Protistology

Description of *Leptomyxa silvatica* n. sp. (Amoebozoa, Tubulinea, Leptomyxida), a new soil amoeba species from Chernevaya taiga soil of West Siberia, Russia

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Summary

During the studies of amoebae from the rare and highly productive soil of Chernevaya taiga (West Siberia, Russia) we have found an isolate of a leptomyxid amoeba showing 18s rDNA sequence significantly different from those of other known species of the order Leptomyxida. Here we describe this isolate as a new species, *Leptomyxa silvatica* n.sp. This species has both morphological and sequence differences from related ones. This finding confirms that reliable morphological differentiation and identification of leptomyxid amoebae is possible only for several remarkable species, while others require molecular data to be correctly labeled or described.

Key words: amoebae, *Leptomyxa*, morphology, phylogeny, Tubulinea

Introduction

The most remarkable character of amoebae belonging to the genus *Leptomyxa* (Amoebozoa, Tubulinea, Leptomyxida) is their ability to adopt two alternative body forms: monotactic (limax-like) form with smooth non-anastamosing pseudopodia (lobopodia) in rapid locomotion and flattened, sometimes expanded branched (ramose or reticulate) form with loboreticulopodia when the cell moves slower (Page, 1987; Smirnov and Goodkov, 1999; Smirnov and Brown, 2004; Smirnov et al., 2005;

2011). Trophozoites of these amoebae form adhesive uroidal filaments along the posterior edge of the cell (Smirnov et al., 2017). The alteration of the body shape may depend on various factors, not yet studied. It is shown that for the species *Flabellula baltica* the body shape depends on the amount of food resources available to the trophozoite (Fenchel, 2009).

Among amoebae of the order Leptomyxida, the most remarkably branched and ramose are species of the genus *Leptomyxa* Goodey, 1915 sensu Sminov et al. (2017). These organisms possess a simple life cycle, which includes subsequent stages of feeding, growth, multiplication and encystment (Goodey, 1915; Pussard and Pons, 1976a, 1976b, 1976c; Page, 1987, 1988; Smirnov et al., 2009; Del Valle et al., 2017). This genus nowadays includes eight species (see list in Smirnov et al., 2017; Del Valle et al., 2017; Smirnov, 2018). The type species of the genus is *Leptomyxa reticulata* Goodey, 1915. This is an irregularly triangular, expanded, large (up to several millimeters), often ramose multinucleate amoeba (usually recognized as a plasmodium) with numerous adhesive filaments along the posterior edge of the moving cell (Goodey, 1915; Page, 1988; Pussard and Pons, 1976a). Other members of the genus are significantly smaller and usually less ramose (Del Valle et al., 2017; Smirnov et al., 2017). Species that were previously classified as *Rhizamoeba*, like *L. flabellata, L. australiensis, L. ambigua* or *L. neglecta* almost do not demonstrate a ramose shape. They are monopodial, clavate or flattened, comet-shaped (Pussard and Pons, 1976c; Chakraborty and Pussard, 1985; Page, 1988; Smirnov et al., 2009; Smirnov et al., 2017).

Until recently, the species differentiation among the genus *Leptomyxa* was mostly based on light-microscopic morphology. However, due to the high level of morphological polymorphism in these amoebae, their recognition is difficult and demands continuous observation of numerous trophozoites in cultures, data on the number of nuclei and the structure of the nucleus, data on encystment and cyst wall structure (e.g., Pussard and Pons, 1976a, 1976b, 1976c). However, even careful study may not result in unambiguous differentiation of closely related species. Nowadays, molecular data are essential for reliable species distinction of leptomyxid amoebae, and often this is the molecular difference that forces investigators to seek for the morphological characters that can further distinguish newly found isolates (Smirnov et al., 2017). Moreover, the above-cited study shows that 18s rDNA gene sequences among leptomyxid amoebae are rather conserved and even a small sequence difference may mark two species with clearly different morphology.

During studies of amoebae from the soil of West Siberia (Russia) we have found an isolate of a leptomyxid amoeba showing a peculiar morphology; its 18s rDNA sequence was significantly different from other known ones. Here we describe this isolate as a new species, *Leptomyxa silvatica* n. sp.

Material and methods

The strain described in the present study was isolated from the sample of the top 10 cm of Chernevaya taiga soil (see Abakumov et al., 2020 for the description of the sampling site) from a location near Tomsk city (Russia, West Siberia, Tomskaya oblast; 56.30693° N, 85.47063° W). To establish enrichment cultures, 0.01 g of mixed soil was placed in a 60 mm sterile Petri dish filled with a 0.025% cerophyl infusion made on PJ medium (Page, 1988; Prescott and James, 1955).

Enrichment cultures were incubated at room temperature and light. After 7−14 days samples were examined using a Nikon TMF 100 inverted microscope. Detected cells were individually transferred into 60 mm Petri dishes filled with the same medium using a tapered-tip Pasteur pipette. One of the successful clones was used to establish the studied culture.

Light microscopic data on living trophozoites and cysts were obtained using Nikon TMF 100, Leica DM2500 and Leica DMI3000 microscopes equipped with phase contrast and DIC optics. Both living cells and cysts were measured using an inverted microscope on the plastic surface of the Petri dish. For detailed study of the nuclear structure trophozoites were transferred to the glass-made object slides, left to adhere for several hours and further fixed with Bouin's solution and stained with iron hematoxylin as described by Page (1988).

For the single-cell PCR, cells were washed in autoclaved PJ medium, collected with the minimal possible amount of medium and placed in 200 mkl PCR tubes using freshly made tapered-tip Pasteur pipette for each cell. Tubes were exposed to several freezing-defreezing cycles (4 cycles from -18 °C to room temperature). Ready PCR mixture was added to the tube content to the final volume of 50 mkl. Primer pairs RibA/S12.2r and S12.2/SB (Pawlowski, 2000) were used for amplification. Thermal cycle parameters were: initial denaturation (10 min at 95

°C) followed by 39 cycles of 30 seconds at 94 °C, 60 seconds at 50°C and 120 seconds at 72 °C, followed by 10 min at 72 °C for the final extension. Amplicons were sequenced directly using the ABI-PRISM Big Dye Terminator Cycle Sequencing Kit with the S12.2, S12.2r, S6f, S4fc, S6ra and S20r primers (Adl et al., 2014; Medlin et al., 1988; Pawlowski, 2000). The resulting sequences were edited and assembled using ChromasPro software (Technilesium).

Obtained sequences were added to the alignment of Leptomyxida 18s rDNA sequences. Sequences were aligned automatically using the Muscle algorithm (Edgar, 2004) as implemented in SeaView 4.0 (Gouy et al., 2010), then the alignment was polished manually. The phylogenetic analysis was performed using the maximum likelihood method implemented in the PhyML program (Guindon and Gascuel, 2003) with the GTR + γ model. The number of invariant sites, alpha parameter and tree topology was optimized by PhyML; 1000 bootstrap pseudoreplicates were used; 1866 sites were selected for the analysis. The Bayesian analysis was performed on the same dataset using MrBayes 3.2.6 run at the CIPRES portal (Miller et al., 2010), the GTR model with gamma-correction for intersite rate variation (8 rate 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 1.7.1 (Rambaut et al., 2018); based on the estimates by Tracer, the first 35% of generations were discarded for burn-in.

The obtained consensus sequence was deposited in GenBank under the number MZ687121.

Results

1. Morphological description of *Leptomyxa silvatica* n. sp.

The general morphology of trophozoites was assessed during observation of cells on the plastic surface of the Petri dish, using an inverted microscope. Smaller and compact amoebae were comet-shaped or elongated, with several hyaloplasmic projections ending with a number of anastomosing lobes forming a small area of loboreticulopodia (Fig. 1, A-D). Rapidly moving amoebae had trapezoid-shaped with a wider frontal edge, which was divided into several lobes. They might carry adhesive filaments on the posterior edge of the cell (Fig. 1, E-J). Stationary and relatively small cells might produce several flattened pseudopodia of irregular shape extending in different directions. When a cell started locomotion, it usually formed one leading pseudopodium extending along the direction of movement, but also retained several lateral lobes (Fig. 1, A; Fig. 2, H). Sometimes the cell extended a pseudopodium or a hyaloplasmic eruption opposite to the direction of locomotion. Furthermore, this pseudopodium retracted and joined the main cytoplasmic body of the cell.

Among larger trophozoites comet-like forms were also documented. These large fan-shaped amoebae formed a kind of palisade of numerous short subpseudopodia directed frontally and laterally as well as a number of wide anterior lobes. Moving cells usually had a number of adhesive filaments along the posterior edge (Fig. 1, E-J; Fig. 2, F, H). Such comet-shaped amoebae were more inclined to fast, direct movement than reticulate forms and rarely formed pseudopodia extending opposite to the direction of movement.

The largest trophozoites were remarkably ramose and reticulate (Fig. 2, A-E). Short subpseudopodia were noticed along the outer contour of moderately branched cells. Amoebae also formed bunches of subpseudopodia or distinct flattened areas carrying short subpseudopodia as the end of major branches (e.g. Fig. 1, K, L; Fig. 2, I). When these amoebae moved, the leading part of the cell had a comet-shaped form, while in the central and posterior parts of the cell one or several elongated branches could be observed.

The length of actively moving trophozoites varied from 66 to 160 μm (mean 146 μm). The maximum width varied from 38 to 188 μm (mean 85 μm) and the minimum width varied from 5 to 94 μm (mean 30 μm).

According to our observations, cells contained numerous elongate vesicular nuclei from 5 to 11 μm in maximal dimension (mean 7 μm) (Fig. 2, F-L). Up to 30 nuclei were seen in large, comet-shaped cells. The nucleolus was rounded or, rarely, oblong, sometimes it had the shape of a thick spindle or of a long drop. This was especially well-visible in stained preparations (Fig. 2, H-I), while in DIC optics it was hard to distinguish nuclei among food vacuoles and other inclusions (Fig. 2, J-L). The refractile index
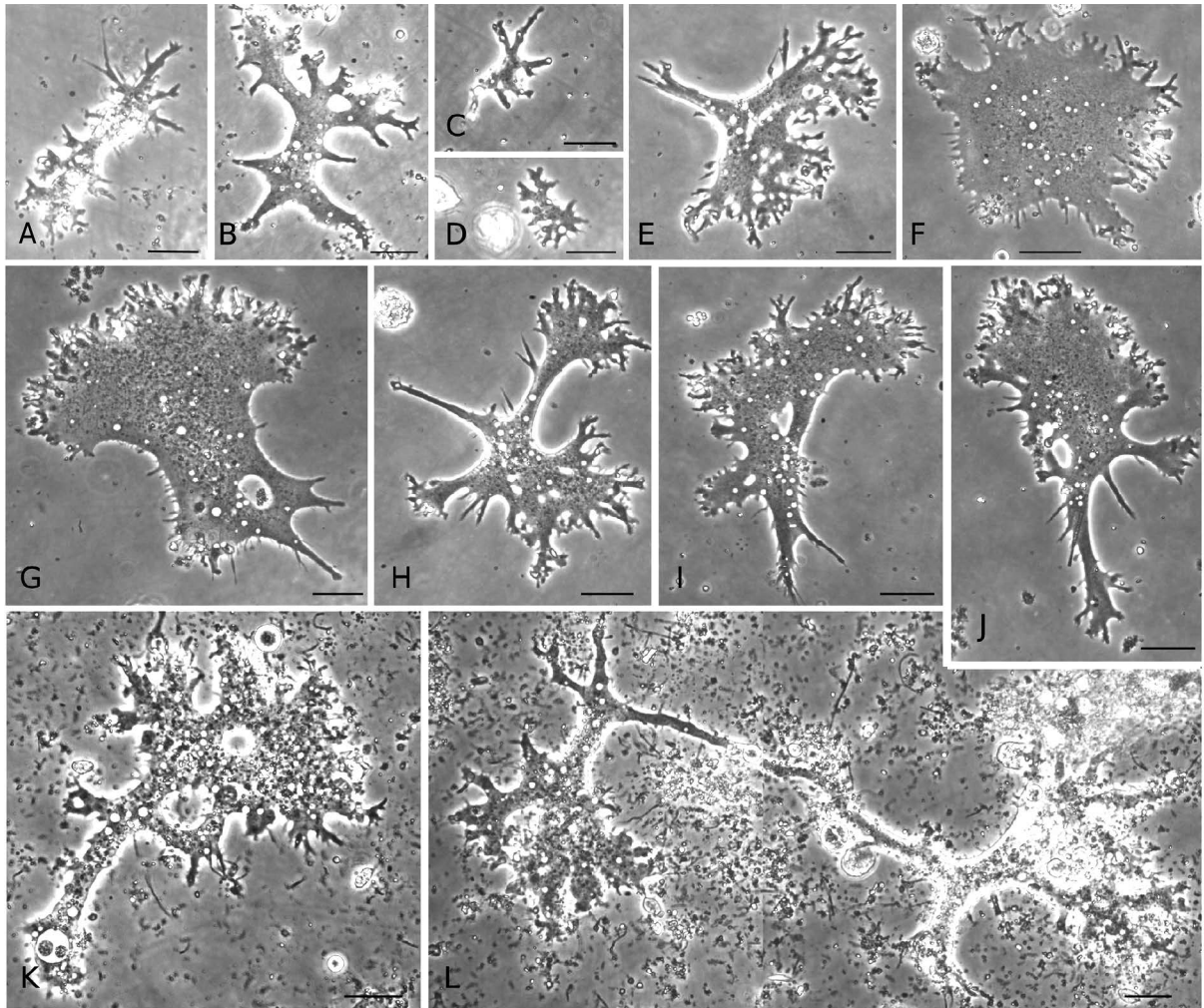
Fig. 1. Light microscopy of *Leptomyxa silvatica* n. sp. (A-D) − Small comet-like trophozoites, (E-J) − large comet-like trophozoites, (K) − an example of intermediate variant between comet-like and ramose forms, (L) − fragmentation in trophozoite with intermediate shape. Phase contrast. Scale bar is 50 µm throughout.

of the nucleolus was probably close to that of the karyoplasm and thus the nucleolus was very lowly contrasted in live cells. Numerous synchronously working contractile vacuoles were visible in the cytoplasm, their number apparently depended on cell volume (Fig. 2, J). The cytoplasm contained a high number of rounded small granules. Neither crystals, nor other opalescent inclusions were documented (Fig. 2, J-L). Sometimes noticeable food vacuoles were observed in the central or posterior part of the granuloplasm of moving cells. In ramose cells vacuoles tended to concentrate in the most massive area of the cytoplasm.

Single-walled cysts were rounded or ovoid, from 24 to 45 µm (mean 34 µm) in the largest dimension. Observed cysts always contained a single nucleus

located in the central part of the cytoplasm (Fig. 2, M-Q).

2. Molecular phylogeny

The 18s rDNA gene sequence of our isolate was always grouped within a clade, containing several environmental sequences, hence showing a certain distance from them. The sister group to this clade, and the closest named relative of our strain was the species *Leptomyxa arborea* (Fig. 3). The support for this position was always from average to low, but the entire clade containing this species and some more environmental sequences was properly supported (94/0.9 BS/PP supports). The entire genus *Leptomyxa*, and all the other genera
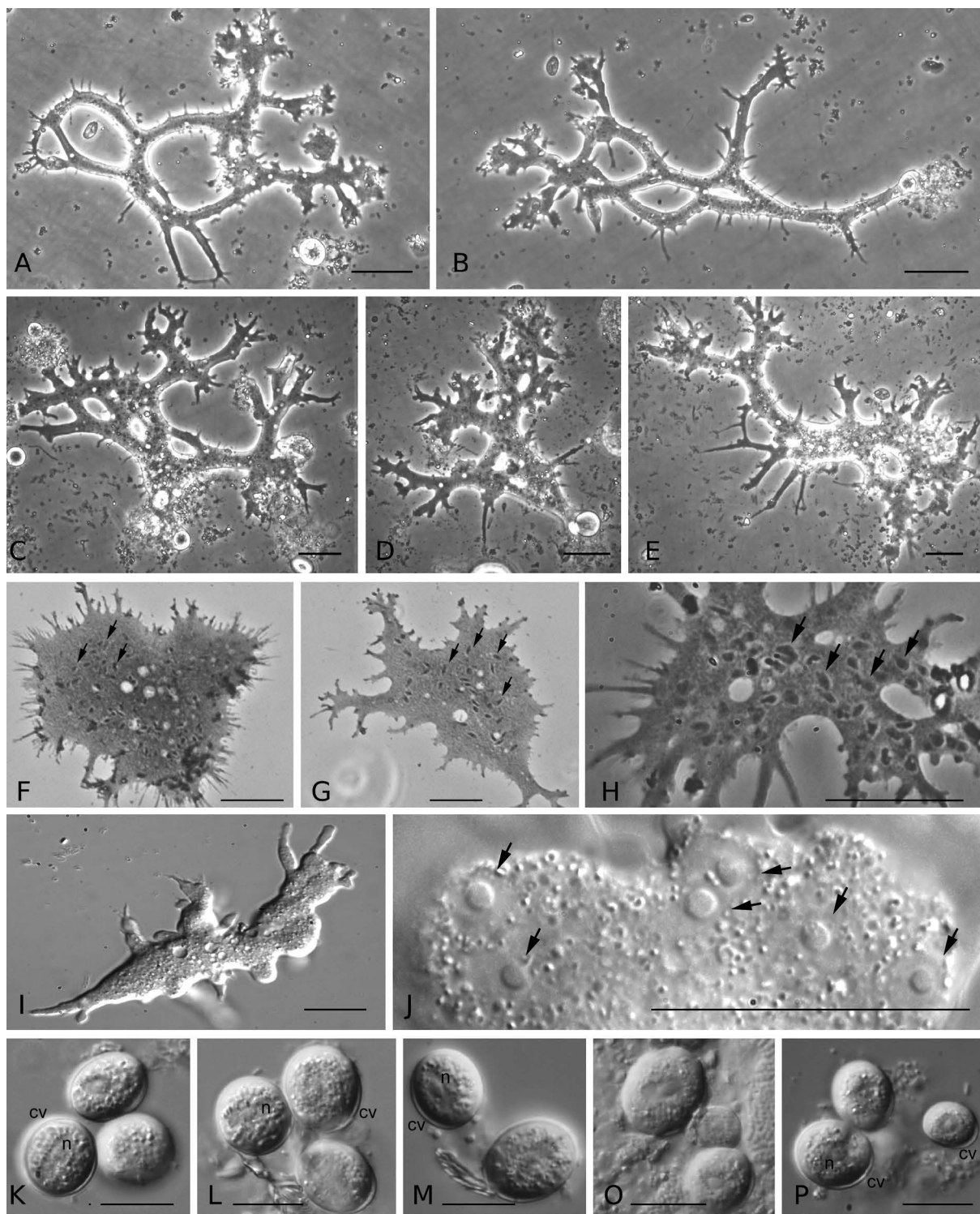
Fig. 2. Light microscopy of *Leptomyxa silvatica* n. sp. (A-E) − Rreticulate plasmodia-like trophozoites. Phase contrast. Scale bar is 50 µm. (F-H) − Stained preparations of trophozoites, iron hematoxylin. Several (not all visible) nuclei are indicated with *arrows*. Phase contrast, scale bar is 50 µm. (I) − Trophozoite in non-directed movement; (J) − higher magnification of the cytoplasm, numerous nuclei are indicated with *arrows*. DIC, scale bar is 25 µm. (M-Q) − Large and small living cysts in aggregations. DIC, scale bar is 25 µm.

of Leptomyxida, were fully supported, as well as the entire Leptomyxida clade. The arrangement of species within the genus *Leptomyxa* generally corresponded to that obtained by Smirnov et al. (2017).

The length of the majority of environmental sequences of leptomyxids available in GenBank rarely exceeds 600 bp. This makes the pairwise comparison of sequences difficult, because the difference in short fragments may not reflect the entire sequence divergence. The level of sequence identity between our isolate and the closest described species *L. arborea* measured in the longest possible fragment (1755 bp shared by both species) is 96.66%. The differences between the sequences of the studied isolate and *L. arborea* are the following (all positions are given in the sequence of *L. silvatica*): 241-314bp – one binucleotide and eight single nucleotide insertions; 756-757 bp CG vs AA in *L. arborea*; 1098-1129 bp – single nucleotide replacement and 26 nucleotides fragment that *L. arborea* lacks. More single and binucleotide replacements are distributed across the sequence. Other neighboring sequences are much shorter. When we perform the comparison in 569 bp fragment shared by the most of nearest neighbors, the level of identity of our isolate with *L. arborea* rises to 97.53%, with the highest level of identity with the sequence FO181391, originating from the soil of the province of Limburg, North-East Belgium (GenBank record data). It counts 99.64% (almost full identity). Further sequences FN394923 (98.38%) and FN394941 (95.77%) follow. This correlates with the position of sequences in the phylogenetic tree.

Discussion

The appearance of flattened extended reticulate trophozoites with posterior adhesive filaments convincingly indicates that the observed strain of naked lobose amoebae belongs to the genus *Leptomyxa* Goodey, 1915 sensu Smirnov et al. (2017).

Both compact and large comet-shaped forms of the studied isolate can be compared with typical trophozoites of *L. variabilis* Geisen et Burberg, 2017, but they produce numerous frontal spine-like subpseudopodia (Smirnov et al., 2017). Ramose forms of the studied isolate may resemble reticulate plasmodia-like trophozoites of *L. arborea* Berney, Geisen et Burger, 2017 and *L. valladaresi* Del Valle, Lorenzo-Morales et Maciver, 2017, but expanded

cells of the studied strain are less branched and often produce short subpseudopodia along the outer contour. Intermediate forms of trophozoites are rather compact and produce frontal lobes but still contain slightly branching parts were never clearly shown in *L. arborea* or *L. valladaresi* (Smirnov et al., 2017; Del Valle et al., 2017). The species *L. valladaresi* was described as primary uninucleate or possessing a few rounded vesicular nuclei (oval only when compressed), well-visible in stained preparations (Del Valle et al., 2017). They are very different in morphology from our pictures. The data on the nuclear morphology of *L. arborea* are not detailed enough, and available images do not clearly show the nucleus.

We have never documented a true limax monopodial form in the studied isolate, which is not a unique case. For example, *L. arborea* and *L. variabilis* are mostly described as expanded and reticulate (Smirnov et al., 2017), while in *L. flabellata, L. valladaresi, L. australiensis* and *L. ambigua* limax-like forms are frequently seen (Chakraborty and Pussard, 1985; Page, 1988; Smirnov et al., 2017; Smirnov, 2018). It seems that in many strains the detection of the limax-like form is a matter of observation duration and specific culture conditions, so it may be difficult to see them in fresh or mixed cultures.

The 18s rDNA sequence identity level of 96.66% is a rather high similarity for an eukaryotic organism. However, among leptomyxids 18s rDNA sequences are very stable, in contrast with many other amoebae lineages. This is the only group of amoebae where 18s rDNA sequences of distant isolates show complete identity and the sequence divergence between morphologically different species may be very low (Smirnov et al., 2009, 2017). For leptomyxid amoebae the difference found in the present study is significant, and if we consider it together with the morphological peculiarities, this all clearly votes for the species status of the studied isolate. Based on enlisted characteristics we consider the studied isolate as a new species and suggest naming it *Leptomyxa silvatica* n. sp. The origin of this species from Chernevaya taiga soil is interesting, because this soil represents a kind of rare, highly productive ecotope, known for its pronounced plant gigantism (see Abakumov et al., 2020). Hence, the closest similarity of its 18s rDNA sequence with several 18s rDNA fragments of unnamed isolates originating from Belgian soil may be evidence of the wider distribution of this species.
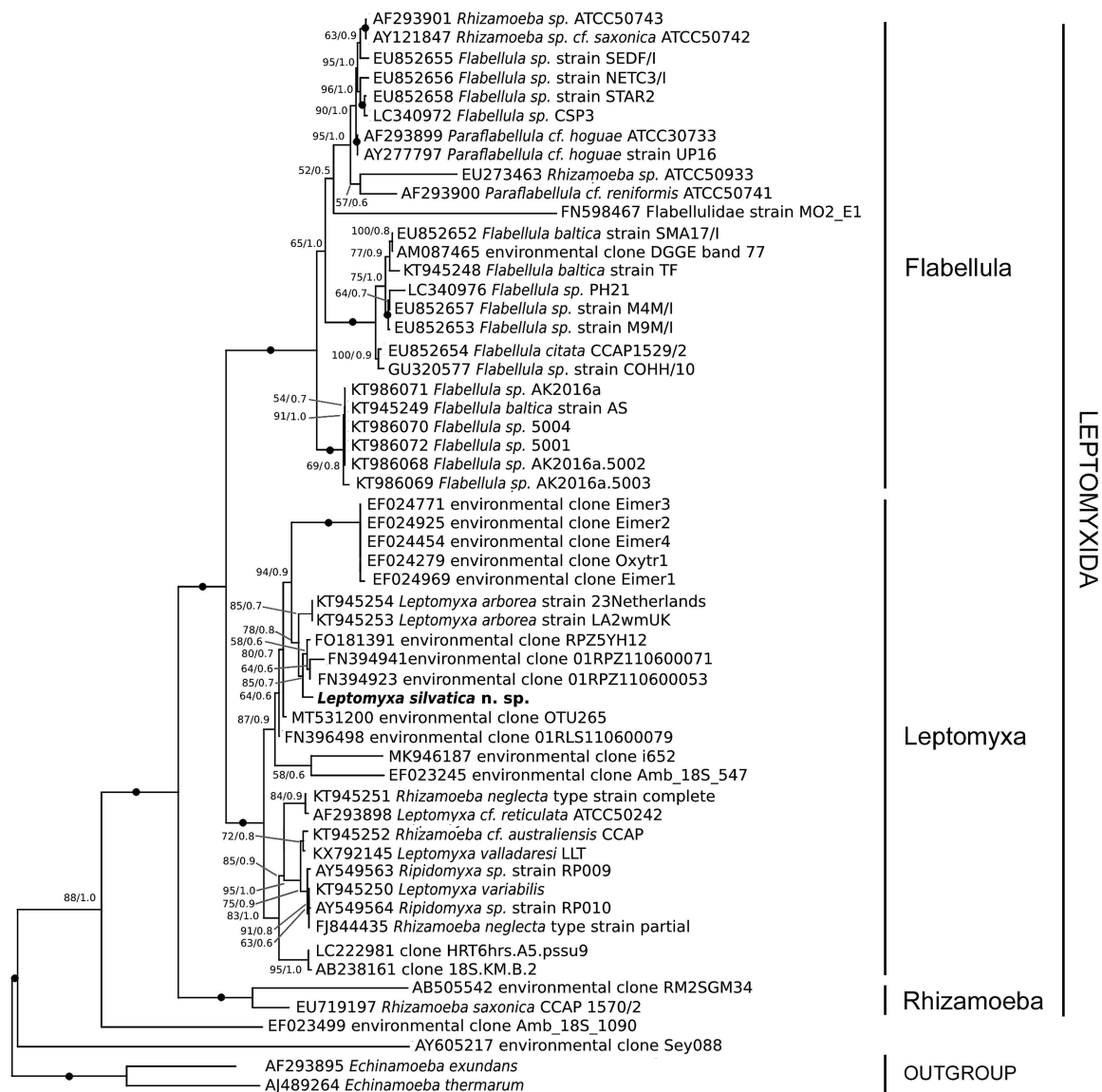
Fig. 3. Phylogenetic tree based on the SSU gene and showing the relationships between available SSU sequences of the order Leptomyxida. Bootstrap support (PhyML) / Posterior probability (MrBayes) are indicated; 1866 nucleotide positions used. Black dots indicate 1.0/100 supports (BS/PP).

Diagnosis: *Leptomyxa silvatica* n. sp.

Flattened, expanded or ramose amoebae. Moving cells form an area of loboreticulopodia on the frontal edge. Moving cells are 60-160 μm across in length and up to 190 μm in width. Trophozoites contain up to 20-30 ovoid vesicular nuclei up to 7 μm in largest dimension. Single-walled rounded or ovoid uninucleate cysts are 24-45 μm across.

Type material: Holotype, permanent preparation No 1049, stained with iron hemotoxylin, deposited in the collection of preparations of the Laboratory of Cytology of Unicellular Organisms, Institute of Cytology, St. Petersburg, Russian Academy of Sciences.

Type location: Chernevaya taiga soil from a location nearby Tomsk city (Russia, West Siberia, Tomskaya oblast; 56.30693° N, 85.47063° W)

Etymology: from latin word silva, forest. The name means "originating from the forest".

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