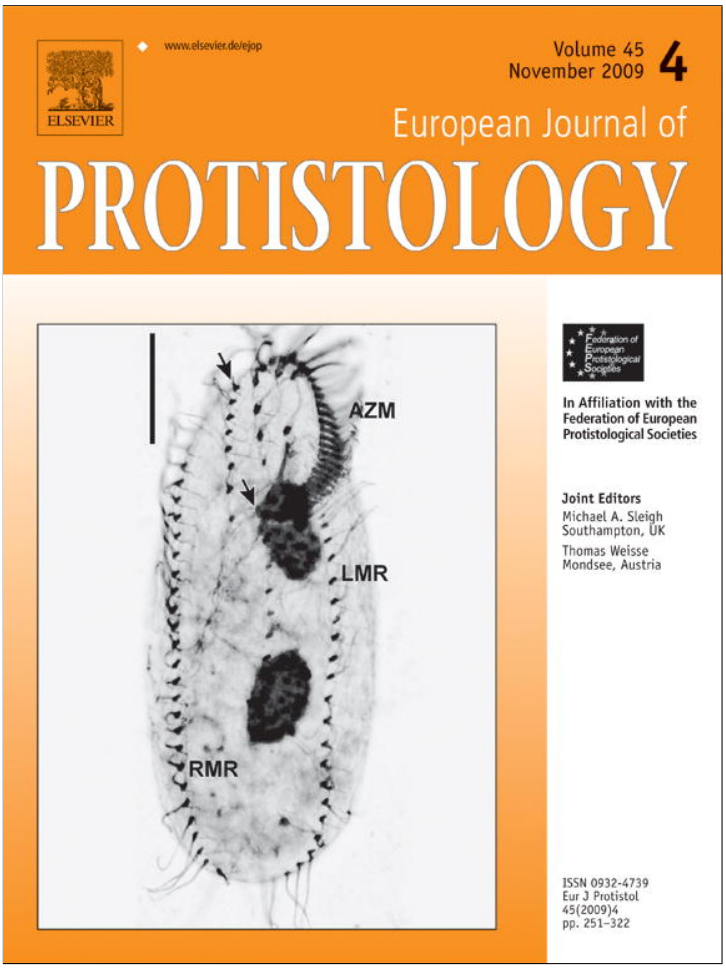

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Rhizamoeba neglecta n. sp. (Amoebozoa, Tubulinea) from the bottom sediments of freshwater Lake Leshevoe (Valamo Island, North-Western Russia), with notes on the phylogeny of the order Leptomyxida

Alexey Smirnov[a,*], Elena Nassonova[b], Jose Fahrni[c], Jan Pawlowski[c]

[a]*Department of Invertebrate Zoology, Faculty of Biology and Soil Sciences, St. Petersburg State University, Universitetskaja nab. 7/9, 199034 St. Petersburg, Russia*
[b]*Laboratory of Cytology of Unicellular Organisms, Institute of Cytology RAS, Tikhoretsky ave. 4, 194064 St. Petersburg, Russia*
[c]*Department of Zoology and Animal Biology, University of Geneva, Sciences III, 1211 Geneva 4, Switzerland*

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Abstract

A new species of Leptomyxida, named *Rhizamoeba neglecta* was found during studies of the amoeba fauna of the inner Lake Leshevoe located at Valamo archipelago (The Lake Ladoga, North-Western Russia). Light-microscopical and ultrastructural studies indicated that it represents a new species of Leptomyxida. The partial 18S rDNA sequence of this amoeba is very similar to that of *Leptomyxa reticulata.*. These organisms, however, are very different in LM morphology and biology. Organisms assigned to the genus *Rhizamoeba* do not form a single clade in the 18S rDNA tree. This may indicate that the genus is an artificial grouping or that a number of studied strains were misidentified. The phylogeny and the systematics of leptomyxids require further investigation.

Keywords: Amoebozoa; Leptomyxida; Systematics; Phylogeny; Ultrastructure

Introduction

The genus *Rhizamoeba* was established to unify amoebae that are monopodial in locomotion and possess adhesive uroidal filaments (Page 1972). At that time it was placed into the family Hartmannellidae. Pussard and Pons (1976) noted that in many respects amoebae of this genus resemble members of the order Leptomyxida and thus suggested to transfer the genus *Rhizamoeba* to this order. Page accepted this suggestion (Levine et al. 1980).

The known representatives of this genus were isolated from marine (Page 1972, 1974) and soil (Chakraborty and Pussard 1985; Goodey 1914; Page 1972) habitats. They were initially placed into different amoebae genera. *Rhizamoeba flabellata* was described as *Leptomyxa* (Goodey 1914); *Rhizamoeba australiensis* as *Ripidomyxa* (Chakraborty and Pussard 1985). The genus *Ripidomyxa* was recognized to be invalid (Page 1988), despite the fact that two recent sequences in the GenBank were named with this generic name (see Smirnov et al. 2008 for details). The ultrastructure is known for *Rhizamoeba saxonica* and *R. flabellata* (Cann 1984; Page 1980).

In the present paper we describe one more species of the genus *Rhizamoeba*, studied by light microscopy

*Corresponding author.
E-mail addresses: lobosea@gmail.com, polychaos@mail.ru (A. Smirnov).

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(LM), transmission electron microscopy (TEM) and 18S rDNA gene analysis and discuss the problems of the phylogeny and systematics of this group of amoeboid protists.

Material and methods

Amoebae were isolated from the samples of the bottom sediments of the Lake Leshevoe (Valamo Island, the Lake Ladoga, North-West Russia). Samples of the uppermost layer of detritus were collected at a depth of 0.2 – 0.5 m along the shore line of the lake between May and September from 1998 to 2007; cells were regularly found in the samples during all these years. The description is mostly based on the results obtained for the samples collected in 1998 and 1999 (LM and TEM) and in 2007 (molecular phylogeny).

In the laboratory the samples were inoculated on 1.5% non-nutrient agar (Page 1988) with an overlay of PJ medium (Prescott and James 1955) or in 0.05% Cerophyl infusion based on PJ medium (Page 1988). Amoebae fed on accompanying organisms while the exact food source remained unclear. Cells were cloned, but the clones obtained were not stable and the cloned cells usually died off after the attempt to transfer them to the fresh medium. Hence the present observations are based not on a single clone, but on a number of clones; all of which were clearly co-specific.

Light micrographs and measurements were done in the year 1998 using a MBI15-2 light microscope (LOMO, Russia). Amoebae were placed on the object slides or photographed right in the culture dish using water-immersion optics. At the same time the fixations for TEM were prepared. An attempt to use conventional glutaraldehyde-osmium tetroxide fixation (4% glutaraldehyde followed by osmium tetroxide postfixation) did not result in an acceptable quality of images. After a number of experiments, amoebae were fixed with the 0.5% osmium tetroxide made in 01.M Na-cacodylate buffer pH 7.3 for 20 min, washed 3 × 10 min in the same buffer, dehydrated in an ethanol series followed by acetone and embedded in Taab 812 resin. Sections were stained with uranil acetate and Reynold's lead citrate.

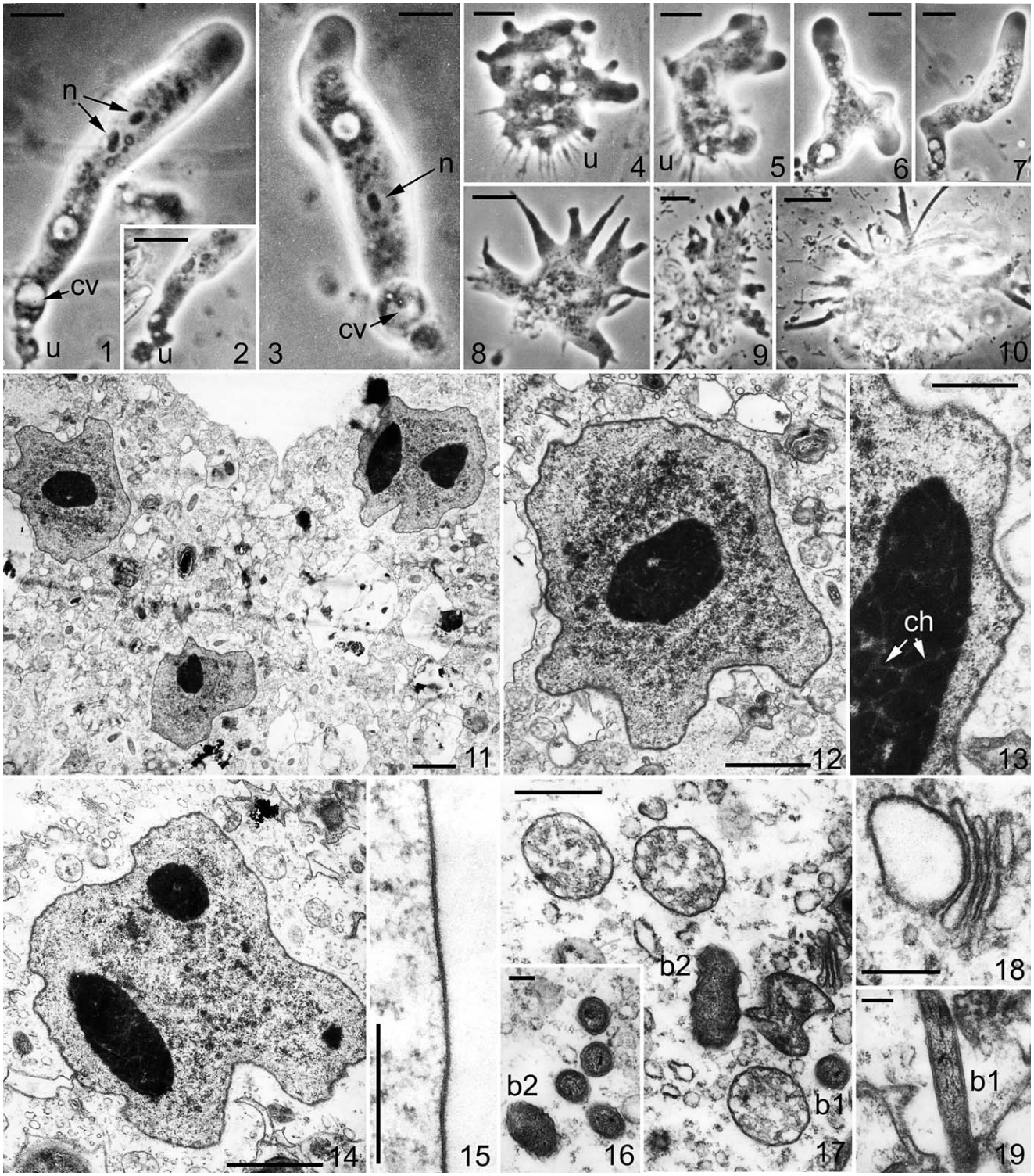
For molecular studies one of the best clonal cultures obtained was transferred to 0.05% cerophyl infusion (Page 1988) in 60 mm Petri dishes. After two weeks of growth it contained approx. 30 cells and appeared to be free from other eukaryotes. The dish was washed off in several changes of the culture medium, then the medium was completely removed from the dish and the bottom of the dish with adhered amoebae was immediately covered with 100 ml of guanidine thyocianate buffer, followed by DNA extraction as described in Pawlowski (2000). Primers RibA (5′> ac ctg gtt gat cct gcc agt <3′) and S20R (5′> gac ggg cgg tgt gta caa <3′) were used for DNA amplification. Thermal cycle parameters were: initial denaturation (5 min. at 95 °C) followed by 40 cycles of 30 s at 94 °C, 90 s at 50 °C and 60 s at 72 °C, followed by 10 min at 72 °C for final extension. The amplification product was purified using a GFX PCR Purification Kit (Amersham Biosciences), ligated into Topo TA Cloning® vector (Invitrogen) and cloned in One Shot® TOP10 *E. coli* ultracompetent cells (Invitrogen). Sequencing reactions used the ABI-PRISM Big Dye Terminator Cycle Sequencing Kit. From the cloned amplicon, a 780 bps fragment was sequenced using S12.2 (5′> gat cag ata ccg tcg tag tc <3′) and S20R (op. cit.) primers. The GenBank number of the obtained sequence is FJ844435.

The obtained sequences were aligned manually (the alignment is available on request) to other leptomyxids available from GenBank. Phylogenetic analyses were done using the maximum likelihood method as implemented in the PHYML program l50 (Guindon and Gascuel 2003) using the GTR + G + I model suggested by Modeltest (Posada and Crandall 1998). The number of invariant sites, alpha parameter and tree topology were optimized by PHYML. The non-parametric bootstrap analysis was performed with 1000 bootstrap pseudoreplicates. The sequences of all leptomyxids were found to be nearly structurally identical, so there was no need to exclude any variable region from the analysis. The alignment was analyzed twice, first using the entire length of all sequences (treating gaps as missing data) and then after cutting it to the length of the *R. neglecta* sequence (780 bps). The configuration of the tree was found to be identical in both cases, only the bootstrap support of some branches differed.

Results

Light microscopy

The locomotive form of this amoeba species always was monopodial, subcylindrical, with a pronounced anterior area of the hyaloplasm (Figs. 1, 3). In some cells it was relatively small, reaching 1/5 – 1/6 of the overall body length, while in others it was large, reaching up to 1/3 of the body length. Most of the cells were clavate, pronouncedly narrowing to the posterior end. Some had adhesive uroidal filaments, while few formed a bulbous or villous-bulbous uroid (Fig. 2), often with several trailing filaments. The moving cell showed a steady cytoplasmic flow with occasional eruptions in the area of the frontal hyaline cap. Sometimes moving cells stopped and the hyaloplasm erupted back along one side; often the cell changed the direction of locomotion after this, but could also continue movement in the



Figs 1–19. LM and TEM of *Rhizamoeba neglecta*. 1, 3 – locomotive forms; 2 – villous-bulbous uroid; 4–7 – successive stages of the shift of stationary amoebae to locomotion. 8 – motionless cell. 9 – a cell that stopped for a while after active movement. 10 – motionless cell covered with fecal pellets. 11 – three nuclei in a single TEM section. 12 – nucleus. 13 – larger view of the nucleolus with the system of channels. 14 – nucleus with two fragments of the nucleolar material. 15 – cell coat. 16 – endobiotic bacteria. 17 – mitochondria and endobiotic bacteria. 18 – dictyosome. 19 – sagital section of a rod-shaped bacterium (type 1). Scale bar is 10 μm (1-10) and 1 μm (11-19). Abbreviations: n – nucleus, cv – contractile vacuole, u – uroid, ch – system of fine channels in the nucleolus, b1 and b2 – two different types of bacteria in the cytoplasm.

initial direction. The length of the locomotive form varied from 70 – 140 μm,its breadth from 10 – 36 μm, the L/B ratio was 5 – 8.

When a cell started locomotion, it produced pseudopodia which were at first small but then became larger and more solid. These pseudopodia were of a narrowly conical or subcylindrical shape, with rounded tips and a large anterior area of the hyaloplasm. The amoebae started to relocate. During this process cells often formed a large adhesive uroid. Finally, the cell converted to the monopodial one and started locomotion (Figs 4–7).

Non-moving (resting) amoebae were flattened and of irregular shape. The cell produced irregular, sometimes branching but never anastomosing pseudopodia that were narrowing towards the tips and consisted mostly of the hyaloplasm (Fig. 8). The length of these pseudopodia reached twice the diameter of the central cytoplasmic mass. In most cases, however, the length did not exceed half of its diameter. The overall size of this kind of cell was up to 100 μm. Sometimes such amoebae produced a number of short pseudopodia directed upwards. Many resting cells in culture were covered with a mixture of fecal pellets, detritus and other material (Fig. 10). Some of the cells were completely covered with such a layer of various particles, and only tips of pseudopodia were visible from beneath. When such cells started to move, the space between two neighbouring pseudopodia (like those in Fig. 8) was often filled by the eruption of the hyaloplasm, which softened the outlines of the cell. (Fig. 4).

An amoeba that was moving but stopped for a while had a different appearance. Normally it was flattened, the main cytoplasmic mass was generally rounded and surrounded with small hyaline lobes and adhesive filaments, resembling the uroidal structures of the moving cell (Fig. 9).

The floating form was of radial type, with numerous tapering conical hyaline pseudopodia (Fig. 20A). The length of these pseudopodia was often nearly equal to the diameter of a central cytoplasmic mass, the longest ones were twice as long as their diameter.

The nucleus was rounded or oblong, often slightly flattened in live specimens with the single central nucleolus. The nucleolus often was flattened, sometimes erythrocyte-shaped. In some cells there were several pieces of the nucleolar material, often closely apposed to each other (Fig. 20B–D). Some cells contained several nuclei; up to 5 per cell were observed. The maximal dimension of the nucleus varied from 6 to 10 μm. Cells had one or (rarely) two contractile vacuoles, numerous opaque inclusions but no crystals. We did never identify cysts in our cultures. It must be noticed, however, that the cultures were never stable enough to ensure a complete observation of the entire life cycle of a cell.

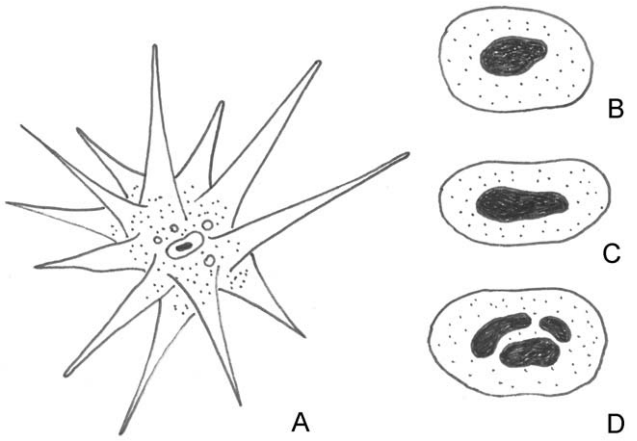


Fig. 20. A – floating form of *R. neglecta*. B-D – different observed variants of the organization of the nucleus and the nucleolus in this species (schemes).

Electron microscopy

The most remarkable characters of the species studied were the nuclei. Fig. 11 illustrates a cell with three nuclei in the field of view, located in the same section. The nuclei in our EM fixations were spherical with large depressions and had one nucleolus or several associated pieces of the nucleolar material (Fig. 12,14). The nucleolus was penetrated with numerous fine channels, forming a complex network of unclear function (Fig. 13). In nuclei with several fragments of nucleolar material each fragment was found to have similar channels.

The cell coat of this amoeba is a layer of thin, amorphous glycocalyx (Fig. 15). There was no evidence for any submembrane structures. The mitochondria had tubular, anastomosing cristae (Fig. 17). The dictyosomes were numerous and consisted of 3 – 6 cisterns; the presumable trans-cistern often was enlarged (Fig. 18). The bacterial endobionts were found in all cells studied. There were not less than two types of bacteria, with distinctive rounded, relatively short and long, rod-shaped morphologies (Figs 16–17, 19). In out TEM images there was no trace of vacuolar membranes surrounding them, but the general preservation of the cytoplasmic matrix under the fixation used does not allow a firm conclusion on this point.

Molecular phylogeny

To clarify the systematic position of our species, we performed a phylogenetic analysis with all named Leptomyxida sequences from GenBank and with environmental sequences that can be reliably assigned to Leptomyxida (Fig. 21). The sequence from our isolate was found to be very close to that of *Leptomyxa*

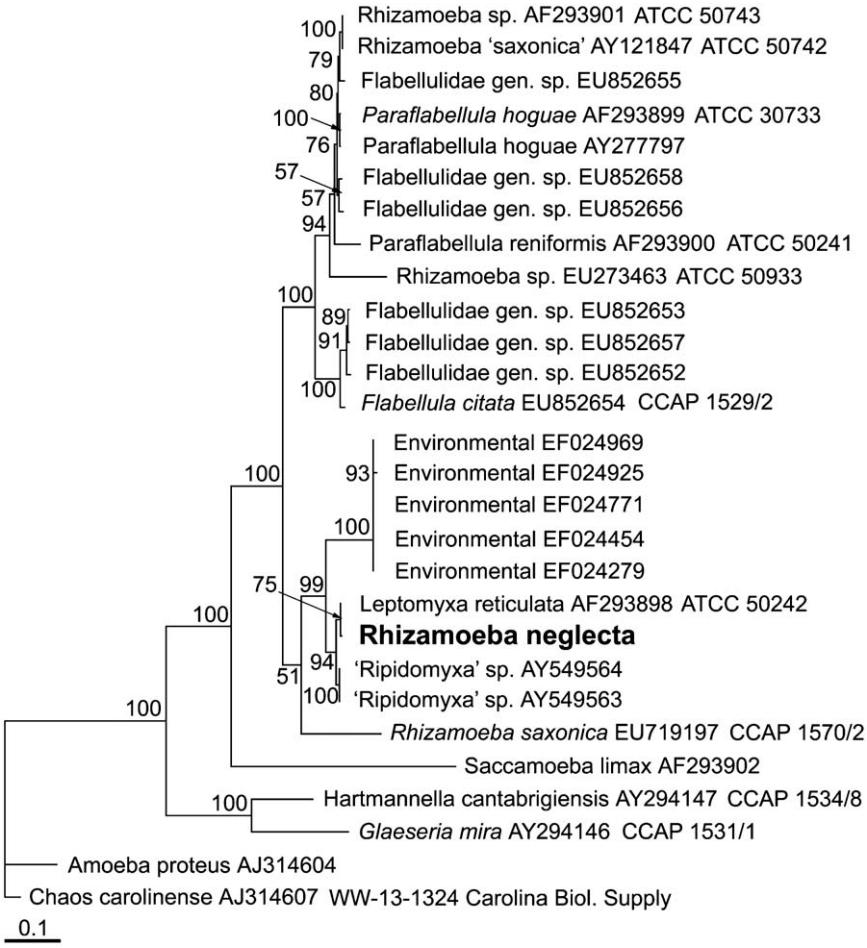


Fig. 21. ML phylogenetic tree of Leptomyxids (780 bps, PhyML; GTR 4 gamma rates, optimized number of invariant sites, gamma alpha parameter and tree topology; 1000 bootstrap pseudoreplicates). New sequence is in bold; sequences originating from the type strains are italicized.

reticulata produced by Amaral Zettler et al. (2000) and two sequences of '*Ripidomyxa*' sp. This group together with a number of environmental sequences forms a well-supported clade (99% bootstrap), to which *R. saxonica* is an outgroup, but with negligible bootstrap support. All these organisms form a sister group to a large, 100%-supported clade consisting of *Flabellula*, *Paraflabellula* and three sequences labeled as belonging to *Rhizamoeba*. Both analyses, i.e. using the entire length of the alignment or using the alignment cut to the length of the *R. neglecta* sequence (780 bps) resulted in identical tree topologies. In some branches, however, the bootstrap support was higher in the tree based on the 780 bps alignment (Fig. 21).

Our isolate differs from *L. reticulata* by minor sequence divergence-four positions of which three are indicated as mismatches in the *L. reticulata* AF293898 sequence (pos. 312 R-G; 456 Y-T; 710 R-A; 721 T-C; positions are indicated in the *R. neglecta* sequence). However, both species are very different in their morphology and biology. Compared to *R. neglecta*, *L. reticulata* is a plasmodial organism with hundreds of nuclei, normally reticulate, of low mobility, showing the monopodial locomotive form only temporarily and under certain circumstances.

Discussion

1. Systematic position and diagnosis of *Rhizamoeba neglecta* n.sp.

The studied species with its monopodial locomotive form, adhesive uroidal filaments and tubular mitochondrial cristae shall be classified into the order Leptomyxida, in agreement with 18S rDNA gene sequence analysis. Among leptomyxids, it shall be assigned to the genus *Rhizamoeba*, since it is the only genus of Leptomyxida that possesses a regular monopodial locomotive form and shows a pattern of locomotion similar to the one described above.

Among known members of the genus *Rhizamoeba*, the present species resembles *R. australiensis*, but shows a number of important differences. According to the published description, the monopodial locomotive form is rare in *R. australiensis*; it exists only for a short time at the air-water interface in culture, and the amoebae rapidly return to their usual flattened form (Chakraborty and Pussard 1985). In our cultures monopodial locomotive forms were common and more persistent; during one of the observations an amoeba moved monopodially on the object slide for more than 3 hours. According to the published photographs, *R. australiensis* has a rather small hyaline cap, not exceeding 1/5 – 1/6 of the cell length (Chakraborty and Pussard 1985; Page 1988). In our species the hyaline cap is well-pronounced and may reach up to 1/3 of the cell length. The length of moving *R. australiensis* is 50 – 180 µm, while in our strain it was 70 – 140 µm. The nucleus of *R. australiensis* is 6.5 – 13 µm (average 10 µm) in diameter and contains an oblong or oval nucleolus 3 – 5 µm in maximal dimension (Chakraborty and Pussard 1985, p. 134). In our species it is oblong, often flattened, 6 – 10 µm in length and often contains several fragments of nucleolar material. We never observed a characteristic flattened form, described for *R. australiensis* (op. cit. p. 135, Figs. 1 and 2). It may be possible that this form exists only in agar cultures without overlay, while the species investigated by ourselves did not grow on agar without overlay despite numerous attempts to establish such cultures. The uroidal structure of *R. australiensis* differs from those in our species.

The ultrastructure of *R. australiensis* was not studied when this species was initially described (Chakraborty and Pussard 1985) but later Cann (1984) studied the ultrastructure of a species which he identified as *Leptomyxa flabellata*. He suggested that this species must be transferred to the genus *Rhizamoeba*. Page (1988) concluded that the strain studied by Cann was misidentified and claimed it to be *R. australiensis*. If we were to accept that the images published by Cann (1984) represent *R. australiensis*, we would conclude that its organization of the nucleus and, especially, of the nucleolus appear to be very different from our strain.

There are a number of early descriptions of *Rhizamoeba* species. Our strain has certain similarities with *Rhizamoeba clavarioide*s (Penard 1902), but it differs well in the nuclear structure and the morphology of resting amoeba and amoeba in non-directed movement (Penard 1902; Siemensma 1987). Schaeffer (1926) described a number of monopodial amoebae with adhesive uroidal structures. He classified them into the genus *Trichamoeba* Fromental 1874. None of these species could be accepted as identical with the present strain.

The 18S r DNA sequence of the present strain differs from that of any known *Rhizamoeba* species; however, the type culture of *R. australiensis* does not exist, so there is no type sequence of this species. We conclude that the strain of *Rhizamoeba* described in the present paper is a new species.

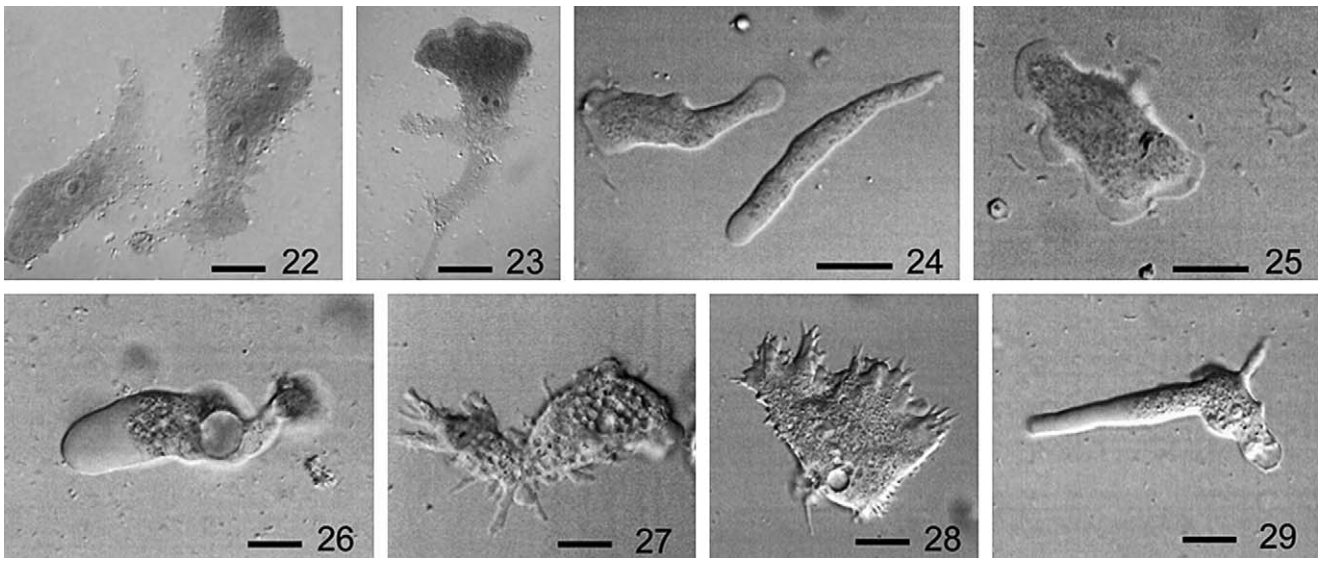
Diagnosis: *Rhizamoeba neglecta* n. sp. Length in locomotion 70 – 140 µm; breadth 10 – 36 µm; the cell is often clavate; adhesive uroidal filaments or villous-bulbous uroid. Frontal hyaline cap occupies up to 1/3 of the total length of locomotive cell. Uninucleate or multinucleate cells. Nucleus 6 – 10 µm in length, oblong and flattened, vesicular or with several fragments of the nucleolar material. The nucleolus contains a system of fine channels, visible in EM. Freshwater. Type location: The Lake Leshevoe, Valamo Island (The Lake Ladoga, North-Western Russia). The type slide is deposited with the collection of slides of the Biological Institute of St. Petersburg State University.

Differential diagnosis: Resembles *R. australiensis* but differs from this species in size and organization of the locomotive form, size and the organization of the nucleus and in the ultrastructure of the nucleolus.

Etymology: From the Latin word neglectus to reflect (a) another, local name of the Lake Leshevoe ('Gluhoe'), which could be translated as "abandoned, forsaken" lake and (b) the long story of the description of this species, found and treated in 1998, but initially considered to be co-specific with *R. australiensis* and stored for almost 10 years among my Valamo records.

2. Systematics and phylogeny of Leptomyxida

The analysis of the present tree indicates that the genus *Rhizamoeba* is polyphyletic; amoebae formally assigned to this genus occupy very different positions in the phylogenetic tree. Taking into account the difficulty of identification of naked amoebae, we must first of all consider the position of the type strains in the phylogenetic tree. Among all sequenced *Rhizamoeba* strains, only *R. saxonica* is a type one. This species is very peculiar in LM morphology, possessing two alternative locomotive forms (long, worm-shaped versus flattened, fan-shaped; Figs 24-25) and ultrastructure. Furthermore, it is the only amoebae species possessing submembrane structures – collosomes (Page 1980). In its LM morphology it is rather similar to the type species of this genus – *Rhizamoeba polyura* (Page 1972). The latter may be also monopodial or flattened and fan-shaped, sometimes even branched into several "arms", each with a fan-shaped end (Page 1972; 1974; see also Figs. 22 and 23). In the 18S phylogenetic trees *R. saxonica* (CCAP 1570) tends to occupy either the basal position to all Leptomyxida (Smirnov et al. 2008) or weakly group with the *Leptomyxa reticulata* - *R.neglecta*-'Ripidomyxa' clade (present study). However, if we cut the alignment according to the length of the *R. neglecta*



Figs 22–29. Other representatives of the genus *Rhizamoeba*. 22–23 – photographs of the stained type preparations of *Rhizamoeba polyura* deposited with the British Museum (Natural History). 24–25 – two alternative forms of *Rhizamoeba saxonica* CCAP 1570/2 strain. 26–27 – *Rhizamoeba flabellata* CCAP 1546/2 strain. 28–29 – photographs of a strain held in CCAP as *Rhizamoeba australiensis*. Scale bar in 10 μm.

partial sequence (780 bps), *R. saxonica* occupies the basal position to Leptomyxids with 100% bootstrap support. Unfortunately, the culture of *R. polyura* was lost, so the type sequence is not available. However, from the LM morphology we can suggest that these two marine species must be closely related, representing "core" *Rhizamoeba*.

A third known marine *Rhizamoeba* – *R. schnepfii* – is relatively poorly described (Kühn 1996/97). The analysis of the published data, especially the notes on its "semi-eruptive" movement and characteristic traces of the eruptive activity visible in the illustrations (Kühn p. 279, Fig. 1–3), as well as the floating form illustrated by Kühn (p. 279, Fig. 4) suggest that it may be a heterolobosean. The presence of trailing uroidal filaments is not unique for *Rhizamoeba*; they may also be present in *Vahlkampfia,* often as a package of short collopodial filaments, or few trailing ones (Page 1983) – very similar to the uroidal structures illustrated and described by Kühn. Ultrastructural studies are necessary to confirm that this species really belongs to the genus *Rhizamoeba*.

The group of freshwater and soil species consisting of *R. flabellata* (Figs 26-27), *R. neglecta* and a strain that was held in CCAP under the name *R. australiensis* (but never officially deposited with the collection; Figs 28-29) show some differences from the "core *Rhizamoeba*" in LM morphology. They also may move as monopodial subcylindrical cells, often pronouncedly clavate. An alternative locomotive form in these species, however, is irregularly triangular, often with numerous conical pseudopodia of different length. It is not yet clear if this difference correlates with phylogeny. We need to have better LM descriptions of sequenced strains to be conclusive on this. However, it is remarkable that both strains of 'Ripidomyxa' and *R. neglecta* very closely group with *Leptomyxa reticulata*. It may be an indication that the SSU gene is too conservative to reliably differentiate these species. A similar situation occurred with several closely related vannellid amoebae species (Smirnov et al. 2007). However, *L. reticulata, 'Ripidomyxa* sp.' and *R. neglecta* are organisms so dissimilar that it raises the question whether the strain ATCC 50242 sequenced by Amaral Zettler et al. (2000) was correctly assigned to the species *L. reticulata*. The images of *L. reticulata* published by Page (1988, 1991) show rather flattened, branched, ramose organisms with expanded sheets of the hyaloplasm at the ends of the pseudopodial "arms". This morphology is very congruent with Goodey's (1914) description and illustrations and with the images that were published by Pussard and Pons (1976). A very similar organism was found in North-Western Russia (illustrated in Smirnov and Goodkov 2000). However, the illustration of *L. reticulata* in Rogerson and Patterson (2000, p. 1046) shows a somewhat different organism that lacks the ramose organization as well as the characteristic flatness at the ends of the pseudopodia. *L. reticulata* is a very characteristic species, but leptomyxid amoebae are very polymorphic. In order to clarify this question, the achievement of further detailed LM data on strain ATCC 50452 remains desirable.

Since *R. saxonica* CCAP 1570/2 is a type strain of this species, the ATCC 50742 strain, very distant in the tree,

cannot be named '*R. saxonica*' and must be described as a separate species. The ATCC strain 50933, deposited to GenBank as *Rhizamoeba* sp. (Tekle et al. 2008) and now named in ATCC as '*Biomyxa* sp.' may be another, as yet undescribed leptomyxid, but not *Biomyxa* – an amoeboid organism of unclear systematic position with fine, branching and sometimes anastomosing pseudopodia (see Leidy 1879). The present analysis also confirms the finding that among five environmental sequences (Fig. 21), assigned in GenBank to Eimeriidae and Ciliophora, some belong to leptomyxids (T. Cavalier-Smith, personal communication).

Finally, the status of the genus *Paraflabellula* remains unclear. The sequence of *P. hoguae* (Sawyer 1975) may be considered a type one, obtained from a strain deposited by T.K. Sawyer. The ATCC annotation says that the strain of *P. reniformis* was also deposited by T.K. Sawyer although this species was described by Schmoeller (1964) from the Baltic Sea and has never been re-isolated since this time (to our knowledge), so the origin of Sawyer's isolate remains unclear. All species of *Flabellula* and *Paraflabellula* form a large, 100%-supported clade, together with three '*Rhizamoeba*' strains, but are mixed within this clade. The primary difference between *Flabellula* and *Paraflabellula* is the presence of lobes and short subpseudopodia on the frontal area of the hyaloplasm, which may be physiological at least in some species. For example, some trophozoites of *Flabellula baltica* may be rather similar with *Paraflabellula* (see Smirnov 1999). The CCAP 1529/2 strain of *F. citata*, after a long period of cultivation on agar without overlay, also resemble *Paraflabellula* when placed in the water drop on the coverslip (observation by A. Smirnov). In conclusion, the systematics of Leptomyxida requires further detailed study.

Acknowledgements

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