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ORIGINAL ARTICLE

A New Freshwater Naked Lobose Amoeba *Korotnevella venosa* n. sp. (Amoebozoa, Discosea)

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18S rDNA; Dactylopodida; molecular phylogeny; scales; systematics; ultrastructure.

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ABSTRACT

A new freshwater species of naked lobose amoebae *Korotnevella venosa* n. sp. isolated from freshwater pond in St. Petersburg, Russia was studied with light and transmission electron microscopy. Basket scales of this species have six vertical columns supporting perforated rim. The latter has tongue-like broadening with membranous region. Vertical columns bifurcate at both ends so that neighboring columns are connected by their bifurcations forming combined structure. Basket scales of *K. venosa* are similar to those of *Korotnevella hemistylolepis* in having six full-length vertical columns and perforated rim. At the same time, they are different in having tongue-like broadening of perforated rim with membranous region and absence of six half-length columns and an intermediate crosspiece. Phylogenetic trees based on 18S rDNA gene placed *K. venosa* either at the base of the whole *Korotnevella* clade, next to *K. hemistylolepis*, or as a sister to the clade comprising *Korotnevella* species with latticework basket in large scales.

THE genus *Korotnevella* Goodkov, 1988 is a member of the family Paramoebidae (Amoebozoa, Discosea, Flabellinea, Dactylopodida) (Smirnov et al. 2011) and comprises scale-bearing dactylopodid amoebae, which lack kinetoplastid symbiont, *Perkinsela amoebae*-like organism, or PLO (Dyková et al. 2008). To date, six freshwater (Schaeffer 1926; Smirnov 1999; Udalov 2015, 2016) and three marine (O'Kelly et al. 2001; Smirnov 1996–97) species of *Korotnevella* are known.

Shape and structure of scales in *Korotnevella* are considered to be species-specific (O'Kelly et al. 2001; Page 1981; Pennick and Goodfellow 1975; Smirnov 1996–97, 1999; Udalov 2015, 2016). Based on scale morphology, all known *Korotnevella* species are combined in three groups (O'Kelly et al. 2001). "Group 1" unifies the most of species: *Korotnevella stella* (Schaeffer, 1926) Goodkov, 1988, *Korotnevella bulla* (Schaeffer, 1926) Goodkov, 1988, *Korotnevella hemistylolepis* (O'Kelly et al. 2001), *Korotnevella monacantholepis* (O'Kelly et al. 2001), *Korotnevella limbata* Udalov, 2015, and *Korotnevella heteracantha* Udalov, 2015. All representatives of this group have two types of scales (large basket scales and smaller dish-shaped scales). "Group 2" includes the only species *Korotnevella nivo* Smirnov, 1997 with uniform crown scales, resembling those of *Paramoeba eilhardi*. "Group 3" includes *Korotnevella discophora* Smirnov, 1999 and recently described *Korotnevella fousta* Udalov, 2016 with uniform ovoid or disk-shaped scales with central cone, resembling a sombrero hat.

Recent molecular phylogenetic study (Udalov 2016) revealed monophyly of the genus *Korotnevella*. However, grouping of its species according to the scale structure was recently doubted; there are data suggesting paraphyly of *Korotnevella* "Group 1", because *K. hemistylolepis* was grouped at the base of *Korotnevella* tree, and was cut off from the rest of this group by *K. fousta*, which has uniform disk-shaped scales.

This paper describes one more *Korotnevella* species with basket and dish-shaped scales named here *Korotnevella venosa* n. sp. This is the same strain that was mentioned by Zlatogursky et al. (2016) under the name "*Korotnevella* sp."

MATERIALS AND METHODS

Isolation and culturing

Upper layer of sediments at 30–40 cm water depth was collected with 500-ml sterile container on January 24,

2011 from the freshwater pool in the greenhouse of the botanical garden of Saint Petersburg State University, St. Petersburg, North-Western Russia (N 59°56′E 30°17′).

Sample was immediately transported to the laboratory and ca. 500 mg of sediment was inoculated in 60-mm Petri dishes with Prescott–James (PJ) medium (Prescott and James 1955). Dishes were examined using a phase-contrast inverted Nikon Eclipse TS 100-F microscope after a week of incubation. Individual cells were collected by tapering Pasteur pipette, washed in fresh sterile medium and transferred to 60-mm dishes filled with 0.025% wheat grass (WG) (Weizengras, Sanatur GmbH, D-78224 Singen) extract made on PJ medium to obtain clonal cultures. The strain was maintained on accompanying bacteria; in addition, nonidentified strain of small vannellid amoeba was added to the culture dishes as a food. Cultures were stored at 15 °C under room light.

Light microscopy

Living cells were observed, photographed, and measured in cultures, in plastic Petri dishes, using inverted Leica DMI3000B microscope (63X lens) equipped with phase-contrast optics. Differential interference contrast (DIC) images were made on the glass object slides using a Leica DM 2500 microscope (100X oil immersion lens).

Transmission electron microscopy

For transmission electron microscopy, cells were fixed in Petri dishes at 4 °C with 2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 40 min, and then postfixed with 1% osmium tetroxide in the same buffer for 1 h. Amoebae were washed with buffer at room temperature three times between fixation steps and prior to dehydration, 5 min each time. After osmium fixation, amoebae were scraped away from the substratum, concentrated by centrifugation at 260 *g* (RCF), and embedded in 2% agar. The pieces of agar containing amoebae were cut out and dehydrated in a graded ethanol series followed by acetone, and embedded in Epon 812 resin (Fluka, Buchs, Switzerland). Silver to light gold sections were made by Leica EM UC6 ultramicrotome with glass knife. Sections were double-stained with 2% uranyl acetate in 70% ethanol for 15 min and Reynold's lead citrate for 2 min. The whole mount preparations of the scales were made by collecting individual cells from cultures, washing them in bidistilled water, placing on the Formvar-coated aperture grids followed by air drying. Sections and whole mounts were observed using a JEOL JEM-1400 electron microscope operated at 80 kV.

Phylogenetic analysis

Sequences of 18S rDNA gene of this strain were deposited in GenBank by Dr. V. Zlatogursky under accession numbers KU681502, KU681489–KU681494. Of them, KU681502 sequence was used for the present analysis. This sequence was added to the dataset used in Kudryavtsev and Pawlowski (2015) paper (courtesy of Dr. A. Kudryavtsev). Sequences were manually aligned using SeaView v. 4.3.3 (Gouy et al. 2010). For the subsequent phylogenetic analysis, 50 sequences including all available *Korotnevella* strains and a number of other Discosea with 1,507 unambiguously aligned nucleotide positions were selected. Maximum likelihood phylogenetic analysis was performed using PhyML (Guindon et al. 2010) using GTR + Γ model of evolution with eight substitution rate categories and 100 independent searches starting from random trees and RaxML (Stamatakis 2006) at CIPRES portal (Miller et al. 2010) with 25 substitution rate categories; model of evolution and number of independent searches starting from random trees were the same as in PhyML analysis. The best-scoring tree in both cases was tested using multiparametric bootstrapping (1,000 pseudoreplicates). Bayesian analysis was performed on the same dataset using MrBayes v. 3.2.2 (Ronquist and Huelsenbeck 2003) with two independent runs of eight MCMC chains for 9,758,000 generations and a burn-in of 25%. Average standard deviation of split frequencies reached 0.001413 at the end of the run.

RESULTS

Light microscopy

Locomotive form excluding the length of the subpseudopodia was 32–75 μm (average 47.4 μm) in length, and 14–41 μm (average 25.2 μm) in width; length/breadth ratio (L/B) was 1.0–4.0 (average 2.0), $n = 59$. Locomotive forms usually had elongated, more or less triangular (Fig. 1A–C) or rectangular (Fig. 1D, E) outlines, also many cells were broad and fan-shaped (Fig. 1C), or nearly squared (Fig. 1D). The hyaloplasm occupied one-ninth to one-fourth of the total cell length. Subpseudopodia formed from the anterior hyaloplasm during directed locomotion were often very short, and resembled short papillate protrusions (Fig. 1B, D). Some of them were more elongated (Fig. 1A, C); but only a few were really long (Fig. 1E). The average length of subpseudopodia was 2 μm ($n = 220$), the longest ones reached 11 μm. Subpseudopodia were usually retracted soon after their formation. However, some of them moved to the posterior end of the cell and then were withdrawn. During this process, these subpseudopodia became longer and thinner (Fig. 1E). No differentiated uroidal structures were observed. Rate of the locomotion on a plastic substrate at 20 °C was 38–63 μm/min (average 47.7 μm/min, $n = 9$) or approximately 1 length of the cell per minute.

Stationary and nondirectionally moving cells usually had irregular shape. Some cells had numerous short papillate subpseudopodia (Fig. 1G); others formed several long and thin subpseudopodia (Fig. 1F).

Mature floating form had more or less spherical or elongated central cell mass (18–41 μm, mean 25.7 μm, $n = 26$) with 4–13 ($n = 20$) pseudopodia. Pseudopodia of the floating form reached up to 30 μm ($n = 30$). They were either straight or curved (Fig. 2).

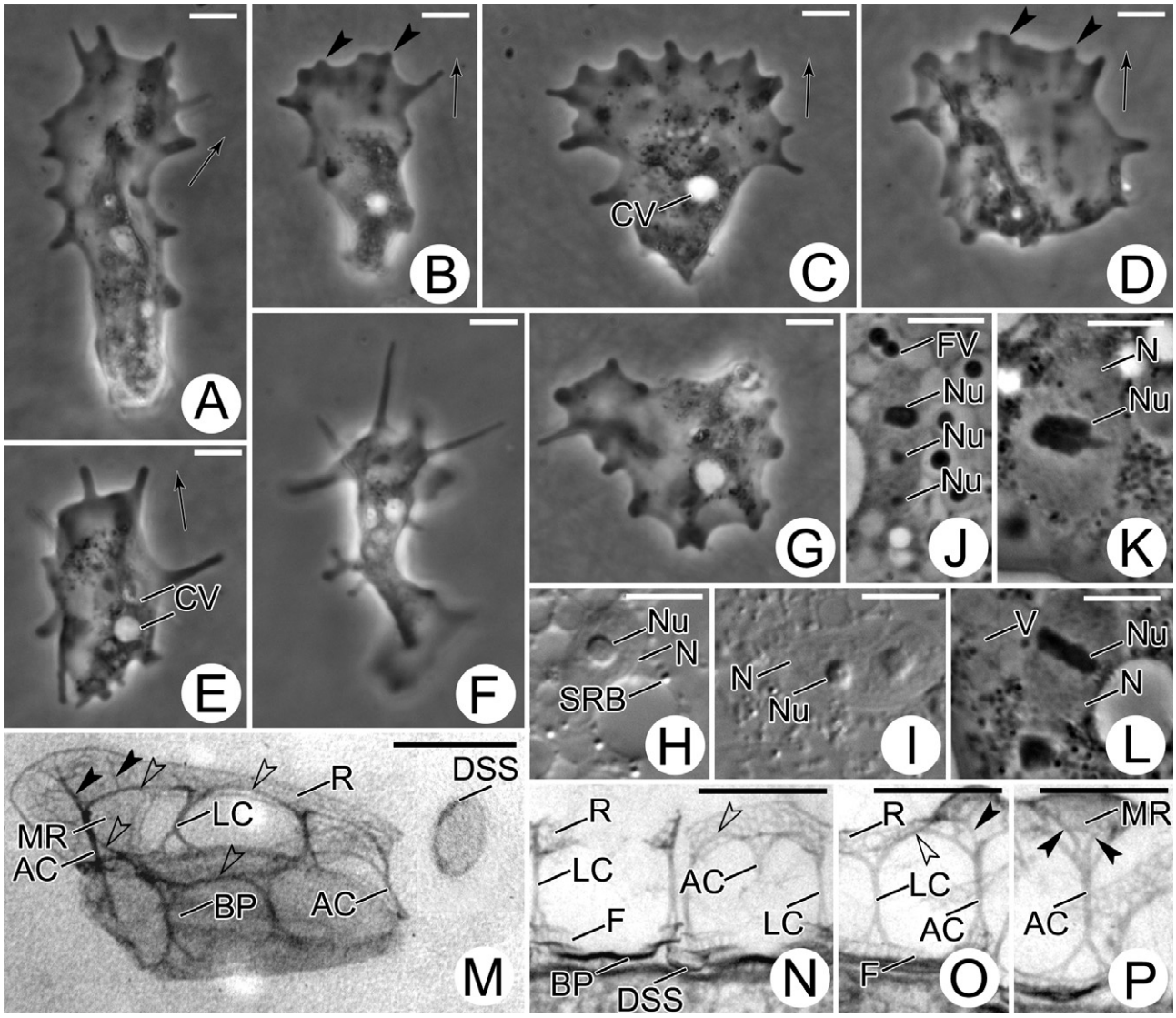

Figure 1 *Korotnevella venosa* n. sp. Light microscopic images (**A–L**). Phase contrast (A–G, J–L) and DIC (H, I). Transmission electron microscopic images (**M–P**). (A–E) Locomotive forms. Arrows indicate the direction of movement, black arrowheads denote short subpseudopodia. (F, G) Stationary or nondirectionally moving cells. (H–L) Cells compressed with a cover slip to visualize the nucleus. (M) The whole mount of scales. Note veins-like branches of apical column (black arrowheads) and connections of two neighboring columns (transparent arrowheads). (N) Cross-section of two basket scales showing a basal plate with a depression (left basket scale) and connections of two neighboring columns (transparent arrowheads). (O, P) Oblique sections of basket scales showing apical column with vein-like branches (black arrowheads) and connections of two neighboring columns (transparent arrowheads). AC, apical column; BP, basal plate; CV, contractile vacuole; DSS, dish-shaped scale; F, fenestrated flange; FV, food vacuole; LC, lateral column; MR, membranous region of perforated rim; Nu, nucleolus; N, nucleus; V, optically transparent vacuole; R, perforated rim; SRB, spherical refractile bodies. Scale bars = 10 μm in (A–L), 0.2 μm in (M–P).

Amoebae had a single ellipsoid nucleus (Fig. 1H–L). The nucleolus was either very small and ball-shaped (Fig. 1H), or represented by two or more ball-shaped or elongated nucleoli of equal (Fig. 1I) or unequal (Fig. 1J) size. Sometimes the nucleolus had elongated (Fig. 1L) or even irregular (Fig. 1K) shape. We observed these variations several times in cultures of various age. Besides a large nucleolus (or nucleoli), the karyoplasm contained small granules (Fig. 1J–L). The nucleus was 7.6–11 μm long (average 9 μm) and 3.8–7.8 μm wide (average 5.5 μm), (n = 21); the diameter of the nucleolus was about 3 μm, (n = 25).

Two contractile vacuoles were present, sometimes they were seen simultaneously (Fig. 1E). The cytoplasm contained dark granules, spherical refractile bodies (Fig. 1H), food vacuoles with a food content (Fig. 1J), and optically transparent vacuoles (Fig. 1L).

We never observed cysts in our cultures.

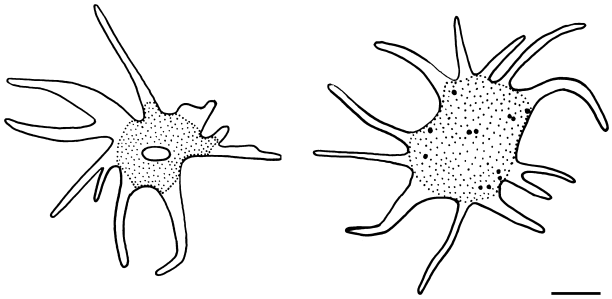

Figure 2 Floating forms of *Korotnevella venosa* n. sp. Line drawings. Scale bar = 10 μm.

Electron microscopy

The plasma membrane of the cells was completely covered with a layer of scales of two types: larger basket scales (Fig. 3B, C) and smaller dish-shaped scales (Fig. 3A). Dish-shaped scales had typical for this scale type structure and were ellipsoid in outline (Fig. 1M, N), 110–154 nm (average 133 nm) (n = 66) long, 57–94 nm (average 76 nm) (n = 66) wide and 19–30 nm (average 24 nm) (n = 22) height.

Basket scales were 393–501 nm (average 447 nm) (n = 34) long, 177–276 nm (average 234 nm) (n = 34) wide and 244–324 nm (average 289 nm) (n = 14) height. Basket scales were measured from the basal plate to the edge of tongue-like broadening of the perforated rim, so it was the maximal height of the scale. The basal plate of the scale was solid, slightly concave, and depressed on the underside (Fig. 1N). From the edges of the basal plate, a fenestrated flange arose (Fig. 1N, O). In cross-section, it was visible that this flange was extended laterally as a ledge (Fig. 1N). From fenestrated flange, six vertical columns arose; two apical columns grow from the ends of the scale (Fig. 1M–P) and four lateral columns were situated along the sides of the scale (Fig. 1M–O). Each column bifurcated at both ends and neighboring columns were joined by their branches (Fig. 1M–O). Thus, all six columns formed a single structure. The upper branches of columns were adjoined to a wide perforated rim (Fig. 1M–O). At one end of the scale, this rim had a tongue-like broadening with a membranous middle region; edges of this broadening were perforated (Fig. 1M, P). At this end of a scale, apical column and its branches gave outgrowths toward the perforated edge of the rim. They were adjacent to the membranous part of the rim and resembled veins of a leaf (Fig. 1M, O, P). Toward this end of the scale its height gradually increased. The opposite end of the scale had a smaller height, no broadening with membranous region and no additional veins-like outgrowths of apical columns (Fig. 1M, N).

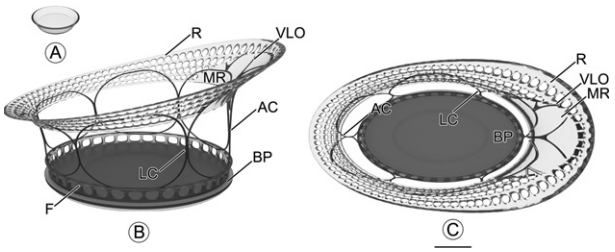

Figure 3 *Korotnevella venosa* n. sp. Diagrams of the structure of dish-shaped scale (**A**) and basket scale (**B, C**). (B) Basket scale, side view. (C) Basket scale, top view. AC, apical column; BP, basal plate; F, fenestrated flange; LC, lateral column; MR, membranous region of perforated rim; R, perforated rim; VLO, vein-like outgrowth of apical column. Scale bar = 0.1 μm.

Phylogenetic analysis

The phylogenetic analysis revealed a clade corresponding to Dactylopodida with high PP but low BS support; within this clade, the genus *Korotnevella* formed a monophyletic assemblage also with high PP but low BS supports (Fig. 4). The sequence of *K. venosa* always grouped among other species of *Korotnevella*, but the position of this sequence was not stable. In ML analysis done with RaxML, it usually occupied the position at the base of the clade comprising all *Korotnevella* species possessing laticework basket in the large scales (*K. monacantholepis*, *K. heteracantha*, *K. limbata* and *K. stella*), hereinafter referred to as "*Korotnevella* crown group". However, this position was newer supported with considerable bootstrap value. In PhyML and Bayesian trees, *K. venosa* branched separately at the base of the entire *Korotnevella* clade, as shown in the Fig. 4. *K. hemistylolepis* formed separate branch next to *K. venosa*, while *K. fousta* occupied a position in the clade with sequences of still undescribed *Korotnevella* strains (*Korotnevella* sp. JVW-2011 JN568811, *Korotnevella* sp. JVW-2015a KP719184, *Korotnevella* sp. JVW-2015b KP719185 and *Korotnevella* sp. JVW-2015b KP719186). This clade was always a sister one to *Korotnevella* crown group. Both these clades had high PP but low bootstrap support.

DISCUSSION

From all nine described *Korotnevella* species, three are marine (*K. hemistylolepis*, *K. monacantholepis* and *K. nivo*), five are known from a fresh water (*K. bulla*, *K. stella*, *K. discophora*, *K. fousta* and *K. limbata*), and one (*K. heteracantha*) was described from soil.

Korotnevella discophora and *K. fousta* are easily distinguishable from *K. venosa* being much smaller, having higher length/breadth ratio, i.e. more elongated locomotive form (Table S1), and most important—in having uniform scales with disk-shaped basement in their cell coat.

Korotnevella nivo differs from *K. venosa* in structure of scales (because they are all of one type, crown-shaped), and it also differs from our isolate in peculiar structure of its nucleus (Table S1).

All the rest of the species of *Korotnevella* have similar scales (at least at first sight) because they all belong to O'Kelly's "Group 1" and have large basket and small dish-

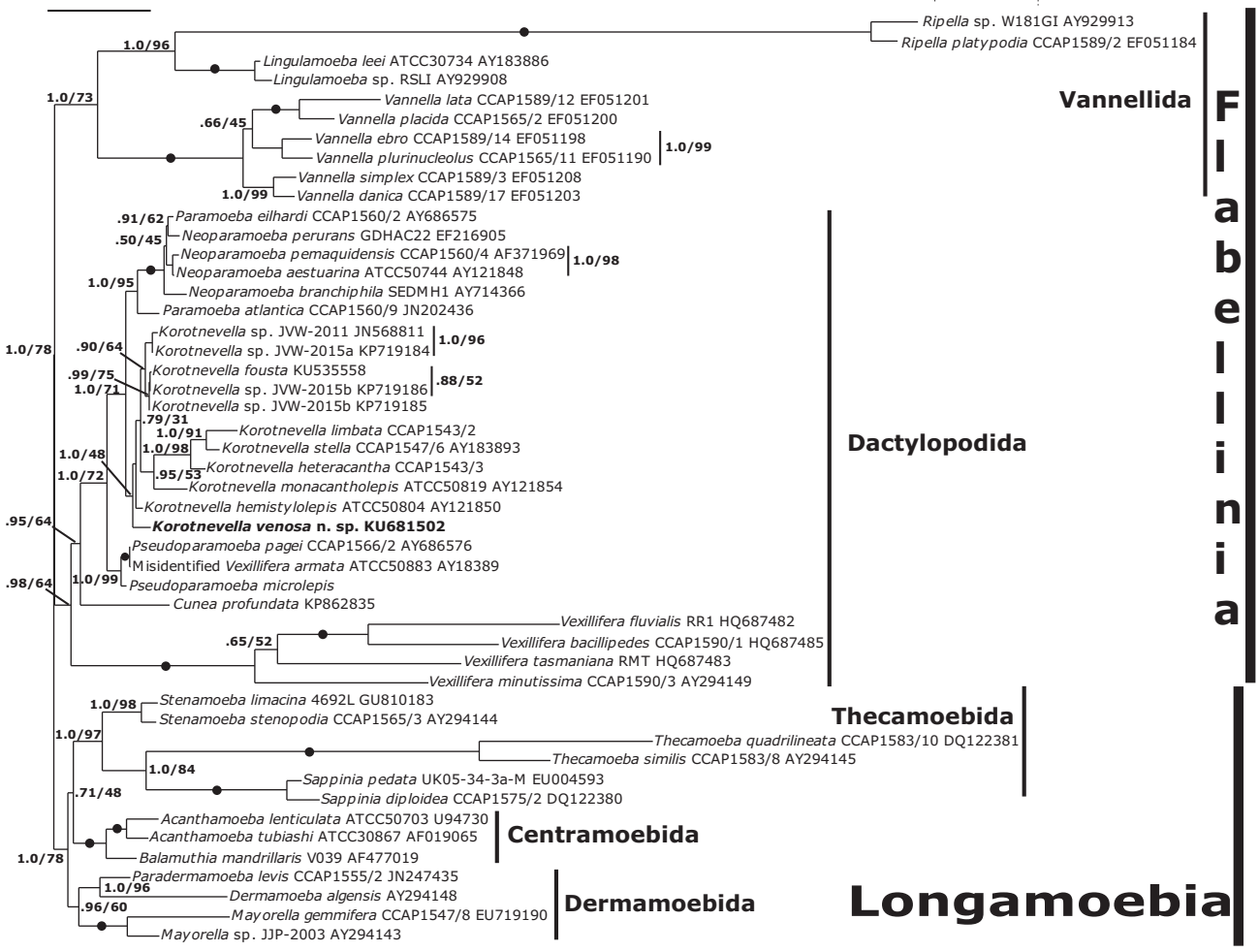

Figure 4 Maximum likelihood tree of a representative set of Discosea based on 18S rDNA gene sequences (GTR + Γ, 1,507 nucleotide positions; 50 taxa) showing the placement of *Korotnevella venosa* n. sp. (in bold) at the base of *Korotnevella* clade. Numbers in nodes indicate Bayesian posterior probability/ML bootstrap support. Indications in nodes without strong support (< 50% BS and < 0.50 PP) omitted. Black circles indicate 1.0/100 support, scale bar = 0.1 substitution/site.

shaped scales in their cell coat. Among them *K. monacantholepis* most strikingly differs from our isolate: it is much larger, has the lowest length/breadth ratio of the locomotive form among korotnevellas (its breadth often is greater, than the length), has peculiar structure of the nucleolus and different structure of basket scales (Table S1). *Korotnevella bulla* is almost twice bigger than *K. venosa* and has completely different structure of basket scales (Table S1). *Korotnevella limbata* has smaller dimensions; at the same time, it has much more elongated, sometimes even vermiform, locomotive morphology, and the highest length/breadth value among *Korotnevella* species (Table S1). Also basket scales of this species have a peculiar structure, which could be easily distinguished from the similar of all other known *Korotnevella* species.

Korotnevella heteracantha and *K. stella* have almost the same size as *K. venosa* (Table S1). But these species have more elongated locomotive form, which is in correlation with their L/B ratio (Table S1). For these species, the shape of the locomotive form characteristic for *K. venosa* (square- or fan-shaped with numerous short papillate pseudopodia) was never described (Page 1981; Schaeffer 1926; Udalov 2015). Neither *K. heteracantha* nor *K. stella* show a polymorphism of a nucleolus, which was observed in *K. venosa*. Finally, these species clearly differ in structure of basket scales (Table S1).

The last species, *K. hemistylolepis* is the most similar one to *K. venosa* in structure of basket scales. It lack laticework basket in both species and have perforated rim and six full-length columns. As in *K. venosa*, at least upper parts of full-length columns of basket scales in *K. hemistylolepis* bifurcate and neighboring columns are connected by these bifurcations. O'Kelly et al. (2001) did not mention this feature of the basket scale structure in the text, but it is clearly visible in their figure (O'Kelly et al. 2001, p. 656 fig. 9). At the same time, *K. venosa* lacks six half-length columns and an intermediate crosspiece, which are present in basket scales of *K. hemistylolepis*. The rim of

K. venosa has tongue-like broadening with membranous region at one end of the basket scale (the rim of basket scales of *K. hemistylolepis* is symmetrical and has small protrusions from both ends) and has no spinules in rim protrusions. Basket scales of *K. venosa* are much larger than those of *K. hemistylolepis* (Table S1). These two species are also similar in forming short and weakly pronounced subpseudopodia during locomotion. However, *K. hemistylolepis* is much smaller and has different structure of the nucleus (Table S1), Y-shaped locomotive form and was isolated from a marine habitat, whereas *K. venosa* is freshwater. Taking into account above considerations, we can conclude that this strain represents a new species named here *K. venosa* n. sp.

Molecular phylogeny confirms that *K. venosa* is undoubtedly a new species. The level of sequence divergence from most similar sequence of *K. hemistylolepis* reaches 11.1%. However, exact relationships of this species remain not clear due to unstable position of the sequence in SSU tree. The position at the base of *Korotnevella* tree, near *K. hemistylolepis* sequence, is generally better supported and seems to be more logic and parsimonious than the position at the base of *Korotnevella* crown group. It is more reasonable because these two species have similarities both at the light and at the electron microscopy level. *K. venosa*'s scales are similar to those of *K. hemistylolepis*: they both lack latticework basket and have perforated rim, as was already noted by Zlatogursky et al. (2016). Also *K. hemistylolepis* and *K. venosa* are similar in tendency to form very short, not elongated, and pronounced subpseudopodia.

The similarity of *K. hemistylolepis* and *K. venosa* basket scales and their neighboring position in *Korotnevella* 18S rDNA molecular phylogenetic tree are in congruence with our early hypotheses that presence of two types of scales and vertical columns in basket scales are probably an apomorphy for a whole *Korotnevella* clade. Absence of a latticework basket is a primitive state of large-scale structure; species, which lack it, occupy a basal position in *Korotnevella* tree, separately from crown group.

TAXONOMIC SUMMARY

Phylum Amoebozoa Lühe, 1913
Subphylum Lobosa Carpenter, 1861
Order Dactylopodida Smirnov et al., 2005
Family Paramoebidae Poche, 1913, Kudryavtsev et al., 2011
Genus *Korotnevella* Goodkov, 1988
Korotnevella venosa n. sp.

Diagnosis

Length of the locomotive is 32–75 μm, breadth 14–41 μm, length:breadth ratio 1.0–4.0. Single ellipsoid nucleus 7.6–11 μm long and 3.8–7.8 μm wide. Nucleolus small and ball-shaped (sometimes elongated, or irregular in shape, or divided into two or more nucleoli of equal or unequal size), nearly 3 μm long. The cell coat consists of two types of boat-shaped scales: larger basket scales and smaller dish-shaped scales. Basket scales have slightly concave basal plate, six vertical columns, unified together by their branches. These branches support perforated rim with tongue-like broadening. Tongue-like broadening has membranous region with vein-like outgrowths of apical columns. No cysts observed.

Etymology

The species group name *venosa* (from the Latin "venosus"; with veins) refers to the outgrowths of the apical column, which are adjacent to the membranous part of the perforated rim and resembled veins of a leaf.

Type material

Type strain, fixed cells embedded in epoxy resin, and a DNA sample are kept with the collection of the "Centre for Culture Collection of Microorganisms" (CCM), Saint Petersburg State University, St. Petersburg, Russia.

Type locality

Fresh water; freshwater pool in the greenhouse of the botanical garden of Saint Petersburg State University, St. Petersburg, North-Western Russia (N59°56′ E30°17′).

DNA sequence data

KU659853–KU659856, KU659858–KU659860 (Cox1 gene), KU681502, KU681489–KU681494 (18S rDNA gene).

Differential diagnosis

Differs from most similar species, *K. hemistylolepis*, in structure of basket scales. The basket scales of *K. venosa* lack six half-length columns, an intermediate crosspiece, spicules in rim protrusions, and have tongue-like broadening with membranous region in perforated rim of basket scales. Also *K. hemistylolepis* is much smaller (16–49 μm, mean 26.3 μm), has Y-shaped locomotive form, vesicular nucleus (with nucleolus sometimes divided into two unequal pieces), and was isolated from a marine habitat, whereas *K. venosa* is freshwater.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Comparative characteristics of described *Korotnevella* species and *Korotnevella venosa* n. sp.