Protistology 16 (1): 42–51 (2022) | doi:10.21685/1680-0826-2022-16-1-5

Protistology

ORIGINAL ARTICLE

Morphology and phylogeny of *Leptomyxa regia* n. sp., isolated from an artificial pond in Izmailovo Park (Moscow, Russia)

Nikita S. Kulishkin[1]*, Alina A. Surkova[1], Yelisei S. Mesentsev[1], Alexey V. Smirnov[1] and Yuri A. Mazei[2]

[1] *Department of Invertebrate Zoology, Faculty of Biology, St Petersburg University, 199034 St. Petersburg, Russia*
[2] *Department of General Ecology and Hydrobiology, Lomonosov Moscow State University, 11999 Moscow, Russia*

| Submitted February 13, 2022 | Accepted March 11, 2022 |

Summary

The order Leptomyxida (Amoebozoa, Tubulinea) unifies species capable of altering their morphology from monopodial, clavate in active locomotion to flattened ramose or branched while feeding or resting. Species belonging to this order are very polymorphic and are hard to distinguish at the morphological level. The molecular data, namely – gene sequences, remain the primary differentiating character, especially within the genus *Leptomyxa*. We have isolated a new representative of this genus, *Leptomyxa regia* n. sp., from the top layer of sediment collected from an artificial pond in Izmailovo Park in Moscow (Russia). A remarkable characteristic of this species is that being monopodial, it moves without forming a pronounced hyaline cap. The latter is visible only at the beginning of locomotion or in non-directed movement. In addition, it differs from other species in the 18S rRNA gene sequence. We provide morphological and molecular data on this remarkable species of Leptomyxida.

Key words: Amoebozoa, Tubulinea, Leptomyxida, morphology, phylogeny, systematics

Introduction

The genus *Leptomyxa* (Amoebozoa, Tubulinea, Leptomyxida) was established by Goodey (1915) to accommodate two amoebae species, first isolated in 1913 from the soil of a cucumber house at Harpender, in Hertfodshire (England, ca. 50 km North from London), and later – from the soil from Edgebaston Birmingham and Great Barr in Staffordshire (Goodey, 1915, p. 6). These organisms were spread out into a thin sheet, branching and anastomosing in *Leptomyxa reticulata* and resembling a fan in outline in *L. flabellata*. Together with the third species described in the same study – *Gephyramoeba delicatula*, these organisms were assigned to suborder Reticulosa (Proteomyxa) according to the classification scheme by Minchin (1912).

All three species mentioned above were reisolated and investigated by Pussard and Pons (1976a, 1976b, 1976c). They provided the formal diagno-

https://doi.org/10.21685/1680-0826-2022-16-1-5

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

sis of the order Leptomyxida and created two families: Leptomyxidae and Gephyramoebidae (Pussard and Pons, 1976a, p. 165). Further, Page (1976) mentioned the order Leptomyxida as a member of the class Acarpomyxea, and in his system from 1987 – as a member of the class Lobosea (Page, 1987). This position of Leptomyxida was retained in the further set of publications (Page, 1988, 1991; Rogerson and Patterson, 2002). Page (1972) suggested the genus *Rhizamoeba* for a marine amoeba species *R. polyura*, possessing adhesive uroidal structures resembling *Leptomyxa* spp. Later, he described one more species of this genus – *R. saxonica* (Page, 1974). More species of *Rhizamoeba* were described by Smirnov et al. (2009) and Mrva (in Smirnov et al., 2017). In addition, Page (1988) transferred the species *L. flabellata* to the genus *Rhizamoeba* and did the same for the species described as "*Ripidomyxa australiensis*" (Chakraborty and Pussard, 1985), thus invalidating the genus "*Ripidomyxa*". As a result, it appeared that all leptomyxid species tending to produce limax-like locomotive form and not forming a reticulate plasmodium should belong to the genus *Rhizamoeba*. In contrast, the genus *Leptomyxa* remained monotypic, containing an expanded, ramose species *L. reticulata*. Details of the complex taxonomic history of leptomyxid genera may be found in Smirnov et al. (2017).

Molecular studies confirmed the monophyly of Leptomyxida (Amaral-Zettler et al., 2000); the only exception was the case with misidentified *Gephyramoeba* strain, now known as *Acramoeba dendroida* and belonging to Variosea lineage (Smirnov et al., 2008). Further revisions of the system of Amoebozoa (Cavalier-Smith et al., 2004; Smirnov et al., 2005) confirmed this finding and led to the placement of the order Leptomyxida into the class Tubulinea (Smirnov et al., 2005, 2011) and, respectively, in Tubulinea lineage in Adl et al. (2005, 2012, 2018). Molecular studies show that sequences of *Leptomyxa* and *Rhizamoeba* are intermingled in the tree, which led to the revision of leptomyxids (Smirnov et al., 2017). In this revision, most of the species described as members of the genus *Rhizamoeba* were transferred to the genus *Leptomyxa*. In contrast, the genus *Rhizamoeba* was retained for three species – *Rhizamoeba saxonica* Page, 1974, *R. polyura* Page, 1972, and *R. matisi* Mrva, 2017 (in Smirnov et al., 2017). This study showed that the locomotive morphology is not decisive in distinguishing *Rhizamoeba* from *Leptomyxa*, making the molecular phylogeny the primary criterion for placing leptomyxid species to either of these genera.

By now, nine representatives of the genus *Leptomyxa* were isolated from freshwater sediment (Smirnov et al., 2009), soil (Geisen and Burberg, 2017 in Smirnov et al., 2017; Del Valle, 2017; Glotova et al., 2021), and leaf litter (Smirnov et al., 2017). This paper describes one more species of this genus studied using light microscopy and SSU phylogeny.

Material and methods

Sampling and cultivation

Amoebae were isolated from the samples of bottom sediments (5 cm of the upper layer of detritus) from an artificial freshwater pond located in Izmailovo Park, Moscow, Russia (55°46'46.8"N; 37°46'09.2"E). To get enrichment culture, we diluted samples 1:1000 with autoclaved 0,025% WG infusion (see Geisen et al., 2014), made on PJ medium (Prescott and James, 1955). About 15 ml of the resulting mix was inoculated in 60 mm Petri dishes filled with wMY agar (Spiegel et al., 1995) and overlayed with the same medium. Cultures were cloned and further maintained by transferring a few cells into new 60 mm Petri dishes with fresh medium.

Light microscopy

Live cells in culture were observed and measured using Leica DMI3000 inverted microscope equipped with phase-contrast and IMC (Integrated Modulation Contrast) optics and Leica DFC295 camera powered by Leica Application Suite (LAS) version 4.11.0 software. Amoebae on glass slides were studied using Leica DM2500 microscope equipped with DIC optics, photographed, and video-recorded using DS-Fi3 Nikon camera powered by NisElements AR software (Nikon).

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

DNA isolation and amplification

To isolate DNA, individual amoeba cells were transferred with tapered tip Pasteur pipette into concave watch glass 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 in fresh, clean watch glass with a new medium. 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 instruction; 12 μl of extraction buffer was added to each tube. For PCR amplification, we used forward RibA (5'>ACCTGGTTGATCCTGCCAGT<3'), which is a second half of the original "Primer A" (Medlin et al., 1988) and reverse RibB (5'>TGA TCCTTCTGCAGGTTCACCTAC<3') primers (Pawlowski, 2000). The PCR program included the following steps: initial denaturation at 94 °C for 2 min; 36 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and elongation at 72 °C for 2.5 min; final elongation at 72 °C for 5 min. PCR products were sequenced without purification using the primers mentioned above and also S6F (forward, 5'-CNGCGGTAATTCCAGCTC-3'), S12.2 (forward, 5'-GATCAGATACCGTCGTAGTC-3'), and S12.2R (reversed, 5'-GACTACGACGGTAT CTGATC-3') primers (Pawlowski, 2000; Glotova et al., 2018) using Big Dye Terminator Cycle sequencing kit and an ABI PRISM automatic sequencer. The consensus sequence was assembled using Chromas Pro software (http://technelysium.com.au/wp/chromaspro/) based on ten sequences with the best trace quality.

Alignment and phylogenetic analysis

The obtained sequence was manually aligned to the comprehensive alignment of leptomyxids used by Glotova et al. (2021). Duplicated and identical sequences were excluded from the analysis. The mask included 1845 unambiguously aligned positions. The phylogenetic analysis was performed using the maximum likelihood method as implemented in PhyML 3.0 (Guindon et al., 2010). GTR+ γ +I model, using 25 random starting trees and a further selection of the best of NNI and SPR trees, was applied. The program optimized other parameters; eight rate categories were used. Clade stability was estimated with 1000 bootstrap pseudoreplicates. Bayesian analysis was performed using MrBayes 3.2.6 (Ronquist et al., 2012), 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 6 mln generations. By that time, it had ceased to converge (final average standard deviation of split frequencies less than 0.01). The first 25% of generations were discarded as burn-in. The pairwise comparison of sequences was performed using "Ident and Sim" tool (Stothard, 2000; https://www.bioinformatics.org/sms2/ident_sim.html).

Results

Light-microscopic morphology

The locomotive form of the studied amoeba was monopodial, subcylindrical, without a pronounced hyaline cap or with very small, crescent-shaped one (Fig. 1, A-E). The latter was pronounced only when the cell changed the direction of movement, at the beginning of the formation of a new pseudopodium (Fig. 1, G, I), or when a flattened slowly moving cell converted to a monopodial one (Fig. 1, J). Most cells were slightly clavate and narrowed to the posterior end in locomotion. Many cells had pronounced, long adhesive uroidal filaments (Fig. 1, B, D), while few formed a bulbous uroid covered by thin short adhesive filaments (Fig. 1, F, O). Moving cells showed steady cytoplasmic flow in the direction of motion with periodical eruptions of the hyaloplasm, usually in the middle of the amoeba body (Fig. 1, F, H). These eruptions often preceded the formation of a new leading pseudopodium in this area, accompanied by the retraction of the previous one. As a result, the cell changed the direction of locomotion and continued movement in a new direction. A few times, we saw hyaline eruptions at the anterior end of the cell, spreading along the lateral side backward. The length of the locomotive form varied from 78 to 104 μm (average 92 μm, n=20), its breadth ranged 16-26 μm (average 21 μm, n=20).

Slowly moving cells were flattened and had multiple conical hyaline subpseudopodia on the anterior end and adhesive lobes and filaments at the posterior end of the cell (Fig. 1, K, M). At the beginning of locomotion, flattened cell showed irregular eruptive bubbling on the periphery of the

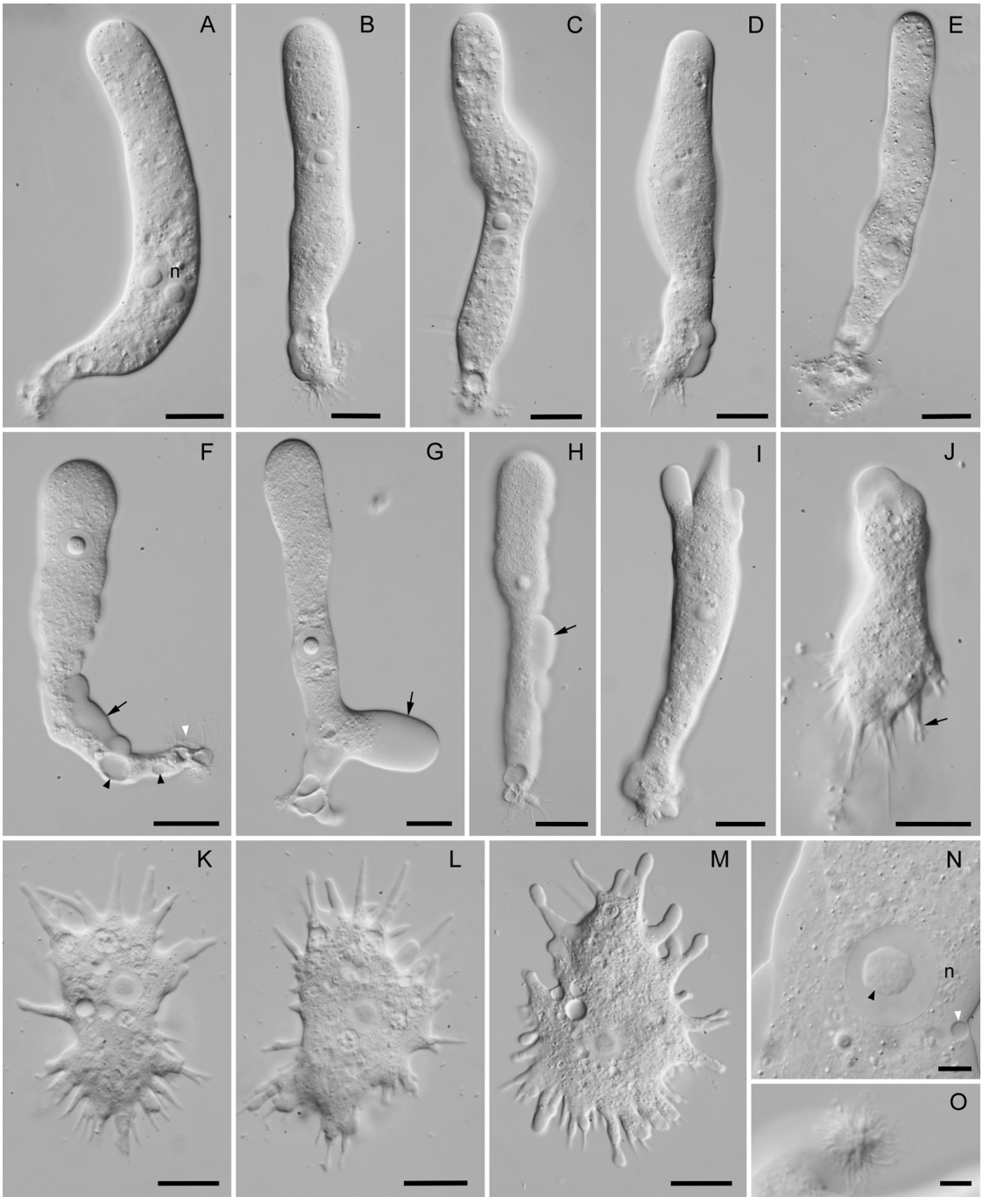

Fig. 1. Light microscopy of *Leptomyxa regia*, DIC. A-E – Monopodial locomotive form (n – nucleus); F – hyaline eruption on the side of the cell (black *arrow* – hyaline eruption, black *arrowhead* – contractile vacuoles, white *arrowhead* – adhesive uroidal filaments); G – the cell with almost formed new leading pseudopodium, showing a pronounced hyaline cap (black *arrow*); H – transitory hyaline eruption on the side of the cell (black *arrow*); I – transitory form of the moving cell, with several short pseudopodia on the frontal end; J – transitory shape, often adopted by the cell at the beginning of locomotion (*arrow* – adhesive filaments,); K – slowly moving flattened cell; L-M – resting, flattened cells; N – higher magnification of the cell showing granuloplasm, nucleus, and cytoplasmic inclusions (n – nucleus, black *arrowhead* – nucleolus, white *arrowhead* – young contractile vacuole); O – bulbous uroid, covered with filaments. Scale bars: 20 μm (A-M) and 5 μm (N-O).

cell, filling the space between subpseudopodia. Some of them acquired rounded ends before merging into the common hyaline mass (Fig. 1, M). After that, the cell started to move. Usually, it formed a large pseudopodium with a pronounced frontal hyaline area and had adhesive filaments at the posterior end. Soon after this, the amoeba adopted a monopodial form and started locomotion.

Non-moving cells, observed in culture, were flattened, with numerous conical hyaline subpseudopodia along the cell periphery. Amoebae tended to show two types of shape: elongated (Fig. 2, A) and tree-like (Fig. 2, B). Sometimes tree-like forms were almost symmetrical (Fig. 2, C). Some of them were covered with a mixture of faecal pellets and detritus. Sometimes we observed cells adhered to the plastic bottom of the Petri dish with uroid, while the rest of the cell was raised above the substrate (Fig. 2, E). These cells were pronouncedly clavate, monopodial, had a broad and massive hyaline cap. The size of non-moving cells was 51-207 μm (average 119 μm, n=58) in the maximal dimension and 17 -101 μm (average 47 μm, n=58) in the perpendicular one.

The floating form was of radial type, with multiple tapering pseudopodia (Fig. 2, D). Nearly a third of each pseudopodium consisted of the granuloplasm. These pseudopodia were approximately three times as long as the diameter of the central part of a floating cell.

The nucleus with the single central nucleolus was rounded, oblong or ovoid and was rather polymorphic (Fig. 1, N; Fig. 2, I-K). In old cultures, we have seen amoebae with two nuclei (Fig. 1, A). Maximal dimensions of the nucleus varied from 8 to 12 μm, of the nucleolus – from 5 to 7 μm. Cells had several small contractile vacuoles that asynchronously merged into one or two larger vacuoles, which further contracted (Fig. 1, F, pointed by black arrowhead). Cells had digestive vacuoles, numerous small granules, and various particles in the cytoplasm. Crystals were not seen.

Cysts were double-walled. Amoebae started to encyst in six-month-old cultures or later (Fig. 2, F-H). The external cyst wall was represented with an irregularly rounded thin layer, receding from the internal cyst wall by a 7-9 μm space. Sometimes, few bacterial cells were present in this space. The internal cyst wall (endocyst) was perfectly round and consisted of one thick layer (approximately 0.5 μm). Sometimes, there was a gap between the wall of the cyst and the body of the amoeba (Fig. 2, G and H: asterisk). The diameter of the endocyst, measured in six specimens, varied from 28 to 35 μm.

Molecular phylogeny

The comprehensive tree of the SSU rRNA sequences of Leptomyxida (Fig. 3) robustly shows the position of our new strain as an independent lineage in the clade, which among non-named environmental isolates contains the species *Leptomyxa varaibilis, L. valladaresi, Leptomyxa* cf. *reticulata*, "*Rhizamoeba* cf. *australiensis*" sequence KT945252, and *Leptomyxa neglecta*. The species composition of this clade is congruent with that published by Smirnov et al. (2017).The species *Flabellula schaefferi* in our tree formed long independent lineage at the base of *F. baltica – F. citata* clade and the entire genus *Flabellula* got split in two clades, as well as the genus *Leptomyxa*. The genetic distances between species in our tree were low or, in some clades, very low, which is also congruent with Smirnov et al. (2017) data. Both kinds of support for the basal branches in the Leptomyxida tree are high, while within the terminal clades, bootstrap (BS) support degraded to average or low values. At the same time, the posterior probability (PP) remained higher. It probably resulted from very low genetic distances between species, often measured with several nucleotides only in organisms, very different morphologically.

The pairwise comparison of sequences shows that the sequence of *L. regia* n. sp. has a high level of identity with all neighbouring sequences and all of them among each other (Table 1). The highest value (99.11% identity) is demonstrated with '*Ripidomyxa*' RP009 sequence AY549563. This strain is not currently available. This level of identity in the 1565 bp fragment corresponds to 14 bp difference, which all are single-nucleotide differences; two of them are single nucleotide deletions. This pattern of differences is congruent with that observed between other species of this clade.

Discussion

Identification of the present isolate

At the morphological level, the studied strain should be classified in the order Leptomyxida Pussard et Pons, 1976 sensu Smirnov, 2017. Characteristic features are the clavate locomotive form, the

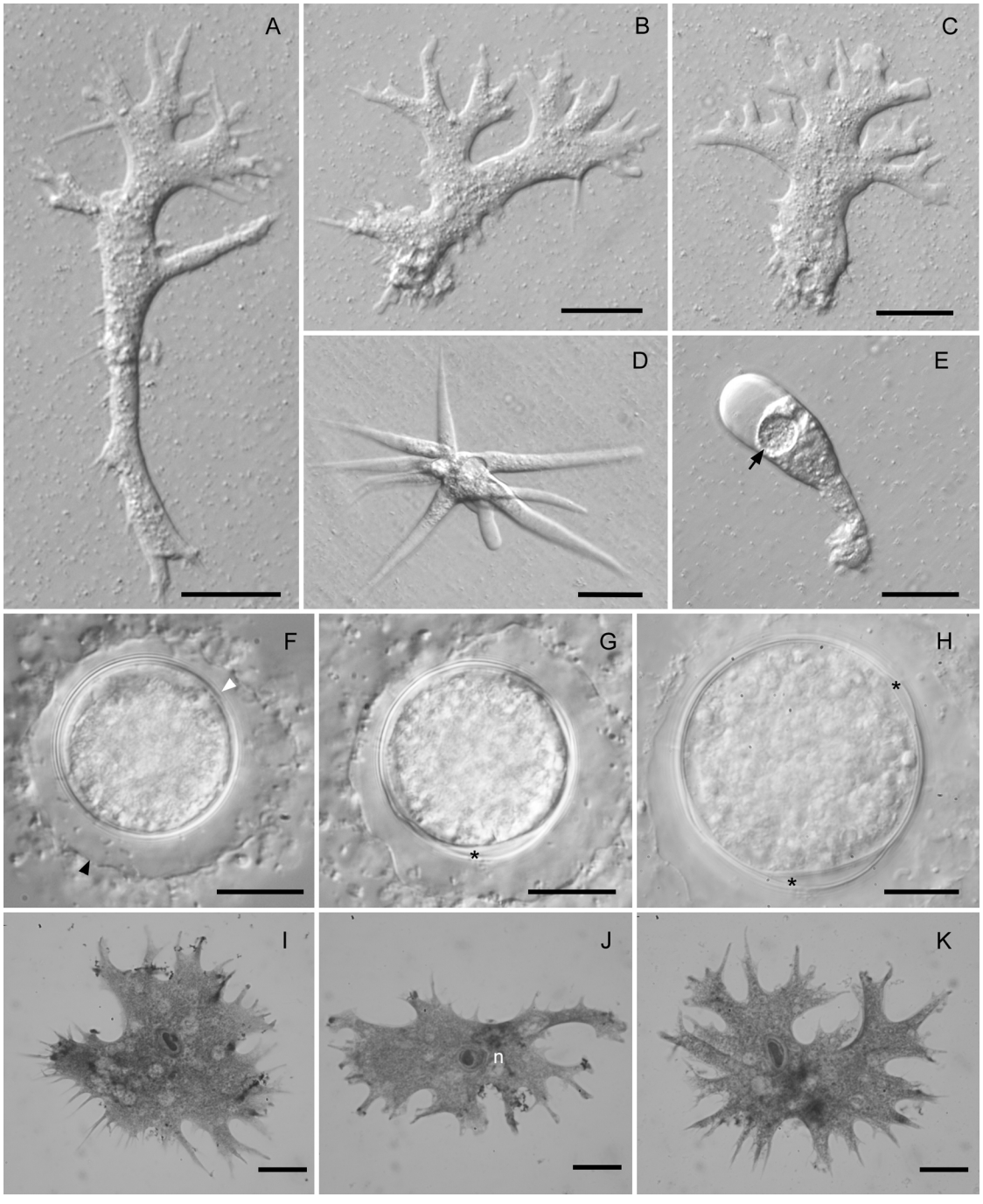

Fig. 2. Light microscopy of *Leptomyxa regia*. A-C – Cells on the plastic surface of a Petri dish. Different resting or feeding forms; D – floating cell; E – cell, adhered to the dish in uroidal part, while the rest is raised over the substratum. This cell demonstrates large contractile vacuole (black *arrow*) and large hyaline area; F-H – cysts of *Leptomyxa regia* (white *arrowhead* – internal cyst layer, black *arrowhead* – external cyst layer; *asterisk* – gap between cyst wall and amoeba body); I-K – permanent hematoxylin-stained preparations of *Leptomyxa regia* (n – nucleus). A-E – IMC, F-H – DIC. Scale bars: A-E, I-K – 20 μm, F-G – 15 μm, and H – 10 μm.

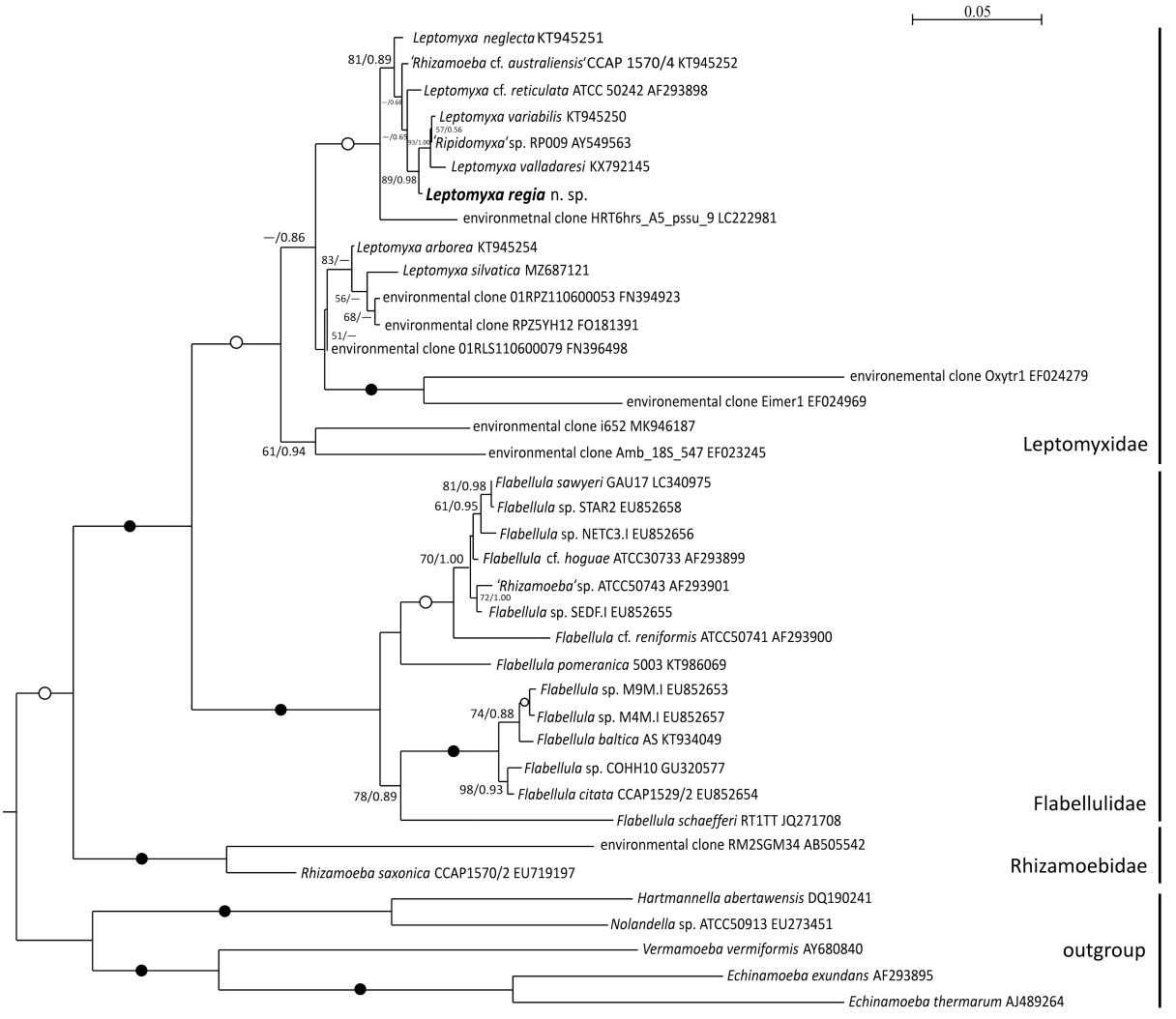

Fig. 3. Phylogenetic tree based on 18S rRNA gene, showing the position of *Leptomyxa regia*. 1845 sites were selected for the analysis; GTR + γ + I was used for ML analysis and GTR + γ with covarion – for Bayesian analysis. Labelling of nodes: ML/PP support. Black circles are used to recognize fully supported nodes (100/1.00 support); white-filled circles are used to label highly supported nodes (both BS > 0.95 and PP > 95). Supports below 50/0.50 are not shown (if both are lower), or are recognised by "-".

presence of adhesive uroidal filaments, shape, and organizations of slowly moving or non-mobile cells, and the remarkable tendency to form eruptions of the cytoplasm, opposite to the direction of locomotion. Other characters of this strain also fit the diagnosis of the order Leptomyxida. Among leptomyxids, our strain should be placed into the genus *Leptomyxa* Goodey, 1915 sensu Smirnov, 2017, based on its ability to adopt flattened, branched form when the cell is not moving.

Within the genus, studied species can be easily distinguished from *L. reticulata, L. arborea, L. variabilis*, and *L. silvatica* because all these species tend to form expanded plasmodia with gaps ("fenestrae" sensu Goodey, 1915) within the perimeter of the cell (Goodey, 1915; Smirnov et al., 2017; Glotova et al., 2021). It is not characteristic of our strain. On the other hand, our strain never produced flattened fan-shaped locomotive forms. By this feature, it can be clearly differentiated from *L. flabellata* (Goodey, 1915).

Representatives of the new strain are in the same size range as *L. ambigua*; however, the present strain is much less clavate in locomotion (Smirnov,

Table 1. The pairwise comparison of sequences belonging to the clade containing *Leptomyxa regia* n. sp. using "Ident and Sim" tool in 1565 bp fragment shared by all sequences. Values indicate the percent of sequence identity.

Species	Leptomyxa regia	Leptomyxa neglecta	Leptomyxa cf. reticulata	"Rhizamoeba cf. australiensis"	Ripidomyxa RP009	Leptomyxa variabilis
Leptomyxa neglecta	98.73					
Leptomyxa cf. *reticulata*	98.41	98.66				
'Rhizamoeba cf. *australiensis"*	98.79	99.11	98.85			
Ripidomyxa RP009	99.11	98.28	98.03	98.34		
Leptomyxa variabilis	99.04	98.22	97.96	98.34	99.87	
Leptomyxa valladaresi	98.60	98.03	97.71	98.09	99.23	99.11

2018, Fig. 1, G, H). It resembles *L. neglecta* and *L. valladaresi* by the shape of resting cells (Smirnov, 2009; Del Valle, 2017). However, our strain forms no pronounced hyaline cap in fast directional locomotion. It is a unique characteristic among amoebae of the genus *Leptomyxa*.

Molecular phylogeny

The sequence of 18s rRNA gene shows several differences with other *Leptomyxa* species. The level of sequence divergence is very low; however, a similar level of 18s rRNA gene sequence divergence is characteristic for the entire clade (Table 1). Simultaneously, it includes species that are very different at the morphological level. Leptomyxida are generally known for their low genetic distances between morphologically different species in 18S rRNA gene tree (Amaral-Zettler et al., 2000; Del Valle, 2017; Smirnov et al., 2017; Glotova et al., 2021). It is the only group of naked amoebae where we have found a complete 18S rRNA gene sequence identity between the geographically distant isolates (Smirnov et al., 2017). The character of nucleotide replacements in the sequence of our strains (single nucleotide replacements and insertions) is the same as in the sequences of other species of this clade. Therefore, we conclude that the present isolate is a new species of the genus Leptomyxa, differing from all known species in the morphology of the locomotive form and the 18s rRNA gene sequence, and name it *Leptomyxa regia* n. sp.

Taxonomic summary

Diagnosis: Locomotive cells of *Leptomyxa regia* n. sp. slightly clavate and show no pronounced hyaline cap in continuous locomotion. Length in locomotion 78–104 μm, breadth 16–26 μm. Predominantly uninucleate, nucleus vesicular, 8–12 μm in maximal dimension, rounded or slightly ovoid. Cysts are double-walled. The external cyst wall is an irregularly rounded thin layer, receding from the internal cyst wall by a distinct space; the diameter of the endocyst is 28-35 μm.

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 1057.

18S rRNA gene sequence of the type strain: GenBank number OM914643.

Type location: Artificial freshwater pond located in Izmailovo Park, Moscow, Russia (55°46'46.8"N; 37°46'09.2"E), known as "Sobachiy Pond".

Etymology: From the Latin word "regius" (royal or regal), after historical owners of Izmailovo Park - the House of Romanovs.

Differences from closely related species: Resembles *L. neglecta, L. valladaresi*, and "*Rhizamoeba* cf. *australiensis*" sequence KT945252, belonging to the strain CCAP 1570/4, but differs from these species by the absence of hyaline cap in locomotive form and by the size of the cell. 18S rRNA gene has sequence differences from the closely related species.

Acknowledgements

Supported by the RFBR project 20-54-53017 to YM (sampling, treatment and LM study of cultures) and RSF project 20-14-00195 to AS (molecular studies). The present study utilized equipment of the Core facility centers "Development of molecular and cell technologies" and "Culture collection of microorganisms" of the Research Park of Saint Petersburg State University. Dedicated to the Year of Zoology 2022 in St. Petersburg State University.

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