slides of the Laboratory of Cytology of Unicellular organisms, Institute of Cytology RAS, under the No 1071. In the present study, the LM description, the 18S rRNA gene sequence, and stained preparations were made from the same strain of *L. neglecta* (LVal). The slide of this strain (Heidenhain's iron hematoxylin-stained permanent preparation made by N. Kulishkin in 2023) is deposited with the collection of slides of the Laboratory of Cytology of Unicellular Organisms, Institute of Cytology RAS, under the No 1072.

Differential diagnosis: Resembles *L. australiensis*, *L. ambigua* and *L. regia* but differs from these species in size and organization of the locomotive form, size and the organization of the nucleus and in the structure of the nucleolus (Chakraborty and Pussard, 1985; Smirnov, 2018). Flabellate cells can be compared with *L. botanica* and *L. monrepos*, but such forms in *L. neglecta* have much thicker hyaloplasmic area and are not that numerous. The 18S rRNA gene sequence differentiate it from the closely related species.

Acknowledgements

Supported by the RSF project 23-74-00050. The present study used equipment of the Core Facility Centers "Development of molecular and cell technologies," "Biobank" and "Culture collection of microorganisms" of the Research Park of St. Petersburg University. We are grateful to Daria Drachko and Yegor Shishkin for their help in collecting a sample, which was a source of the strain LVal, from Lake Nikonovskoe, Valamo Island, Lake Ladoga.

References

Adl S.M., Simpson A.G.B., Farmer M.A., Andersen R.A. et al. 2005. The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. J. Eukaryot. Microbiol. 52 (5): 399–451. https://doi.org/10.1111/j.1550-7408.2005.00053.x

Adl S.M., Simpson A.G.B., Lane C.E., Lukeš J. et al. 2012. The revised classification of Eukaryotes. J. Eukaryot. Microbiol. 59 (5): 429–514. https://doi.org/10.1111/j.1550-7408.2012.00644.x

Adl S.M., Habura A. and Eglit Y. 2014. Amplification primers of SSU rDNA for soil protists. Soil Biol. Biochem. 69: 328–342. https://doi.org/10.1016/j.soilbio.2013.10.024

Adl S.M., Bass D., Lane C.E., Lukeš J. et al. 2018. Revisions to the classification, nomenclature, and diversity of Eukaryotes. J. Eukaryot. Microbiol. 66 (1): 4–119. https://doi.org/10.1111/jeu.12691

Amaral Zettler L.A., Nerad T.A., O'Kelly C.J., Peglar M.T. et al. 2000. A molecular reassessment of the Leptomyxid amoebae. Protist. 151 (3): 275–282. https://doi.org/10.1078/1434-4610-00025

Bolivar I., Fahrni J.F., Smirnov A. and Pawlowski J. 2001. SSU rRNA-based phylogenetic position of the genera Amoeba and Chaos (Lobosea, Gymnamoebia): the origin of gymnamoebae revisited. Mol. Biol. Evol. 18 (12): 2306–2314. https://doi.org/10.1093/oxfordjournals.molbev.a003777

Cavalier-Smith T., Chao E.E.-Y. and Oates B. 2004. Molecular phylogeny of Amoebozoa and

the evolutionary significance of the unikont *Phalansterium*. Eur. J. Protistol. 40 (1): 21–48. https://doi.org/10.1016/j.ejop.2003.10.001

Chakraborty S. and Pussard M. 1985. *Ripidomyxa australiensis* nov. gen. nov. sp., a mycophagous amoeba from Australian soil. Protistologica. 21 (1): 133–140.

Edgar R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research. 32 (5): 1792–1797. https://doi.org/10.1093/nar/gkh340

Fahrni J.H., Bolivar I., Berney C., Nassonova E. et al. 2003. Phylogeny of lobose amoebae based on actin and small-subunit ribosomal RNA genes. Mol. Biol. Evol. 20 (11): 1881–1886. https://doi.org/10.1093/molbev/msg201

Geisen S., Weinert J., Kudryavtsev A., Glotova A. et al. 2014. Two new species of the genus *Stenamoeba* (Discosea, Longamoebia): cytoplasmic MTOC is present in one more amoebae lineage. Eur. J. Protistol. 50 (2): 153–165. https://doi.org/10.1016/j.ejop.2014.01.007

Glotova A.A., Loiko S.V., Istigichev G.I., Kulemzina A.I. et al. 2021. Description of *Leptomyxa silvatica* n. sp. (Amoebozoa, Tubulinea, Leptomyxida), a new soil amoeba species from Chernevaya taiga soil of West Siberia, Russia. Protistology. 15 (4): 312–320. https://doi.org/10.21685/1680-0826-2021-15-4-7

Goodey T. 1915. A preliminary communication on three new proteomyxan rhizopods from soil. Arch. Protistenkd. 35: 80–102.

Kulishkin N.S., Surkova A.A., Mesentsev Y.S., Smirnov A.V. et al. 2022. Morphology and phylogeny of *Leptomyxa regia* n. sp., isolated from an artificial pond in Izmailovo Park (Moscow, Russia). Protistology. 16 (1): 42–51. https://doi.org/10.21685/1680-0826-2022-16-1-5

Kulishkin N.S., Smirnov A.V. and Patterson D.J. 2023. New species of the genus *Leptomyxa* (Goodey, 1915): *Leptomyxa botanica* n. sp. and *Leptomyxa monrepos* n. sp., from Northwestern Rus sia. Eur. J. Protistol. 91: 126028. https://doi.org/10.1016/j.ejop.2023.126028

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

Miller M.A., Pfeiffer W. and Schwartz T. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Gateway Computing Environments Workshop (GCE), New Orleans, LA, USA, pp. 1–8. https://doi.org/10.1109/GCE.2010.5676129

Page F.C. 1972. *Rhizamoeba polyura* n. g., n. sp., and uroidal structures as a taxonomic criterion for amoebae. Trans. Am. Microsc. Soc. 91 (4): 502–513. https://doi.org/10.2307/3225479

Page F.C. 1974. A further study of taxonomic criteria for limax amoebae, with descriptions of new species and a key to genera. Arch. Protistenkd. 116: 149–184.

Page F.C. 1976a. A revised classification of the Gymnamoebia (Protozoa: Sarcodina). Zool. J. Linn. Soc. 58 (1): 61–77. https://doi.org/10.1111/j.1096-3642.1976.tb00820.x

Page F.C. 1976b. An illustrated key to freshwater and soil amoebae. Freshwater Biol. Ass. Ambleside.

Page F.C. 1988. A new key to freshwater and soil gymnamoebae. Freshwater Biol. Ass. Ambleside, Cumbria.

Pawlowski J., Bolivar I., Fahrni J.F., Cavalier-Smith T. et al. 1996. Early origin of foraminifera suggested by SSU rRNA gene sequences. Mol. Biol. Evol. 13 (3): 445–450. https://doi.org/10.1093/oxfordjournals.molbev.a025605

Pawlowski J. 2000. Introduction to the molecular systematics of foraminifera. Micropaleontology. 46: 1–12. https://www.jstor.org/stable/1486176

Prescott D.M. and James T.W. 1955. Culturing of *Amoeba proteus* on *Tetrahymena*. Exp. Cell. Res. 8 (1): 256–258. https://doi.org/10.1016/0014-4827(55)90067-7

Pussard M. and Pons R. 1976a. Etude des genres *Leptomyxa* et *Gephyramoeba* (Protozoa, Sarcodina). II. *Leptomyxa flabellata* Goodey, 1915. Protistologica. 12 (2): 307–319.

Pussard M. and Pons R. 1976b. Etude des genres *Leptomyxa* et *Gephyramoeba* (Protozoa, Sarcodina). I. *Leptomyxa reticulata* Goodey, 1915. Protistologica. 12 (1): 151–168.

Ronquist F., Teslenko M., van der Mark P., Ayres D.L. et al. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61 (3): 539–542. https://doi.org/10.1093/sysbio/sys029

Smirnov A.V. and Goodkov A.V. 1996. Systematic diversity of gymnamoebae in the bottom sediments of a freshwater lake in Karelia (Lobosea, Gymnamoebia). Zoosyst. Rossica. 4: 201–203.

Smirnov A., Nassonova E., Berney C., Fahrni J. et al. 2005. Molecular phylogeny and classification of the lobose amoebae. Protist. 156 (2): 129–142.

https://doi.org/10.1016/j.protis.2005.06.002

Smirnov A.V., Nassonova E.S. and Cavalier-Smith T. 2008. Correct identification of species makes the amoebozoan rRNA tree congruent with morphology for the order Leptomyxida Page 1987; with description of *Acramoeba dendroida* n. g., n. sp., originally misidentified as '*Gephyramoeba* sp.' Eur. J. Protistol. 44 (1): 35–44. https://doi.org/10.1016/j.ejop.2007.08.001

Smirnov A., Nassonova E., Fahrni J. and Pawlowski J. 2009. *Rhizamoeba neglecta* n. sp. (Amoebozoa, Tubulinea) from the bottom sediments of freshwater Lake Leshevoe (Valamo Island, North-Western Russia), with notes on the phylogeny of the order Leptomyxida. Eur. J. Protistol. 45 (4): 251–259. https://doi.org/10.1016/j.ejop.2009.04.002

Smirnov A.V., Chao E., Nassonova E.S. and Cavalier-Smith T. 2011. A revised classification of naked lobose amoebae (Amoebozoa: Lobosa). Protist. 162 (4): 545–570. https://doi.org/10.1016/j.protis.2011.04.004

Smirnov A., Nassonova E., Geisen S., Bonkowski M. et al. 2017. Phylogeny and systematics of leptomyxid amoebae (Amoebozoa, Tubulinea, Leptomyxida). Protist. 168 (2): 220–252. https://doi.org/10.1016/j.protis.2016.10.006

Smirnov A. 2018. Fine structure of *Leptomyxa ambigua* n. sp. CCAP 1546/2 strain, formerly known as "*Rhizamoeba flabellata*" (Amoebozoa, Tubulinea, Leptomyxida). Eur. J. Protistol. 62: 95–100. https://doi.org/10.1016/j.ejop.2017.12.001

Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 30 (9): 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Stothard P. 2000. The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. Biotechniques. 28 (6): 1102–1104. https://doi.org/10.2144/00286ir01

Tekle Y.I., Grant J., Anderson O.R., Nerad T.A. et al. 2008. Phylogenetic placement of diverse amoebae inferred from multigene analyses and assessment of clade stability within 'Amoebozoa' upon removal of varying rate classes of SSU-rDNA. Mol. Phylogenet. Evol. 47 (1): 339–352. https://doi.org/10.1016/j.ympev.2007.11.015