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Korotnevella novazelandica n. sp. (Amoebozoa, Discosea, Dactylopodida) — a new freshwater amoeba with unusual combination of scales

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Summary

A new freshwater species of naked lobose amoebae, *Korotnevella novazelandica* n. sp. (Amoebozoa, Discosea, Flabellinia, Dactylopodida), from New Zealand was studied and described. This species has sombrero-shaped as well as dish-shaped scales, a combination previously not known in *Korotnevella*. Phylogenetic analysis based on 18S rRNA gene placed it in a clade along with *Korotnevell*a species possessing uniform sombrero-shaped scales: *K. pelagolacustris, K. jeppesenii* and *K. fousta*. At the level of light microscopy, *K. novazelandica* lacks clear distinctions from the above-mentioned species, but it could be easily distinguished from them in electron microscopy by the presence of dish-shaped scales. The presence of dish-shaped scales may be considered as a primitive character for the *K. pelagolacustris* + *K. jeppesenii* + *K. fousta* + *K. novazelandica* clade, which were secondarily lost in the most of species in this clade. The sombrero-shaped scales could have evolved from basket scales or developed de novo after the loss of basket scales.

Key words: 18S rRNA gene, *Korotnevella*, molecular phylogeny, scales, systematics, ultrastructure

Introduction

The genus *Korotnevella* encompasses flattened amoebae of dactylopodial morphotype (Smirnov and Goodkov, 1999; Smirnov and Brown, 2004), which have the ability to form finger-shaped subpseudopodia (dactylopodia). In the current system of Amoebozoa this genus belongs to the order Dactylopodida, family Paramoebidae (Smirnov et al., 2011).

The cell surface of amoebae of this genus is completely covered with scales, which are considered to be species-specific (Pennick and Goodfellow, 1975; Page, 1981; Smirnov, 1999; O'Kelly et al., 2001; Udalov, 2015, 2016; Udalov et al., 2016; Zlatogursky et al., 2016; Van Wichelen et al., 2016). The only exception is *Korotnevella nivo*, which has scales almost identical to those of a species from another genus, *Paramoeba eilhardi* (Smirnov, 1996/97).

Korotnevella species can be categorized in three groups based on the scale structure (O'Kelly et al., 2001). Most known species of the genus have two types of scales: large basket-shaped scales and small

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dish-shaped scales. This group was designated as a "Group 1" by O'Kelly et al. (2001) and unites five freshwater (Schaeffer, 1926; Pennick and Goodfellow, 1975; Page, 1981; Udalov, 2015; Udalov et al., 2016) and two mesohaline species (O'Kelly et al., 2001). Recently it was shown that this group is not monophyletic (Udalov, 2016).

The only species of the genus known from marine (not just mesohaline) habitats, *Korotnevella nivo*, has uniform crown-shaped scales and was designated by O'Kelly and coauthors as belonging to "Group 2". Interestingly, these scales are nearly identical in structure with those of *Paramoeba eilhardi* (Smirnov, 1996/97). This suggests that the species could be a PLO-free *Paramoeba eilhardi* or its close relative (O'Kelly et al., 2001), especially since a PLO-free strain of *Paramoeba eilhardi* has been described (Grell and Benwitz, 1970).

The last group of *Korotnevella* species designated by O'Kelly and coauthors was labeled as "Group 3," and to date this group includes four freshwater species with very similar morphology: *Korotnevella diskophora* (Smirnov, 1999), *K. fousta* (Udalov, 2016), *K. jeppesenii* and *K. pelagolacustris* (Van Wichelen et al., 2016). The cell surface of these species is covered with uniform sombrero-shaped scales, which were initially termed as "disk-shaped" (Smirnov, 1999; O'Kelly et al., 2001), because of the incorrect interpretation of the oval basal part of the scale in transmission electron-microscopic images as round in outline (Udalov, 2016).

Here, we describe one more freshwater *Korotnevella* species named *K. novazelandica* n. sp. This species has sombrero-shaped scales, which are typical for species of O'Kelly's "Group 3", but it also has small dish-shaped scales. The presence of the latter scales was believed to be an exclusive characteristic of a "Group 1" until now.

Material and methods

Isolation and culturing

The sample containing *Korotnevella novazelandica* n. sp. was collected by Alexander Khalling from a creek at the foot of the mountain in Mount Cook National Park, New Zealand (S 43°42′51.5″, E 170°05′02.6″) in January, 2016.

The sample was transported to a laboratory and ca. 500 mg of sediment was inoculated in 60 mm Petri dishes with 0.025% wheat grass (WG) (Weizengras, Sanatur GmbH, Germany) extract made of Prescott-James (PJ) medium (Prescott and James, 1955; Page, 1988).

After 7 days of incubation samples were examined with a phase-contrast inverted Nikon Eclipse TS 100 microscope. Individual cells were collected by tapered-tip Pasteur pipette, washed in fresh sterile medium and transferred to 60 mm dishes filled with the same medium to obtain clonal cultures. The strain was maintained on the accompanying bacteria. Cultures were kept at +15 °C under room light.

Light microscopy

Living cells of amoebae were observed and photographed in plastic Petri dishes using a phase-contrast inverted Leica DMI3000B microscope (63× lens) or on the glass object slides using a Leica DM 2500 microscope (100× oil immersion lens) equipped with differential interference contrast (DIC). In both cases we used a Nikon DS-Fi1 digital camera with the accompanying software (Nis-Elements, Nikon Corporation, Tokyo, Japan). Measurements of living cells were taken from micrographs made in cultures, on the plastic surface with an inverted Leica DMI3000B microscope using ImageJ Ver. 1.46r software (Abramoff et al., 2004).

Scanning and transmission electron microscopy

For scanning electron microscopy (SEM) cover slips were placed in Petri dishes containing amoebae. Then a fresh sterile medium was added to intensify the growth of culture. Two or three days later, the cover slips with amoebae were fixed for 45 min in the Párducz's fixative: a mixture of a 1% solution of osmium tetroxide and a 1% solution of a mercuric chloride in double distilled water (Párducz, 1966; Paulin, 1992). After the fixation, the cover slips were washed three times for 10 min in double distilled water and then dehydrated in a graded ethanol series (10 to absolute 100%) for 10 min in each solution. Then the cover slips were critical point dried with liquid CO_2 using Quorum K850 Critical Point Drier (Kent, UK), sputter coated with platinum using Quorum Q150R Sputter Coater (Kent, UK) for 60 seconds and observed using a Zeiss Sigma FE-SEM scanning electron microscope.

For the whole mount preparations of scales, individual cells were collected from the culture, washed in double distilled water, placed in the drops

of water on the Formvar coated aperture grids and air dried. Whole mounts were observed using a JEOL JEM-1400 electron microscope operated at 80 kV. Measurements of scales were made on the images of the whole mounts using ImageJ Ver. 1.46r software.

DNA extraction, PCR-amplification and sequencing

Genomic DNA was extracted from the cell culture using the guanidine thiocyanate method (Maniatis et al., 1982, p. 189). 18S rRNA gene was amplified as single segment with primers RibA 5'-ACCTGGTTGATCCTGCCAGT-3' and RibB 5'-TGATCCTTCTGCAGGTTCACCTAC-3' (Medlin et al. 1988). The PCR program included the following steps: initial denaturation at 95 °C for 2 min; 40 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 60 s and elongation 72 °C for 2 min; and a final elongation at 72 °C for 10 min. The obtained product was purified from agarose gel using Cleanup mini Purification Kit (Evrogen, Moscow, Russia) and then cloned in an E. coli JM107 strain, using the pTZ57R/T vector and InsTAclone PCR Cloning Kit (Thermo Fisher Scientific Inc). Sequencing was performed on an ABI Prism 3500 xl sequencer (Applied Biosystems, U.S.A.).

Phylogenetic analysis

Chromatograms (traces) were manually edited using ChromasPro v. 1.6 software (Technelysium Pty Ltd, South Brisbane, Queensland, Australia). The 18S rRNA gene sequence of *K. novazelandica* was added to the dataset used in the Kudryavtsev and Pawlowski (2015) paper (courtesy of Dr. A. Kudryavtsev) and manually aligned using SeaView v. 4.3.3 (Gouy et al., 2010). For the phylogenetic analysis 52 sequences including all available *Korotnevella* strains and a number of other Discosea with 1,509 unambiguously aligned nucleotide sites were selected.

For sequence identity and sequence difference count matrixes 1797 bp fragment of 18S rRNA gene of *Korotnevella pelagolacustris, K. jeppesenii, K. fousta* and *K. novazelandica* was used. Both matrixes were estimated using BioEdit v. 5.0.9 (Hall, 1999).

Maximum likelihood analysis was performed using RaxML (Stamatakis, 2006) installed on CIPRES portal (Miller et al., 2010) using GTR + γ model of evolution with 25 substitution rate categories and 100 independent searches starting from the random tree. The model of evolution was suggested by the program Modeltest (Posada and Crandall, 1998). The best-scoring tree was tested using multiparametric bootstrapping (1000 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 10,000,000 generations and a burnin of 25%. Average standard deviation of split frequencies reached 0.001811 at the end of the run.

Results

Light microscopy

The body of the locomotive form was 21–39 µm (average 29.0 µm) in length and 4–13 µm (average 9.4 µm) in width; the length: breadth ratio (L/B) was 2.0–4.8 (average 3.3), n = 50.

The locomotive form had the shape of an irregular tetragon or a triangle with the base directed anteriorly. The most of cells in locomotion were elongated (Fig. 1A, B, D), but some individuals were wide and flattened even during locomotion (Fig. 1C). Some cells showed a bulbous uroid (Fig. 1D). The hyaloplasm occupied 1/7–1/4 of the total cell length. Amoebae usually formed thin and long finger-shaped subpseudopodia during locomotion (Fig. 1B, C). When cells were stationary or randomly moving they had irregular shape with several subpseudopodia (Fig. 1E).

Amoebae had a single elongate nucleus (Fig. 1F). The nucleus was 4.3–6.6 µm (average 5.2 µm) in length and 3.2–4.3 µm (average 3.8 µm) in width, n = 25. The nucleus contained very small, spherical nucleolus (Fig. 1F). The diameter of the nucleolus was 1.6–1.8 µm (average 1.7 µm, n = 25).

One or two contractile vacuoles were usually present in the cytoplasm (Fig. 1C, D). Other cytoplasmic inclusions were food vacuoles with bacteria, numerous small granules and spherical refractive bodies. No crystals were observed.

The floating form had a more or less spherical central mass of the cytoplasm with long and, as a rule, straight radiating pseudopodia (Fig. 2A, B). Rarely we observed floating cells, where some of pseudopodia were slightly curved.

Amoebae readily produced cysts in cultures. Before the encystment amoebae usually formed very large aggregations, which often floated in the water

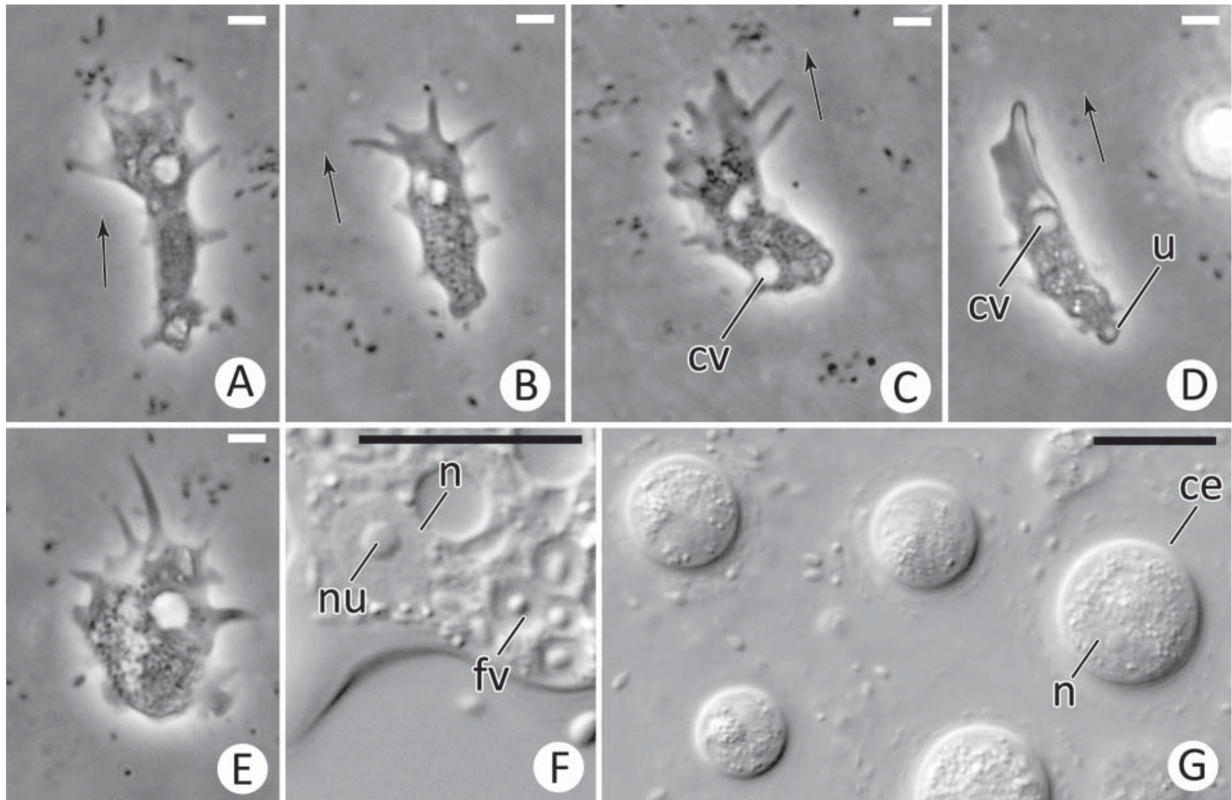

Fig. 1. *Korotnevella novazelandica* n. sp. light (A–G) and scanning electron (H–K) micrographs. A–D – Locomotive forms in a Petri dish, phase contrast; *arrows* indicate the direction of cell's locomotion; E – non-directionally moving cell in a Petri dish, phase contrast; F – nucleus, the cell is compressed with a cover slip, DIC; G – an aggregate of cysts, DIC. *Abbreviations*: contractile vacuole (cv), cyst envelope (ce), nucleolus (nu), nucleus (n), uroid (u). Scale bars: 10 µm.

column (Fig. 1G). Cysts consisted of a spherical cell mass enclosed in a thick envelope (Fig. 1G). The diameter of the cytoplasmic body of the encysted cell was 6–13 µm (average 10.0 µm) while the diameter of a cyst including its envelope was 8–16 µm (average 12.6 µm), n = 51.

Scanning and transmission electron microscopy

The most of studied cells kept native form under fixation and critical point drying, some were apparently fixed during locomotion (Fig. 3A). Aggregates of trophic cells and cysts were also observed (Fig. 3B). The plasma membrane of the trophic cell was always covered with a layer of scales. Two types of scales were observed: large sombrero-shaped scales and small dish-shaped scales (Fig. 3C). The sombrero-shaped scales had an elongated basal plate with irregular margins on critical point dried preparations (Fig. 3C). Often the ends of the basal plate were pointed, while its margins were convoluted on its dorsal side. In SEM preparations they did not form a continuous layer, their margins were bended upwards to the different degree and scales themselves had holes in the basal plate, looking as being slightly damaged (Fig. 3C). Some of dish-shaped scales were covered with the

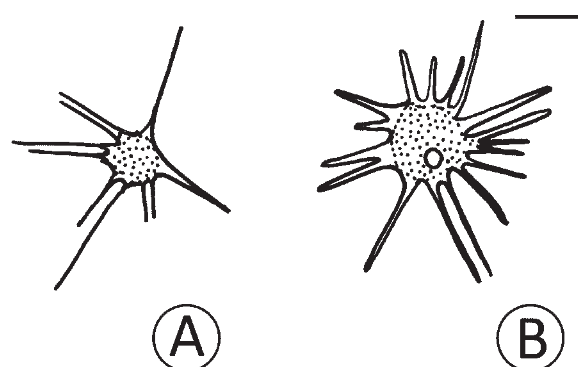

Fig. 2. Floating forms of *Korotnevella novazelandica* n. sp. Line drawings. Scale bar: 10 µm.

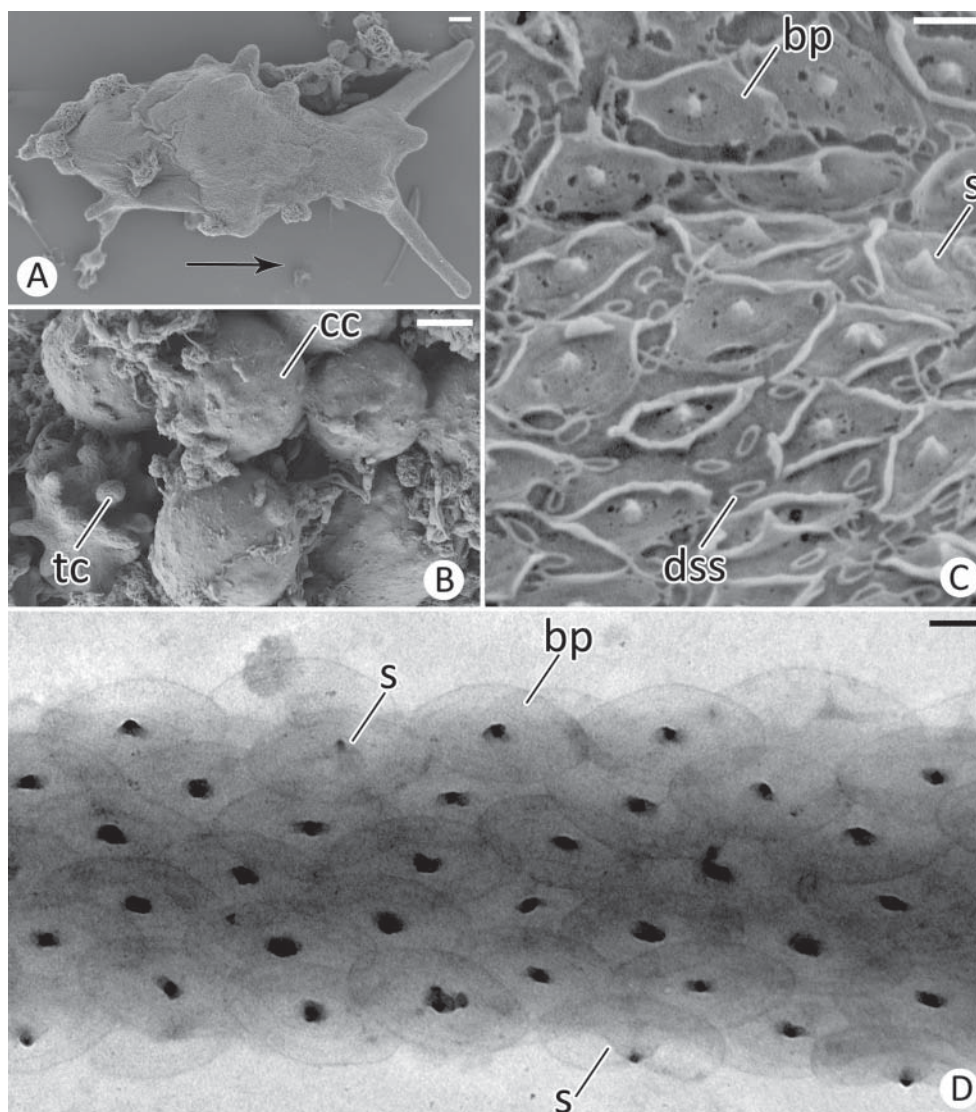

Fig. 3. *Korotnevella novazelandica* n. sp. scanning (A–C) and transmission (D) electron micrographs. A – Critical point dried locomotive form, an *arrow* indicates the direction of cell's locomotion; B – a fragment of aggregate which includes cysts and trophic cells; C – a fragment of a cell coat of a trophic amoeba showing scales; D – whole mount preparation of air dried cell. *Abbreviations*: basal plate (bp), dish-shaped scale (dss), cyst cell (cc), spine (s), trophic cell (tc). Scale bars: 1 µm (A), 0.1 µm (B, C), 0.2 µm (D), 3 µm (E).

margins of sombrero-shaped scales. In the whole mount preparations sombrero-shaped scales were located much closer to each other so that the margins of scales overlapped and small scales were not visible (Fig. 3D). In these preparations sombrero-shaped scales had regular, flat margin and were oval in outline (Fig. 3D). Thus, we cannot completely exclude the possibility that the curved shape of a basal plate in sombrero-shaped scales revealed with SEM could be in fact an artifact of a fixation and critical point drying of the flexible scale material. The basal plate had concentric, ring-like markings on its surface. A spine tapering from its base to the apex arose from the middle part of the basal plate (Fig. 3C, D). Sombrero-shaped scales were 320–406 nm (average 366 nm) in length and 178–249 nm (average 208 nm) in width, n = 46. The height of the spine was 43–83 nm (average 57 nm, n = 27). The diameter of the spine in its basal part was 49–103 nm (average 67.4 nm, n = 27). The ratio of the spine's height to the spine's base width was 0.6–1.2 (average 0.9, n = 27).

Small dish-shaped scales were 45–81 nm (average 67 nm) in length, 43–112 nm (average 75 nm)

in width and 9–20 nm (average 14 nm) in height, n = 25.

The cyst envelope consisted of a homogeneous meshwork material; on the surface of this layer the scales of the trophic stage were sometimes seen. Specialized cyst scales were not observed in the SEM images.

Phylogenetic analysis

The results of the phylogenetic analysis (Fig. 4) revealed a clade corresponding to the order Dactylopodida with high posterior probability (PP), but with low bootstrap (BS) value. The genus *Korotnevella* according to our analysis was monophyletic, but the corresponding clade also had high PP and low BS support.

The 18S rRNA gene sequence of *Korotnevella novazelandica* n. sp. always grouped with sequences of *K. jeppesenii, K. pelagolacustris* and *K. fousta* (Fig. 4). The cell surface of the three above-mentioned species is covered with sombrero-shaped scales. The sequence identity level of *K. novazelandica* counted in 1797 bp fragment shared by all these four sequences varied from 0.919 with *K. pelagolacustris* to 0.975 with *K. fousta* (Table S2). However, in terms of the nucleotide difference this means 144 bp differing with *K. pelagolacustris* and 44 bp difference with *K. fousta* (Table S3). The clade consisting of these four species occupied a position next to *K. hemistylolepis* (which was the most basal *Korotnevella* sequence) and was a sister to the clade uniting sequences of all remaining *Korotnevella* species (*K. venosa, K. monacantholepis, K. heteracantha, K. limbata* and *K. stella*).

Among amoebae with sombrero-shaped scales, *K. novazelandica* was sister to *K. fousta*; both sequences were sister to one of two *K. jeppesenii* sequences, while *K. pelagolacustris* occupied the most basal position in this clade (Fig. 4). The position of *K. novazelandica* is supported with the marginally appropriate PP value (0.77), but at the same time it has no bootstrap support (49%).

Discussion

Identification of *Korotnevella novazelandica* n. sp.

Within the genus *Korotnevella* there are six species whose locomotive form size and L/B ratio are similar to that of *K. novazelandica*. These species are *K. hemistylolepis, K. limbata, K. diskophora, K. fousta, K. jeppesenii* and *K. pelagolacustris*. The remaining species (*K. bulla, K. stella, K. heteracantha, K. monacantholepis, K. venosa* and *K. nivo*) are much larger and (or) have a lower L/B ratio (Table S1).

Based on the light microscopic evidence *K. hemistylolepis* differs from *K. novazelandica* by a characteristic Y-shaped locomotive form (O'Kelly et al., 2001). The other five species do not significantly differ in shape of their locomotive form from our isolate and among themselves.

K. limbata differs somewhat from *K. novazelandica* (and also from *K. diskophora* and *K. fousta*) in the structure of the nucleus. The nucleolus in *K. limbata* is often divided into two parts and contains an optically transparent lacuna (Udalov, 2015), whereas in *K. novazelandica, K. fousta, K. diskophora, K. jeppesenii* and *K. pelagolacustris* the nucleus normally contains a single spherical nucleolus without a lacuna (Udalov, 2016; Van Wichelen et al., 2016). However, taking into account that the structure of the nucleus can vary in different strains of the same species, for example in *Korotnevella stella* (Page, 1972, 1981, 1988, 1991), this character cannot be considered as a reliable one.

Korotnevella jeppesenii and *K. pelagolacustris* are very similar to *K. novazelandica* both in the outline of the locomotive form and in their morphometric characteristics (Table S1). Thus, these species hardly can be differentiated from our isolate using light microscopy. Moreover, *Korotnevella jeppesenii* and *K. pelagolacustris*, as well as *K. novazelandica* have a tendency to form aggregates of cells floating in the water column (Van Wichelen et al., 2016), although the authors observed encystment several times only in *K. pelagolacustris*.

Our species could be easily distinguished from all other *Korotnevella* species by the structure of scales. Large scales of *K. limbata* and *K. hemistylolepis* are typical basket-shaped scales; besides a basal plate, they also include a perforated flange and a rim along the upper edge of the scales (O'Kelly et al., 2001; Udalov, 2015). The scales of *K. diskophora, K. jeppesenii, K. pelagolacustris* and *K. fousta* are very similar in structure to the large sombrero-shaped scales of *K. novazelandica*. They also consist of a basal plate and a spine that extends from its central part (Smirnov, 1999; Udalov, 2016; Van Wichelen et al., 2016). However, these four species have uniform sombrero-shaped scales and do not have small dish-shaped scales.

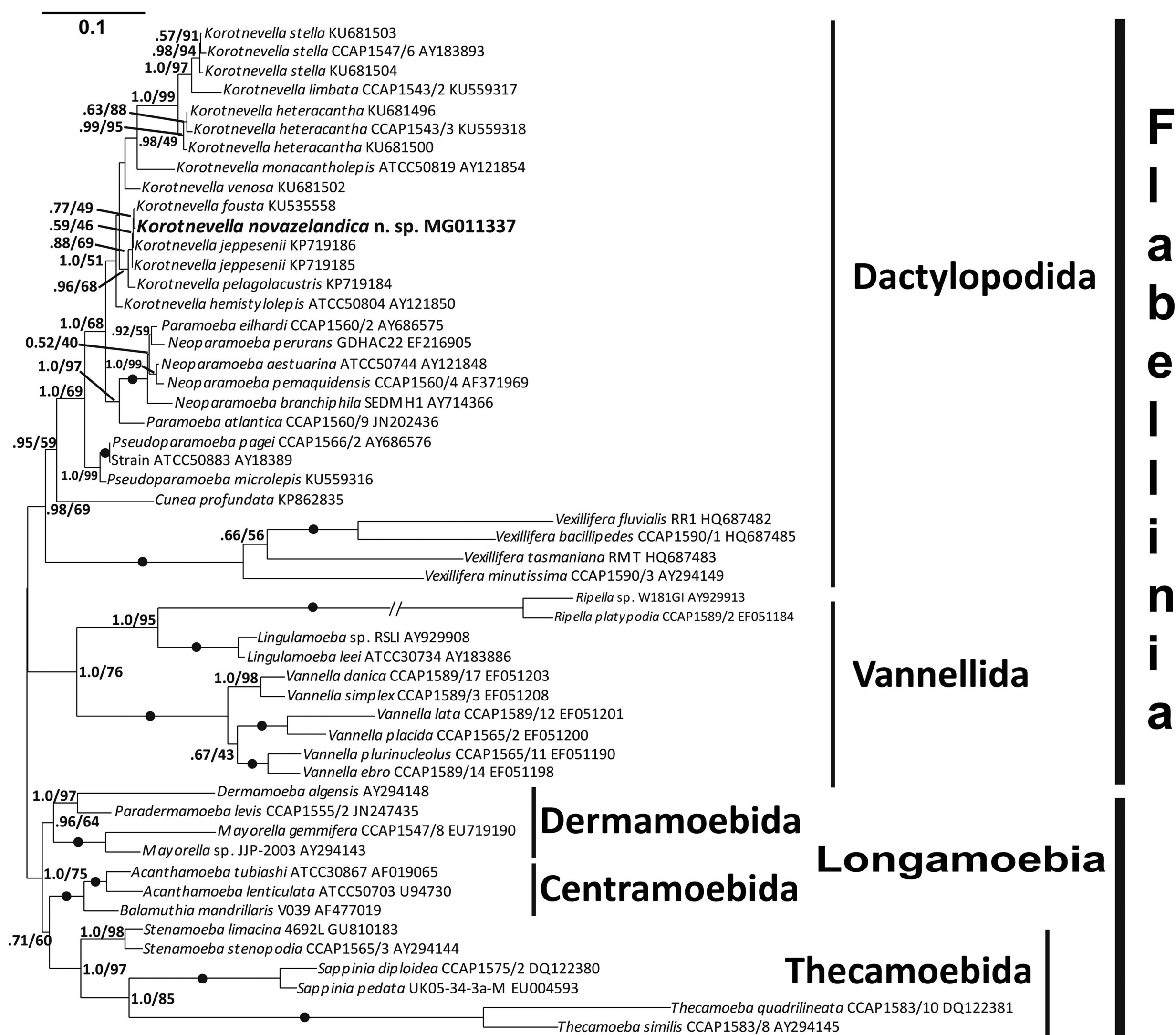

Fig. 4. A phylogenetic tree showing the placement of *Korotnevella novazelandica* n. sp. (in bold). 18S rRNA gene, 1,509 positions; 52 sequences. Black dots = 1.0/100 supports (PP/BS), values below 0.50/50 are not specified. Crossed branch was shortened in half. Scale bar: 0.1 substitution/site.

At the level of 18S rRNA sequences these species show relatively high level of sequence identity (0.919–0.975). These levels fall within the limits of intra-species variability in some other amoebae genera, like *Vannella* (Smirnov et al., 2002; Nassonova et al., 2010). This means that the border between morphospecies in *Korotnevella* may be shadowed at the level of 18S rRNA sequence and more sensitive markers are desirable to clarify relationships of this sort. COX1 gene could be an appropriate candidate for this, as shown by Nassonova et al. (2010) and Zlatogursky et al. (2016). However, evident differences in the scale structure support the point that these are different morphospecies with relatively low genetic distance from each other at the level of 18S rRNA gene.

Diagnosis

Korotnevella novazelandica n. sp. Length in locomotion 21–39 µm; breadth 4–13 µm; L/B ratio 2.0–4.8. Single elongate vesicular nucleus with the single spherical nucleolus. Length of the nucleus 4.3–6.6 µm; width 3.2–4.3 µm. Nucleolus 1.6–1.8 µm in diameter. The cell coverings include two types of scales: large sombrero-shaped scales and small dish-shaped scales. Basal plate of large scales is oval in outline. The size of the basal plate in sombrero-

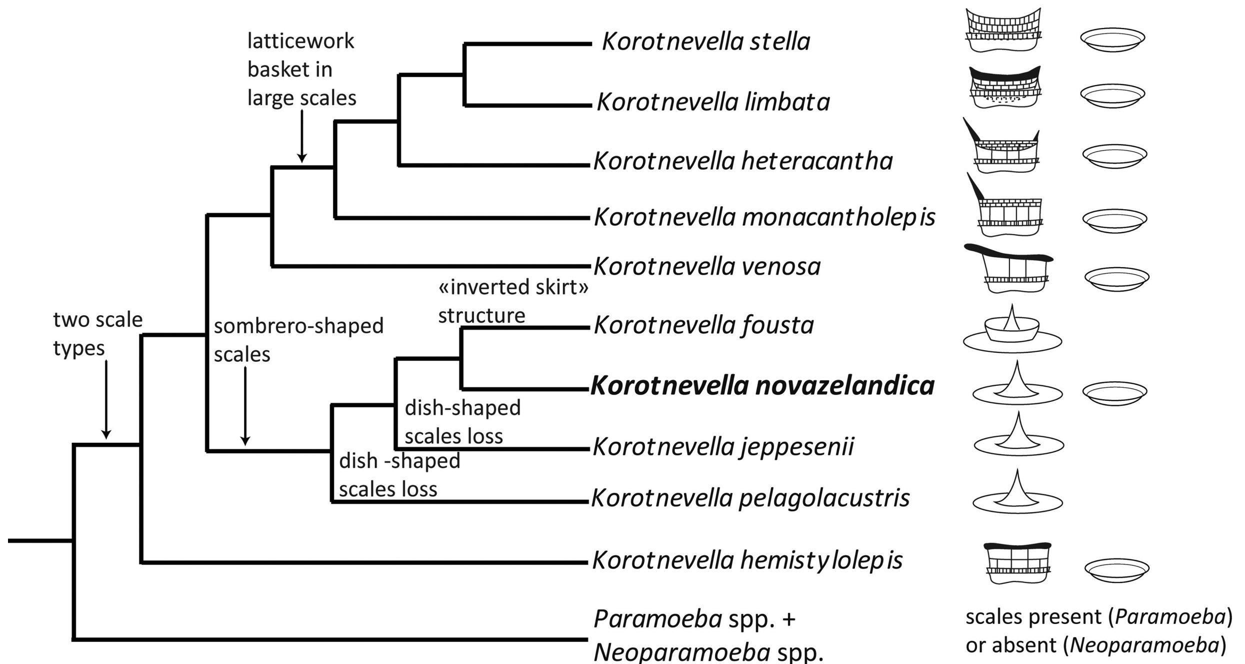

Fig. 5. Data on scale structure of *Korotnevella* species mapped on the 18S rRNA gene phylogenetic tree. A new sequence of *K. novazelandica* n. sp. is in bold.

shaped scales is 320–406 nm in length and 178–249 nm in width. The height of the central spine is 43–83 nm. Dish-shaped scales are 45–81 nm in length, 43–112 nm in width and 9–20 nm in height.

Differences from closely related species: differs from closely related species (*K. diskophora, K. pelagolacustris, K. jeppesenii* and *K. fousta*) and from remaining *Korotnevella* species in presence of dish-shaped scales among sombrero-shaped scales.

Etymology: the species group name *novazelandica* (from the Latin "Nova Zelandia"; New Zealand) refers to type locality of the species.

Type material: type strain and a DNA sample from this strain are kept with the collection of the "Centre for Culture Collection of Microorganisms" (CCM), Saint Petersburg State University, St. Petersburg, Russia under the reference number CCMA0019.

Type locality: fresh water; creek at the base of the mountain in Mount Cook National Park, New Zealand (S 43°42′51.5″, E 170°05′02.6″).

DNA sequence data: GenBank accession number: MG011337

NEW DATA ON THE EVOLUTION OF *KOROTNEVELLA* SCALES

All species with sombrero-shaped scales formed a monophyletic assemblage in our tree (Fig. 5). Thus, the presence of sombrero-shaped scales could be treated as a morphological autapomorphy for a clade comprising *K. novazelandica, K. fousta, K. jeppesenii* and *K. pelagolacustris*. *K. novazelandica* is the only species of this group that has dish-shaped scales, similar to those of the representatives of a "Group 1" sensu O'Kelly et al. (2001). We can suggest that they are homologous to the dish-shaped scales of the species of a "Group 1", but the structure of these scales is very simple and we cannot exclude the possibility that they are just analogous. This problem requires further studies. At the same time, *K. novazelandica* is a sister to *K. fousta* and occupies the crown of a clade, which unites species with sombrero-shaped scales, while the basal part of this clade consists of sequences of *K. jeppesenii* and *K. pelagolacustris* (Fig. 5). It could mean that the presence of dish-shaped scales is a primitive character, which was retained in *K. novazelandica* and secondarily lost in other species with sombrero-shaped scales (Fig. 5). Sombrero-shaped scales could have appeared as a modification of basket-shaped scales or de-novo after basket scales loss; an "inverted skirt" structure appeared in *K. fousta de novo* (Fig. 5). However, alternatively, we cannot exclude that the ability to form either basket- or sombrero-shaped scales is just the matter of a simple gene regulation and that cells may switch from one

type of scales to another, as lately observed in the genus *Cochliopodium* (Kudryavtsev and Völcker, pers. comm.).

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