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High Genetic Diversity of Amoebae Belonging to the Genus *Mayorella* (Amoebozoa, Discosea, Dermamoebida) in Natural Habitats

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Abstract. Amoebae of the genus *Mayorella* are widespread in marine, freshwater and soil habitats. These amoebae have relatively low number of morphological characters allowing species differentiation, so the number of valid species remains rather small. Representatives of the genus *Mayorella* are hard to maintain in culture, the very most of them are polyphagous and carnivorous. To live and multiply they require a variety of food objects, including other protists. Thus they are difficult objects for molecular studies. Only two sequences of *Mayorella* were available until recently in GenBank. For the present study we isolated eight strains of *Mayorella* from different locations worldwide, documented them with the light microscopy and obtained complete or partial sequences of their 18S rRNA gene. Results show that all members of the genus *Mayorella* form a robust clade within the order Dermamoebida (Amoebozoa: Discosea). The vast majority of our strains probably represent new species; this evidences that diversity of the genus *Mayorella* in natural habitats is high and that this genus is rather species-rich, comprising no less than 11 species. Our results show that nowadays sequence data are necessary for reliable identification of *Mayorella* species.

Key words: Amoebozoa, *Mayorella*, diversity, phylogeny, systematics

INTRODUCTION

The genus *Mayorella* was established by Schaeffer (1926) with the type species *Mayorella bigemma* (formerly *Amoeba bigemma*) Schaeffer, 1918. It was named in the honour of Alfred G. Mayor, curator of natural sciences of the Brooklyn Institute of Arts and Sciences, the founder and the first director of the Tortugas Marine Laboratory where Schaeffer worked at that time (see Colin 1980, fig. 1a).

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The taxonomic history of this genus was rather complex (Page 1972, 1981, 1983, 1976, 1987). Amoebae possessing somewhat similar morphology may have thick, multi-layered cell coat – "cuticle" (Page and Blackey 1979), but may also have scales on the surface of the cell membrane (Page 1981, 1982, 1983, 1983a). Page (1981) attempted to establish the genus *Hollandella* for *Mayorella*-like amoebae with cuticle; however electron microscopy showed that *Holandella* (former *Mayorella*) *riparia* – the species that he made as a type for this genus had scales, not a cuticle. Further it was synonymized with the species *Korotnevella stella*. Hence the genus *Hollandella* was abandoned (Page 1983; see also O'Kelly *et al.* 2001). Species with cuticle were left in the genus *Mayorella*, while those

with scales were segregated to the genus *Dactylamoeba* Korotneff, 1879. The generic name *Dactylamoeba* was later formally replaced with the name *Korotnevella* Goodkov, 1988 (Goodkov 1988; see also Smirnov 1996).

The diversity of *Mayorella*-like amoebae in natural habitats is high, while species distinction among amoebae of this genus is difficult and requires electron microscopy of the cell surface (Page 1982, 1983, 1988). Many species described solely by light microscopy remain hardly recognizable and cannot be reliably re-isolated basing on the available descriptions (see Bovee 1985). More or less properly characterized species (including electron microscopy of the cell coat) are *Mayorella cantabrigiensis*, *M. vespertilioides* and *M. penardi* (Page 1983a); *M. viridis* (Cann 1981); *M. kuwaitensis* (Page 1983, Smirnov 1999); *M. gemmifera* (Page 1983, Dykova *et al*. 2008) and *M. dactylifera* (Goodkov and Buryakov 1988). The differences between these species concern their size characteristics (including the size of the nucleus), morphology of the floating form, shape and kind of the cytoplasmic inclusions and the structure of the cell coat (Page 1983, 1988). However the latter was shown to be polymorphic even between strains, assigned to the same species (Page 1983, 1983a).

There are twenty-two amoeba species mentioned in the literature as potentially belonging to the genus *Mayorella* (Penard 1902, Bovee 1970, Page 1976, Shaeffer 1926, Hollande *et al.* 1981). However many of these descriptions are incomplete and do not contain electron-microscopic data; in many cases they are limited to several line drawings and brief size data. So we cannot consider these species names as valid ones and, respectively, cannot rely on these descriptions for identification purposes. Species of two more amoebae genera are in some respects similar to *Mayorella,* those are *Oscillosignum* Bovee, 1953 and *Subulamoeba* Bovee, 1953, but none of these genera can be called valid without re-isolation and re-description of their respective representatives because of the insufficient information and absence of any electron-microscopic data on these organisms.

The genetic diversity of amoebae belonging to the genus *Mayorella* is virtually not studied. Amoebae of this genus are polyphagous and cannot be maintained in pure culture, thus they are difficult objects for molecular work. There are only two sequences of *Mayorella* available in GenBank – that of *Mayorella gemmiferra* and of unnamed *Mayorella* strain isolated from Gurre Lake in Denmark by A. Smirnov (Fahrni *et al*. 2003, Dykova *et al.* 2008). This produces an evident shortage of sequences in this part of the 18S rRNA trees. The aim of the present study was to increase the number of available *Mayorella* 18S rRNA gene sequences in order to estimate the potential species diversity within this amoeba genus and provide data for obtaining better resolution in this part of the phylogenetic tree. Using controlled starvation of cells followed by the single-cell PCR we obtained partial sequences of eight *Mayorella* strains; the most of them represent species, potentially new for science.

MATERIAL AND METHODS

Mayorella strains used in the present study were isolated from different locations in North America, Europe and Far East of Russia. The positions of sampling sites are provided in the Figures S1-S3. Details on isolated strains are provided in the Table 1.

For isolation of amoebae, 0.5–1 ml of sediment and water from the sample were placed into 90 mm Petri dishes and incubated in 0.025% Cerophyl infusion made on PJ medium (Page 1988, Smirnov and Brown 2004) or PJ medium with addition of 1–2 rice grains (Prescott and James 1955). After 7–10 days of incubation samples were examined using a Nikon TS100F inverted microscope; detected *Mayorella* cells were transferred to fresh medium using a tapered-end Pasteur pipette. Strains were cloned and further maintained in 60 mm Petri dishes in 0.025% Cerophyl infusion made on PJ medium. All inoculation and cloning procedures were performed with sterile instruments and under sterile conditions. Living trophozoites were observed, measured (50 or more specimens were measured, if other is not indicated in the text) and photographed using Nikon TS100F, Leica DM2500 and Leica DMI3000 microscopes equipped with phase contrast and DIC optics at 40–100× magnification.

For the single-cell PCR, cells were transferred from the culture to the Petri dish filled with filtered medium (0.45 μm syringe filters, Orange Bioscience), after 15–20 min – to the second dish with the same medium. Then they were left to starve for 2–3 days. Cells were transferred every day to the fresh dish with freshly filtered medium. All transfers were performed using freshly made pipettes. After starvation, cells were collected with the minimal possible amount (1–2 μl) of medium and placed in 0.2 ml PCR tubes. Tubes were subsequently exposed to several rapid freezing-defreezing cycles (4–5 cycles from –18°C to room temperature), ready PCR mixture was topped on the tube content to the final volume of 50 μl. Primer pairs RibA/S12.2r and S12.2/SB (Pawlowski 2000; S12.2R sequence is 5'> gac tac gac ggt atc tra tc <3') were used for amplification of each species, the same primers were used for sequencing. Genbank numbers of newly obtained sequences are listed in Table 1. 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 purified using Cleanup mini Purification Kit (Eurogene) and sequenced using ABI-PRISM Big Dye Terminator Cycle Sequencing Kit.

Table 1. Sampling sites and sequence GenBank numbers corresponding to observed *Mayorella spp.* strains.

Strain	Isolation site	Genbank number	Sequence length, bp
Mayorella sp. strain FE_mz241	the Zea river, Blagoveshchensk city area, Russia, 50°17′N, 127°32′E	MG428632	627
Mayorella sp. strain Amur_2	the Amur river, Blagoveshchensk city area, Russia, 50°17′N, 127°32′E	MG428630	1750
Mayorella sp. strain FE_2	the Ginchin river, Muraviovski natural resort, Russia, 49°50′N, 127°38′E	MG428626	482
Mayorella sp. strain FE 16	the Ginchin river, Muraviovski natural resort, Russia, 50°09′N, 128°20′E.	MG428628	855
Mayorella sp. strain 2Th5	the Thames river, UK, 51°41′N, 2°01′W	MG428627	574
Mayorella sp. strain Germany	Pfaffen lake, Germany, 49°09′, N9°13′E	MG428629	485
Mayorella sp. strain Oklahoma	lake in Ardmore city area, Oklahoma, USA, 34°11′N, 97°07′W	MG428633	684
Mayorella sp. strain Belaya	the Belaya river, Adygea, Russia, 44°14′N, 40°1′E.	MG428631	881
Mayorella sp. strain JJP2003	Gurre Lake, Denmark, 56°1′N, 12°29′E	AY294143	2131

Obtained sequences were added to our general eukaryotic alignment. First, sequences were automatically aligned using the Muscle algorithm (Edgar 2004) as implemented in SeaView 4.0 (Gouy *et al*. 2010); alignment was later refined manually. The phylogenetic analysis was performed using maximum likelihood method as implemented in PhyML program (Guindon and Gascuel 2003) with GTR + γ model suggested by jModeltest program (Darriba *et al*. 2012); 1390 sites were selected for the analysis; the number of invariant sites, alpha parameter and tree topology were optimized by PHYML. To improve the dataset, both available culture-derived *Mayorella* sequence were used as a BLAST query; if top 20 results contained sequences not present in our alignment, those were added to the general eukaryotic alignment and analyzed using PhyML as described above. Sequences showing affinities to *Mayorella* were mounted in the alignment.

The final ML phylogenetic tree (Fig. 3) was obtained using RaxML program (Stamatakis 2006). A GTR + γ + I model of evolution was applied for the analysis. First, 100 MP starting trees were generated; for the tree with the highest likelihood the stability of clades was assessed using a non-parametric bootstrap with 1000 pseudoreplicates. All model parameters were estimated from the data. The set of species used for the analysis included all *Mayorella* sequences and a set of outgroups. Several sets of sites were tried for analysis; the minimal one (the most conservative) contained 1206 sites, while the maximal one contained 1632 sites. Highest bootstrap supports (BS) for most of branches were obtained using 1554 sites set and this set was used for further analysis. Bayesian analysis was performed using MrBayes 3.1.2 program (Ronquist and Huelsenbeck 2003), GTR model with gamma correction for intersite rate variation and the covarion model. 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 Tracer 1.6 software (Rambaut *et al*. 2014); based on the estimates by Tracer, the first 30% of generations were discarded for burn-in. For the *Mayorella*-only tree with few outgroups (Fig. 4), a fragment containing 1414 sites shared by more than two sequences was used; all other settings for the analyses were the same as above.

RESULTS

1. Brief descriptions and illustrations of studied strains

***Mayorella sp.* strain Belaya.** Locomotive cells were elongate or triangular (Fig. 1A–E), with smooth edge of the frontal hyaline area or with several subpseudopodia, which continued in hyaline ridges on the dorsal surface of the cell (Fig. 1 D). Length of locomotive form was 55–90 μm (average 69.7 μm), width 20–50 μm (average 33.1 μm), L/B ratio 1.4–2.2 (average 2.2). No differentiated uroidal structures were observed.

The granuloplasm contained food vacuoles, contractile vacuoles, numerous crystals of irregular shape, sometimes aggregated in patches and the single spherical nucleus about 8 μm in diameter with the single central nucleolus about 7 μm in diameter. The floating form had 8–10 subpseudopodia radiating from the rounded central mass of the cytoplasm (Fig. 1E). No cysts were found in our cultures.

***Mayorella sp.* strain 2Th5.** Locomotive cells were irregularly triangular and usually were wider in the frontal part (Fig. 1F–J). They produced 3–5 conical pseudopodia. Cells usually had morulate or bulbous uroid (Fig. 1H). Length of the locomotive form was 40–100 μm (average 71.9 μm), breadth 15–40 μm (average 27.2 μm), L/B ratio 1.4–5 (average 2.8).

The granuloplasm contained food vacuoles, contractile vacuole, numerous small paired crystals and the single spherical nucleus about 8 μm in diameter with the central nucleolus about 6 μm in diameter. Floating form had 4–5 radiating conical pseudopodia with nar-

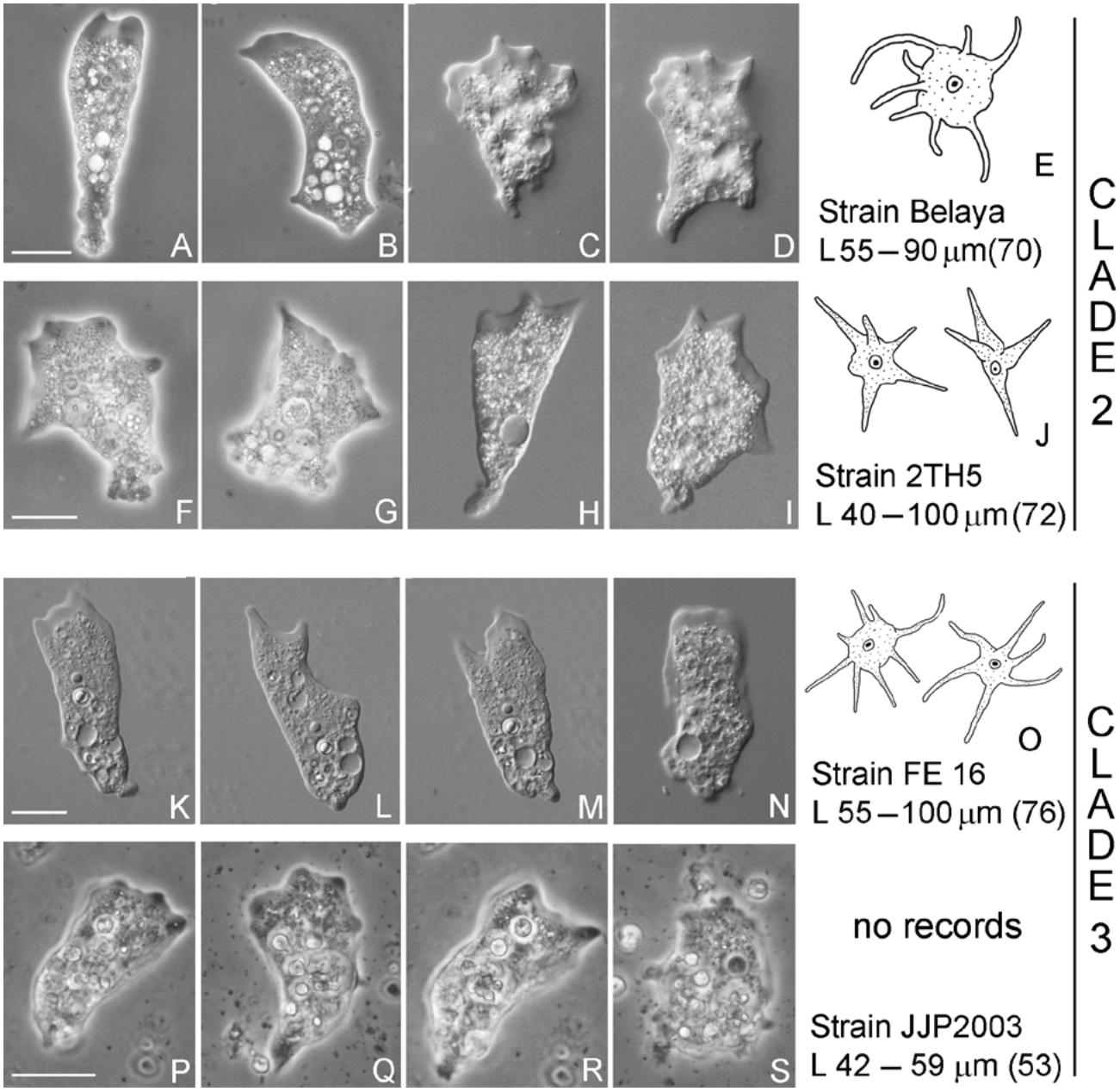

Fig. 1. Diversity of locomotive and floating forms of studied strains: A–E – *Mayorella sp.* strain Belaya; F–J – *Mayorella sp.* strain 2Th5; K–O – *Mayorella sp.* strain FE 16; P–S – *Mayorella sp.* strain JJP2003. DIC and phase contrast images, scale bar is 20 μm.

row conical hyaline ends (Fig. 1J). No cysts were found in our cultures.

***Mayorella sp.* strain 16.** Locomotive cells were elongate or triangular in outlines, sometimes with 2–3 conical frontal pseudopodia (Fig. 1K–O) and/or several short subpseudopodia. Cells had small bulbous uroid (Fig. 1K–M) or no differentiated uroidal structures. Length of the locomotive form was 55–100 μm (average 76.8 μm), breadth 15–55 μm (average 27.8 μm), L/B ratio 1.3–6.7 (3.1).

The granuloplasm contained food vacuoles, one to three contractile vacuoles, small crystals and granules and the single spherical nucleus about 8 μm in diameter with the central nucleolus about 7 μm in diameter.

The floating form had 6–8 radiating pseudopodia with conical parts consisting of the hyaloplasm (Fig. 1O). No cysts were found in our cultures.

***Mayorella sp.* strain JJP2003.** Locomotive cells usually had a shape of irregular triangle with the base directed anteriorly (Fig. 1P–S). Sometimes cells formed 1–3 short conical pseudopodia. Moving cells often had small bulbous uroid (Fig. 1 R–S). Length of the locomotive form was 42–59 μm (average 53 μm), breadth 23–35 μm (average 27.5 μm), L/B ratio 1.4–2.57 (1/97). Amoebae of this strain were measured from archive digital images; the number of measured cells was eight.

The granuloplasm contained numerous food vacuoles, a few crystals of irregular shape and the single spherical nucleus about 8 μm in diameter with the central nucleolus about 5 μm in diameter. The floating form was not found in available images and records. No cysts were recorded for this strain.

***Mayorella sp.* strain Amur_2.** Locomotive cells were of variable shape, usually wider in the frontal part (Fig. 2A–D). Sometimes cells produced subpseudopodia, originating from the frontal hyaline zone (Fig. 2C–D). Length of the locomotive form was 35–85 μm (average 68.3 μm), breadth 10–45 μm (average 20 μm), L/B ratio 1.9–8.5 (average 4.2). Dorsal ridges sometimes occurred in moving cells (Fig. 2D). No differentiated uroidal structures were observed.

The granuloplasm contained food vacuoles, from one to two contractile vacuoles, small rounded crystals and the single spherical nucleus about 5 μm in diameter with the central nucleolus about 4 μm in diameter. Floating form commonly had three radiating conical pseudopodia with tapering distal ends consisting of the hyaloplasm (Fig. 2E). No cysts were found in our cultures.

***Mayorella sp.* strain FE_2.** Locomotive cells usually resembled an irregular triangle with the base directed forward (Fig. 2F–I). Some of observed cells were elongate, sometimes with 3–4 large triangular frontal pseudopodia (Fig. 2G–H). Length of the locomotive form was 53–95 μm (average 75.3 μm), breadth 12–50 μm (average 23.5 μm), L/B ratio was 1.5–7 (average 3.5). No differentiated uroidal structures were observed.

The granuloplasm contained food vacuoles, from one to five contractile vacuoles, non-numerous crystals, sometimes paired or aggregated in patches and the single spherical nucleus about 8 μm in diameter with the central nucleolus about 6 μm in diameter. The floating form had 6–8 radiating pseudopodia with conical hyaline ends (Fig. 2J). No cysts were found in our cultures.

***Mayorella sp.* strain Germany.** Locomotive cells were irregularly triangular (Fig. 2K–N), with two-three conical pseudopodia and sometimes – with several frontal subpseudopodia. Some of observed cells had small bulbous uroid, but the most had no differentiated uroidal structures. Length of locomotive form was 55–100 μm (average 74.7 μm), breadth 10–50 μm (average 23.2 μm), L\B ratio 1.5–9 (average 3.9).

The granuloplasm contained food vacuoles, contractile vacuoles, numerous crystals of various size and shape and the single spherical nucleus about 9 μm in diameter with the central nucleolus about 7 μm in diameter. The floating form had 5–6 radiating wide conical pseudopodia with conical hyaline ends (Fig. 2O). No cysts were found in our cultures.

***Mayorella sp.* strain FE_mz241.** Locomotive cells were elongate, with the pronounced anterior hyaline border (Fig. 2P–S). Sometimes they formed wide conical pseudopodia (Fig. 2R–S). Length in locomotion was 50–115 μm (average 78.4 μm), breadth 15–35 μm (average 25.2 μm), L/B ratio 1.7–5.8 (average 3.3). Cells had no differentiated uroidal structures.

The granuloplasm contained food vacuoles, one to three contractile vacuoles, spherical crystals and the single spherical nucleus about 7 μm in diameter with the central nucleolus about 5 μm in diameter. Floating amoebae had 4–6 radiating pseudopodia; distal parts of these pseudopodia were narrow conical and consisted of the hyaloplasm (Fig. 2T). No cysts were found in our cultures.

***Mayorella sp.* strain Oklahoma.** Locomotive cells were irregularly triangular, with 2–3 conical pseudopodia and distinct frontal and lateral hyaline areas (Fig. 2U–X). Length of the locomotive form was 35–75 μm (average 58.3 μm), breadth 10–35 μm (average 18.5 μm), L\B ratio 1.3–5.8 (average 3.5). No differentiated uroidal structures were observed.

The granuloplasm contained food vacuoles, contractile vacuoles, several paired or spherical crystals and the single spherical nucleus about 6 μm in diameter with the central nucleolus about 5 μm in diameter. Floating form had several thin radiating pseudopodia with conical hyaline ends and, usually, several shorter projections (Fig. 2Y). No cysts were found in our cultures.

2. Molecular phylogeny

The phylogenetic tree based on the 18S rRNA gene sequence revealed the genus *Mayorella* with high BS and full PP support (Fig. 3). The neighboring clade consisted of *Dermamoeba algensis* and *Paradermamoeba*

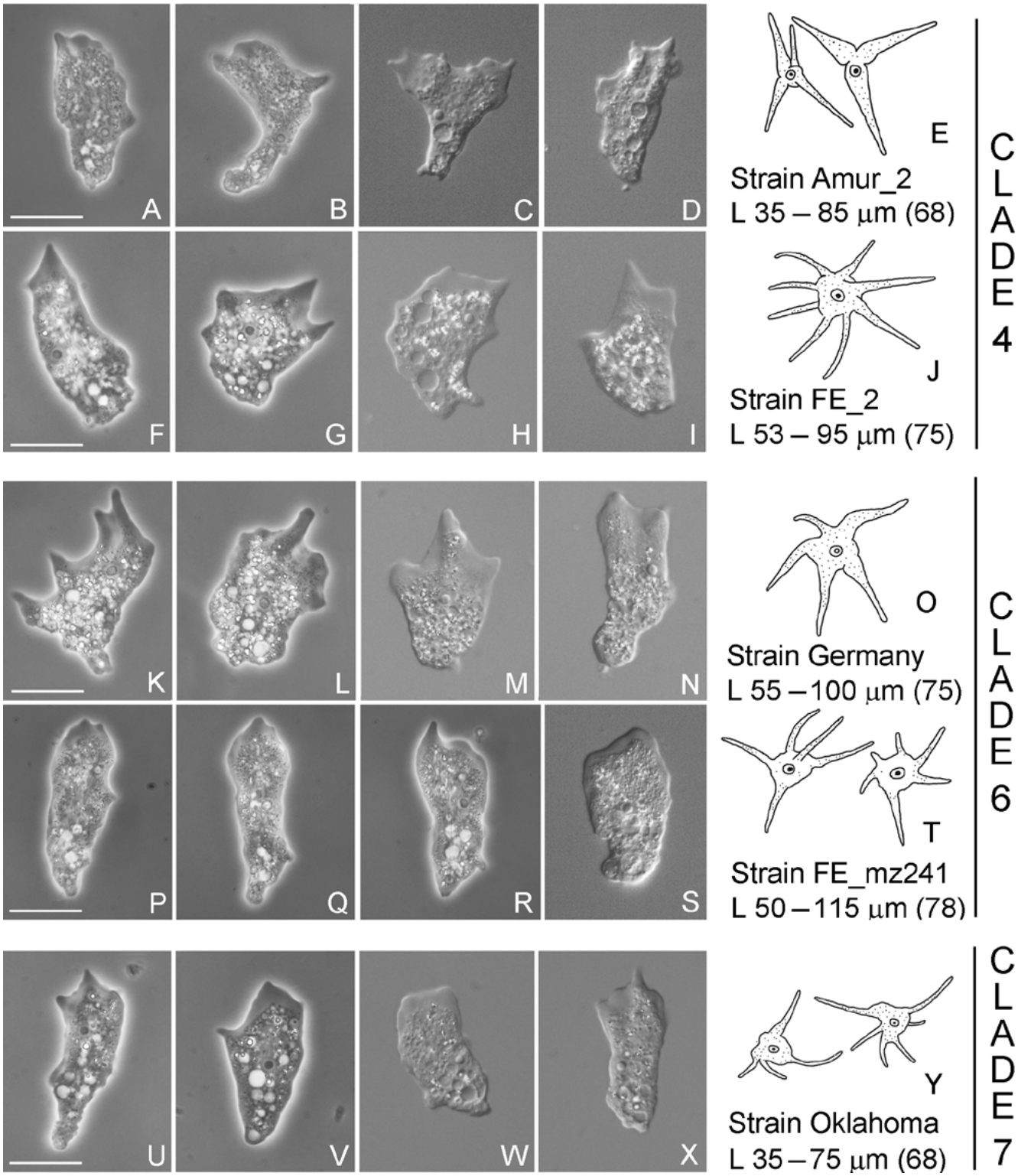

Fig. 2. Diversity of locomotive and floating forms of studied strains: A–E – *Mayorella sp.* strain Amur_2; F–J – *Mayorella sp.* strain FE_2; K–O – *Mayorella sp.* strain Germany; P–T – *Mayorella sp.* strain FE_mz241; U–Y – *Mayorella sp.* strain Oklahoma. Scale bar is 20 μm.

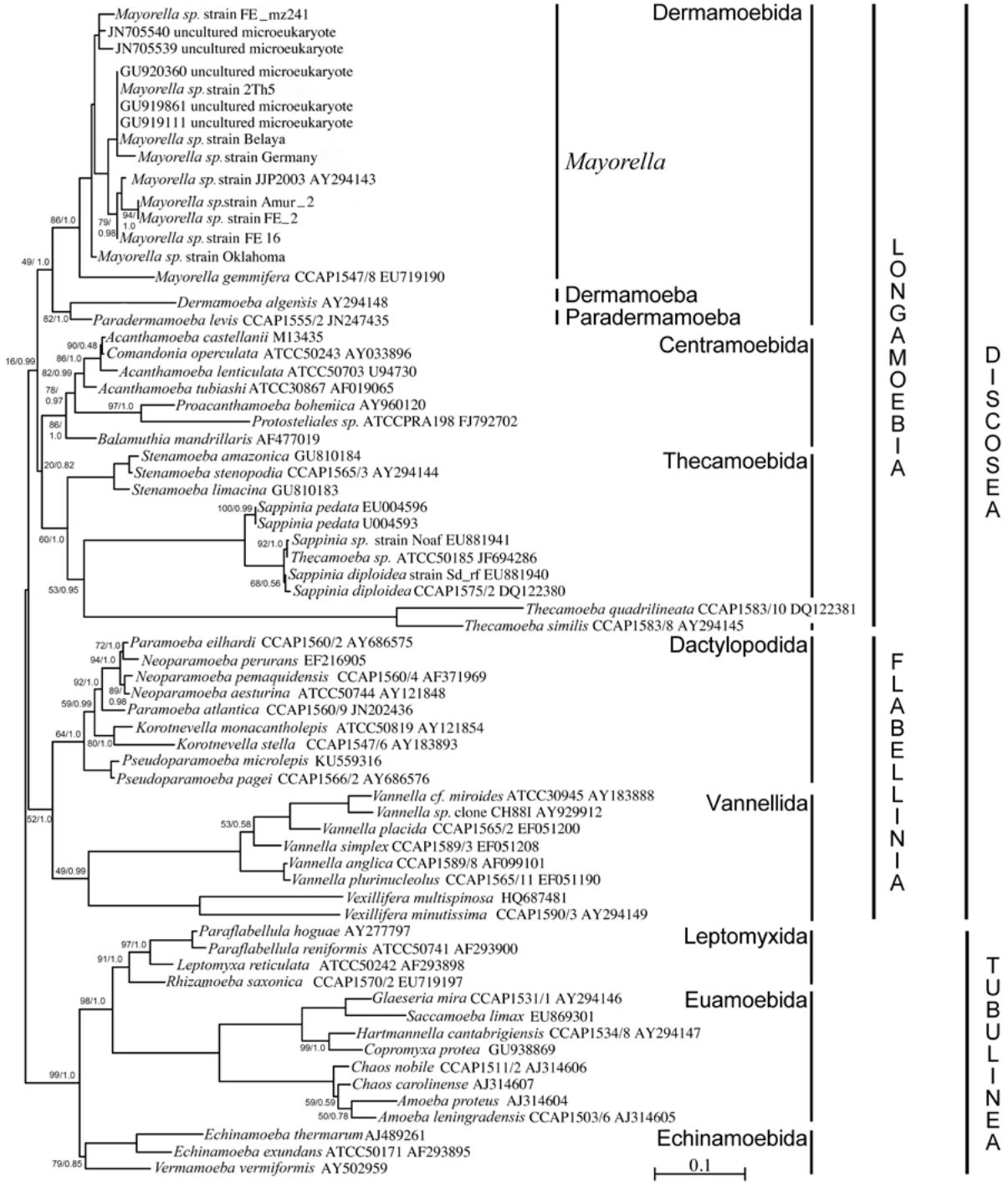

Fig. 3. Phylogenetic tree based on SSU rRNA gene sequences for all available *Mayorella* sequences and a wide sample of Discosea and Tubulinea taxa. Supports are indicated as PP/BS; black dots indicate 1.0/100 PP/BS support. GTR + γ + I model of evolution; 1554 sites.

levis, also grouped with each other with high support, altogether forming the clade corresponding to the order Dermamoebida. The neighbors to Dermamoebida were clades consisting of species belonging to the orders Acanthamoebida and Thecamoebida. Both these clades had high BS and full PP support, while their joint grouping was only moderately supported. This assemblage in total formed the group, formally corresponding to the subclass Longamoebia *sensu* Smirnov *et al*. (2011), also we aware that this assemblage may be invalid (Kang *et al.* 2017, Tekle and Wood 2017). The rest of the tree shows usual arrangement of amoebae lineages.

In the narrower phylogenetic tree containing the *Mayorella* sequences and a few outgroups, strains of *Mayorella* form seven well-supported clades (Fig. 4). Four environmental sequences (GU920360, GU922590, GU921517, GU919111) grouped in Clade 1. Clade 2 included two newly observed strains *Mayorella* strain Belaya and *Mayorella* strain 2Th5 and environmental sequence GU919861. Clade 3 unified *Mayorella* sp. strain FE 16 and *Mayorella* sp. strain JJP2003. Clade 4 consisted of *Mayorella* sp. strain FE_2 and *Mayorella* strain Amur_2. Clade 5 represented a separate branch formed by the strain *Mayorella gemmifera* CCAP 1547/8 (this is the type strain of this species). Clade 6 included *Mayorella sp*. strain Germany and *Mayorella sp.* FE_mz241. Clade 7 consisted of *Mayorella* sp. strain Oklahoma and environmental sequences JN705540 and JN705539. The entire *Mayorella* clade in this tree was fully supported, while the supports for internal branching were moderate to low (this could be attributed to the relatively high level of the SSU sequence similarity among mayorellas in general). Only two clades – clade 3 and clade 4 were fully supported; both unify very highly similar sequences.

DISCUSSION

The wide list of *Mayorella*-like amoebae species mentioned in the literature without detailed description indicates that light microscopy does not provide reliable data for identification of *Mayorella* species (Bovee 1985, Page 1988, see also Goodkov and Buryakov 1988 and Smirnov 1999). Transmission electron microscopy revealed a characteristic structure of cell coat ('cuticle') in *Mayorella,* which is a genus-specific character, but this finding did not solve the problem of species identification entirely because of the variability of cell coat structure under different fixation procedures (Page 1983, 1988). From the published data we can reasonably suggest that species diversity within the genus *Mayorella* is much higher than described presently, and high molecular diversity of *Mayorella*-like amoebae recovered in the present study confirms this suggestion.

Among the studied strains we observe a good correlation between the branching pattern formed at the molecular tree and the morphological data on studied species. Strains of *Mayorella* belong to seven clades, provisionally recognized in Figure 4.

Clade 1 unifies four environmental sequences obtained from activated sludge by pyrosequencing (source – GenBank data). Of them, three (GU920360, GU922590 and GU921517) look identical in our tree and, if the full length of available fragment is considered, the differences between these sequences do not exceed 7–8 bp, all are nucleotide indels, evenly distributed across the molecule and present both in the variable and in the conservative areas of the sequenced fragment. The polymorphism of this kind potentially can be due to pyrosequencing problems. All these three are identical in the variable region 138–177 bp (counted in GU919111 sequence). Thus we can suggest that all three of them represent the same *Mayorella* species.

GU919111 sequence forms a sister group to the three above mentioned ones. In the shared fragment, 380 bp in length, the level of the sequence identity between this sequence and GU920360 (taken as an exemplar from the above mentioned group of three sequences) is 0.968. These sequences show certain pattern differences in the variable region 138–177 bp (counted in GU919111 sequence). We can suggest that GU919111 sequences probably belong to different *Mayorella* species rather than the group formed by GU920360, GU922590 and GU921517 sequences, that may represent a single species. Hence, Clade 1 contains two putative *Mayorella* species.

The second clade – Clade 2 unifies one environmental sequence GU919861 (its origin is identical to the mentioned above sequences of the Clade 1 – it is from activated sludge) and two of our new organism-derived sequences belonging to *Mayorella sp.* strain Belaya and *Mayorella sp.* strain 2Th5. Of them GU919861 sequence shows the level of sequence identity 0.942 and 0.952 with *Mayorella* strain Belaya and strain 2Th5, respectively in the 480 bp region shared by all three sequences. This sequence has pronounced differences in the nucleotide pattern in the variable region 185–228 bp from both other isolates belonging to this clade and

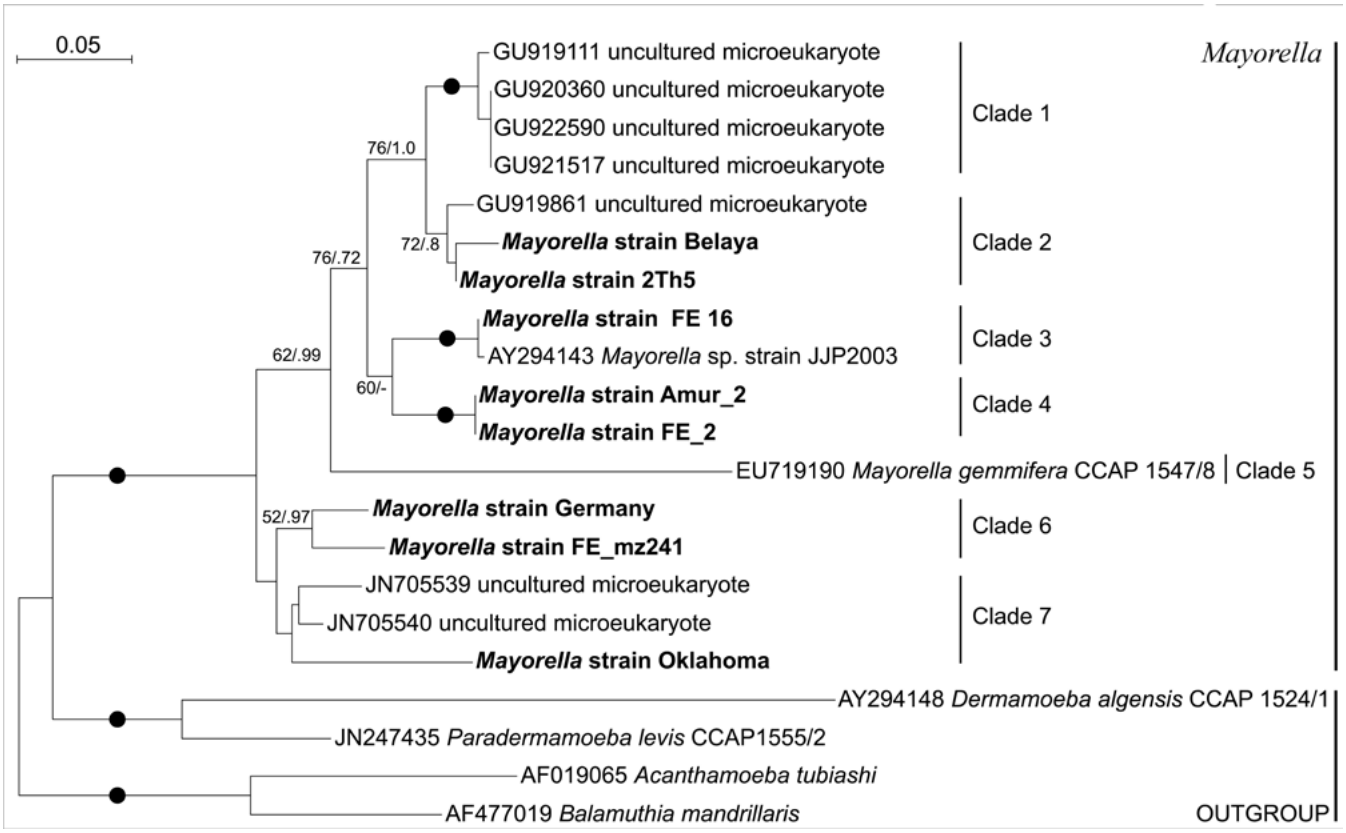

Fig. 4. Phylogenetic tree based on SSU rRNA gene sequences for *Mayorella* species only, with few outgroups. Supports are indicated as PP/BS; black dots indicate 1.0/100 PP/BS support. GTR + γ + I model of evolution; 1414 sites.

can safely be considered as representing an individual species. The level of sequence identity between *Mayorella sp.* strain Belaya and *Mayorella sp.* strain 2Th5 is 0.978 (10 differing bp), and these two sequences are quite similar in the variable region mentioned above (one bp difference and complete structure identity). At the morphological level they show rather similar appearance of the locomotive form, also cells of the strain Belaya show more pronounced tendency to move as a whole, without forming discrete conical pseudopodia. However, these two strains are drastically different in the organization of the floating form, which has pronounced central cytoplasmic mass and thin, curved, very narrow conical hyaline pseudopodia in the strain Belaya versus thick conical pseudopodia with the large granuloplasmic base in the strain 2Th5. The interpretation of this case is difficult, also further in the text we will consider the case when species have even higher sequence identity but different floating forms (Clade 3). Taking into account all mentioned differences, to the moment more parsimonious solution looks to recognize these two strains as different species. Hence, Clade 2 probably contains three different species in total.

Clade 3 consists of two sequences – that of *Mayorella* sp. strain FE 16 and AY294243 sequence from JJP2003 strain, obtained by Fahrni *et al.* (2003). These two sequences show nearly complete identity (1bp difference in 674 bp fragment in the position 1882 counted in AY294243 sequence) and group on the phylogenetic tree with full support. The comparison of the light-microscopic images found in our archive materials with the data FE 16 strain shows that they are rather similar in the locomotive morphology (also JJP2003 strain looks a little wider, which may be related to the conditions of observation, because this the only strain that was photographed in cultures, using inverted microscope in the year 2002). These strains have noticeable size differences – strain FE 16 is considerably longer in maximal dimension than JJP2003 strain (100 μm vs 59 μm, respectively), but we should take into account that

the number of measured cells for JJP2003 strain is negligible (8 cells in total – all available from digital photographs), so it may be not representative. Weighting results taken altogether the most parsimonious seems to suggest that FE16 strain is identical to the JJP2003 strain, originally isolated by A. Smirnov from Gurre Lake in Denmark.

Clade 4 unifies sequences obtained from two of our strains – *Mayorella* sp. FE_2 and *Mayorella* Amur_2. They have nearly identical SSU sequences (the difference is just 3 bp in one and the single place in 380 bp fragment). They group together on the phylogenetic tree with full support. On the morphological level these two strains are similar in size and general appearance of the moving cell; the nuclear structure and sizes are also nearly identical (average length is 75 and 68 μm, respectively); the nuclear structure and sizes are also nearly identical, but they drastically differ in the appearance of the floating form. The developed floating form in the strain Amur_2 has few thick conical pseudopodia virtually without the pronounced central cytoplasmic mass, while in the strain FE_2 floating form is radial, with the pronounced central mass of the cytoplasm and numerous narrow conical pseudopodia. The reasons for these differences are not clear yet. Page (1981, 1983) show different floating forms in different strains, apparently belonging to the same morphological species of *Mayorella*, so we can assume that this is a strain-specific character. Alternatively we can suggest that the resolution of the 18S rRNA gene sequence is not sufficient in this case, however this is not likely because all other morphological species in our tree are segregated with quite distinguishable genetic distances. In contrast with the above-described case (strains Belaya and 2Th5), very high level of the sequence identity lends strong credence to recognizing them as belonging to the same species. So by now we consider these two strain as representing one species. This conclusion is supported with the fact that they originate from one geographic region (Far East of Russia).

Clade 5 (in fact, branch) includes the sequence of *M. gemmifera* CCAP 1547/8 (type strain), which is quite distant from all other strains. This is the only marine species in our tree, also this is the longest sequence – one of two nearly complete SSU sequences of *Mayorella* available to the moment. Probably that is why its positon on the more general amoeba tree, where less sites are included in the analysis (Fig. 3), differs from that in the Fig. 4. In the Fig. 3 *M. gemmifera* forms the most basal branch to the entire *Mayorella* clade.

Clade 6 is formed with the sequences of the strain *Mayorella sp.* Germany and its neighbor, strain FE_mz241. They have the level of sequence identity 0.95, which corresponds to 19 differing bp in the 380 bp fragment, also these species have well visible differences in the nucleotide pattern in variable regions (e.g. positions 83–115, counted in *Mayorella sp.* strain Germany sequence). At the morphological level these strains have relatively similar sizes, but differ in the morphology of the moving cell (strain Germany has much more pronounced tendency to form large conical pseudopodia in locomotion) and in the morphology of the floating form (in the strain Germany it usually has solid conical bases of pseudopodia while in the strain FE_mz241 narrow pseudopodia start right from the central cytoplasmic mass). It is reasonable to conclude that they probably represent different species.

Among sequences forming Clade 7, that of *Mayorella sp*. strain Oklahoma stays well aside in the tree (sequence identity level compared to the other members of this clade is 0.906–0.903, which corresponds to 36–37 bp difference in the 380 bp fragment shared by all sequences). Two environmental sequences belonging to this clade originate from the same study (Lin *et al*. 2012) but show evident differences in variable regions (e.g. positions 256–406 and 438–601 in JN705540 sequence) and the sequence identity level is 0.88 in the 1557 bp fragment, which corresponds to 172 differing positions). This allows us to suggest that they also belong to different species. Thus Clade 7 probably includes no less than three *Mayorella* species; of them one is a morphologically described organism, while for two of them we have the sequence data only.

In the published literature there are descriptions of 22 species, which may be considered as belonging to the genus *Mayorella*. Among them there are only four properly described freshwater species, documented with the light microscopy and TEM data – *M. viridis*, *M. cantabrigiensis*, *M. vespertilioides* and *M. penardi* (see Cann 1981; Hollande *et al*. 1981; Page 1983a, 1988; Brown and Smirnov 2004) and four marine species – *M. gemmifera*, *M. dactylifera*, *M. kuwaitensis* and *M. pussardi* (see Page 1983, Goodkov and Buryakov 1988, Smirnov 1999). Since all our samples originate from freshwater habitats, it is possible to exclude marine species from the analysis, as there are no indications for existence of euryhaline *Mayorella* species. Among freshwater and soil-dwelling species, *M. cantabrigiensis* Page, 1983 and *M. viridis* (Leidy, 1874) Harnisch, 1983 are considerably larger than any of our strains and can probably

be excluded from the comparison as well. The species *M. vespertiliodes* may be compared in size range with strains Amur_2 and FE_2, but it usually forms much better pronounced conical pseudopodia during locomotion, more mamilliform than those seen in both our strains. The floating form in *M. vespertilioides*, as described by Page (1983a, 1988), rarely has long radiating pseudopodia. The same differences are valid if we compare strain Belaya and 2Th5 strain, which also fit the same size range. So we cannot reliably identify any of our strains as *M. vespertiliodies*. The species *M. penardi* has larger maximal size, also its average size is similar to that in four of our mentioned strains, but, according to Page (1983a), it has stronger tendency to form dactylopodia-like pseudopodia and its floating form may only be compared with that of FE 16 strain.

If less studied, but formally named *Mayorella* species are considered, it is possible to mention that in size and proportions (L/B ratio) the strain *Mayorella* sp. strain Germany, as well as strain FE_2 and strain Amur_2, resembles *Mayorella limacis* Bovee, 1970. However in strains Germany and FE_2 amoebae form frontal conical pseudopodia during locomotion and both these strains have a floating form with pronounced conical radiating pseudopodia, but not with thin hyaline pseudopodia characteristic for *M. limacis*. The strain Amur_2 has the floating form very different from that of *M. limacis*.

Above considerations, even based on the formal characters, are evidently too weak to announce the reliable co-specificity of any of our isolates with either of the named species. However the same is true for differences, so we have to conclude that it may be very problematic to classify a strain as belonging to a named species, even if the latter is properly studied. This is the reason why we do not name strains in this study – taking into account relatively high level of the SSU sequence identity between different *Mayorella* species, taxonomic naming would be appropriate when longer (preferably – complete) SSU sequences and (ideally) some other molecular markers will be obtained and TEM data on the cell coat of these strains will become available. This will allow us to compare all available data and will provide more solid base for identification of some (if any) isolates as known species. It is highly possible that re-description of older species will require establishing neotypes, since no type culture or any other documentation remain available for the most of them. However this work would unavoidably grow in a lasting study, much limited with the availability of material, while the genus *Mayorella* to the moment remains one of the least represented in molecular trees, which, among other matters, limits the possibility to identify new sequences of *Mayorella* in the environmental studies. So we think it is rational to make the present dataset available now. Further these isolates will be named with reference to the strain names used in the present study.

High genetic and morphological diversity of the genus *Mayorella* recovered in the present study evidences that this is a species-rich amoebae genus, like many other amoebae genera, e.g. *Vannella* (Smirnov *et al.* 2002, 2007); *Cochliopodium* (Kudryavtsev 1999, 2000, 2005, 2006; Kudryavtsev and Smirnov 2006) or *Flamella* (Michel and Smirnov 1999, Kudryavtsev *et al.* 2009, Shmakova *et al*. 2016, Glotova and Smirnov 2017) where most of the diversity is not yet recovered. It is now evident that the genus *Mayorella* completes the list of amoebae genera where DNA sequencing is obligate for precise species identification. However, as with many other amoebae taxa, the present study shows relatively high level of the SSU rDNA gene polymorphism in this amoeba genus (Nassonova *et al.* 2010, Zlatogurski *et al.* 2016). Thus proper species description of *Mayorella* species, besides the sequence, still requires light- and electron-microscopic data.

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Figs. S1–S3. Sampling sites located in North America, Europe and Far East of Russia (schemes).

Fig. S1


Baltic Sea
Estonia
Latvia
Moscow
Москва
North Sea
United
Kingdom
Lithuania
Ireland
Netherlands
Poland
Belarus
Germany
Belgium
Prague
Paris
Czech Rep
Slovakia
Ukraine
Austria
Moldova
Hungary
France
Romania
Croatia
Serbia
Black Sea
Italy
Rome
Barcelona
Bulgaria
Georgia
Portugal
Madrid
Istanbul
Azerbaij
Tyrrhenian Sea
Spain
Greece
Turkey

Fig. S2

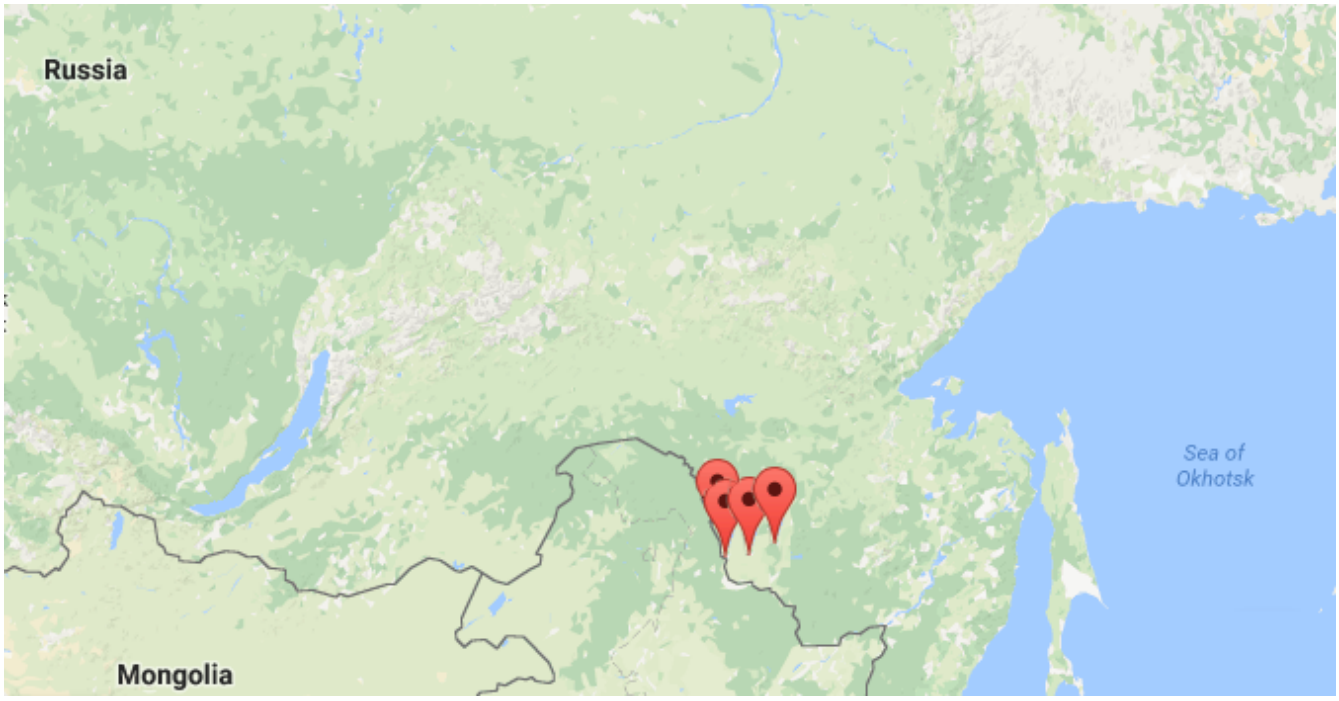
Russia
Sea of
Okhotsk
Mongolia

Fig. S3