ISSN 0095-4527, Cytology and Genetics, 2023, Vol. 57, No. 6, pp. 567–578. © Allerton Press, Inc., 2023.
Ukrainian Text © The Author(s), 2023, published in Tsitologiya i Genetika, 2023, Vol. 57, No. 6, pp. 60–72.

Phylogenetic Relationships among Naked Amoebae Found in Natural Biotopes

M. Patsyuk*

Ivan Franko Zhytomyr State University, Zhytomyr, Ukraine

**e-mail: kostivna@ukr.net*

Received January 23, 2023; revised May 25, 2023; accepted November 13, 2023

Abstract—Using morphological traits and molecular-genetic research methods, the authors have identified 24 species of naked amoeba from natural biotopes. The 18S rRNA gene sequences were obtained for the following species of naked amoeba: *Amoeba proteus* isolate AP07 (ON907618), *Saccamoeba limax* isolate SLU_22 (OP894078), *Saccamoeba limax* isolate SL_Uk19 (OQ520144), *Saccamoeba* sp. strain IDL777 (MZ079370), *Thecamoeba striata* isolate THS19 (OQ134482), *Thecamoeba striata* isolate THS20 (OQ134483), *Thecamoeba similis* isolate Prut river (OL604177), *Thecamoeba similis* isolate Baggersee Innsbruck (Baggersee Rossau) (OL604178), *Thecamoeba quadrilineata* isolate THQD2 (ON398269), *Thecamoeba quadrilineata* isolate THQA1 (ON398268), *Thecamoeba* sp. strain THS203 (MZ079371), *Stenamoeba stenopodia* isolate UKSS7 (OP375108), *Stenamoeba stenopodia* isolate POLSS7 (OP419588), *Korotnevella stella* isolate KSD2 (ON398267), *Korotnevella stella* isolate KSA1 (ON398266), *Vexillifera bacillipedes* isolate river Dnepr (OK649262), *Vannella lata* isolate Kamenka river (OL305063), *Vannella lata* isolate Varta river (OL305064), *Vannella* sp. strain VLS303 (MZ079372), *Vannella simplex* isolate Black Sea (OM403052), *Vannella simplex* isolate Mediterranean Sea (OM403053), *Ripella* sp. strain RPL100 (MZ079369), *Mayorella vespertilioides* isolate MV_7 (OP739500), *Mayorella* sp. isolate MY_7 (OP729930), *Acanthamoeba* sp. strain ATM123 (MZ079366), *Acanthamoeba* sp. isolate river Elbe (OK649261), *Acanthamoeba polyphaga* isolate AcPoly01 (ON908497), *Acanthamoeba polyphaga* isolate AcPoly15 (ON908496), *Acanthamoeba griffini* isolate Black sea (OM522832), *Acanthamoeba griffini* isolate Mediterranean Sea (OM522833), *Cochliopodium actinophorum* strain COP101 (MZ079367), *Cochliopodium minus* isolate river Stokhid (OK649264), *Cochliopodium* sp. strain COP102 (MZ079368), *Vahlkampfia avara* isolate VA7 (OP179657), *Willaertia magna* isolate river Teterev (OK649263). All of the naked amoebae species on the phylogenetic tree constructed based on the 18S rRNA gene are located within Amoebozoa and grouped with Tubulinea and Discosea. There are separate groups of freshwater, marine, and terrestrial biotopes; these groups are sister species relative to one another with low results of bootstrap analysis, which shows a low accuracy in the distances of particular amoeba species isolated from different natural biotopes.

Keywords: naked amoeba, morphology, the 18S rRNA gene, phylogeny, natural biotopes

DOI: 10.3103/S0095452723060063

INTRODUCTION

Naked amoebas are the most widespread group of protists in marine and freshwater bodies and soils, and some of them are endoparasites. These organisms are often isolated from natural biotopes, but their biodiversity remains poorly studied. To answer the question about the specificities in the spread of naked amoebae, long-term studies should be undertaken, comprising numerous samples from different biotopes and considering the fact that the composition of species may vary due to different seasons. These studies are of a particular importance since a considerable part of the amoeboid fauna remains unstudied and we regularly identify new species in our samples.

The question concerning the phylogeny of naked amoebae also remains unanswered since the group includes taxa differing in morphological traits. Phylogenetic relationships among the naked amoeba species cannot be determined without faunistic studies on the diversity of these protists. Descriptions of new species enable us to understand the evolutionary pathways of this group of heterotrophic organisms. Many studies discussing the systematics of naked amoebae typified the latter by the morphological traits that did not reflect their phylogenetic similarity (Leidy, 1879; Butschli, 1880–1882; Delage et al., 1896; Calkins, 1901; Penard, 1902). Description and identification of naked amoebae is a complex process requiring the isolation of protists into a culture and the application of light and electron microscopy. An organism is correspondingly identified at the strain level. The introduction of molecular genetic methods allowed researchers to identify new species on a regular basis. A new spe-

cies cannot currently be described while the earlier described species cannot be identified without analyzing morphological data. A contemporary study on the fauna of naked amoebae must combine the light microscopic and molecular genetic methods. Comparing current data with results of earlier conducted studies, we can observe changes in the fauna of naked amoebae even in any well-studied biotope, as well as improve its characteristics, using molecular data. In addition, molecular genetic research methods enable us to identify relationships within the Amoebozoa group (Sims et al., 1999; Amaral Zettler et al., 2000).

We should note that the first systems of naked amoebae for depicting phylogenetic relationships among different groups of these protists were developed by a group of researchers (Cavalier-Smith et al., 1998, 2004, 2009, 2016; Peglar et al., 2003; Tekle et al., 2008; Lahr et al., 2015). These systems were based on both morphological and molecular data of amoeboid organisms. Apart from naked amoebae, the systems on phylogenetic trees showed other members of protists. In our studies, we use the current system for naked amoebae described in (Cavalier-Smith et al., 2016).

The objective of our study was to identify phylogenetic relationships among different species of naked amoebae collected by us from different biotopes within the 2013–2022 combined studies on the fauna of these protists.

MATERIALS AND METHODS

The utilized specimens of naked amoebae were isolated from samples taken from freshwater bodies of Ukraine, Poland, the Czech Republic, Germany, Austria, and Switzerland. In total, we investigated over 1000 samples and prepared nearly 500 amoeba cultures. Specialists prefer not to manipulate with natural material, choosing laboratory strains of amoebae. We evenly distributed 5-mL samples across 100-mm Petri dishes with non-nutrient agar (NNA) according to the Page methods with the addition of grains of rice (Page, 1988). Amoebae were maintained in cultures under laboratory conditions at +20°C with nonregulated light. Each dish with the sample was observed once per 8 days using a Lomo MBR-3 microscope. To determine the species of amoebae, one cell was isolated from each dish with a long Pasteur pipette onto D 50 mm Petri plates with 1.5% NNA (Page, 1988), which was prepared on the Prescott-James (PJ) mineral medium and further multiplied. The PJ medium has the following composition (Page, 1988):

Prepare three main solutions (each diluted with 100 mL water).

	Main solution A
$CaCl_2 \cdot 2H_2O$	0.433 g
KCl	0.162 g
	Main solution B
K_2HPO_4	0.512 g
	Main solution C
$MgSO_4 \cdot 7H_2O$	0.280 g

A portion of 1 mL from each resulting solution was mixed with 997 mL of distilled water.

The species were identified using an Axio Imager MI microscope (Animalia Center for Collective Use of Scientific Instruments, Schmalhausen Institute of Zoology, National Academy of Sciences of Ukraine) with differential interference contrast, placing a water drop with live cells on a slide. The main morphological traits included sizes of locomotor forms (cell width (W)—distance measured perpendicular to the direction of the widest part of the cell; length of the cell (L)—distance between the anterior and posterior ends of the mobile cell; ratio between the length of the cell and its width (L/W)) and the diameter of the nucleus of the cell and the cyst (Page and Siemensma, 1991). The measurements were performed on intact cells or microphotographs. At least 50 amoebae from each strain were measured. The nuclei were measured at least in 50 amoebae from each strain. The cells were measured with an ocular micrometer (×40).

To typify naked amoebae, the following traits conventional for this group were used: the morphology of the locomotor form, considering their morphotype, the morphology of the uroid and pseudopodiae, the character of cytoplasm streaming, and the floating form development (Page, 1983; Page and Siemensma, 1991).

DNA isolation. DNA is nonisolable from the majority of amoebae since, apart from the very amoebae, other eukaryotes (such as fungi, animal-like organisms) on which amoebae feed are present in amoeba cultures. Therefore, prior to isolating DNA and to cleaning amoebae from other eukaryotic contaminants, they were maintained on hungry agar. Genomic DNA was isolated using the guanidine-isothiocyanate method (Maniatis et al., 1982). The 18S rRNA gene was amplified using universal eukaryotic primers (RibA 5'-ACCTGGTTGATCCTGCCAGT-3' (Medlin et al., 1988). The same primers were used for sequencing for each species. The obtained DNA sequences (Table 1) were compared with the GenBank data using the BLAST (NCBI) software (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The obtained sequences were automatically aligned using the MUSCLE algorithm realized under the MEGA 10.0 software program. Based on the alignment structured by the 18S rRNA gene sequences for the representatives of the genera *Amoeba* Bory de St. Vincent, 1822, *Saccamoeba* Bovee, 1972, *Cochliopodium* Hertwig and Lesser, 1874, *Mayorella* Schaeffer, 1926, *Vannella* Bovee, 1965, *Vexillifera* Schaeffer, 1926, *Korotnevella*

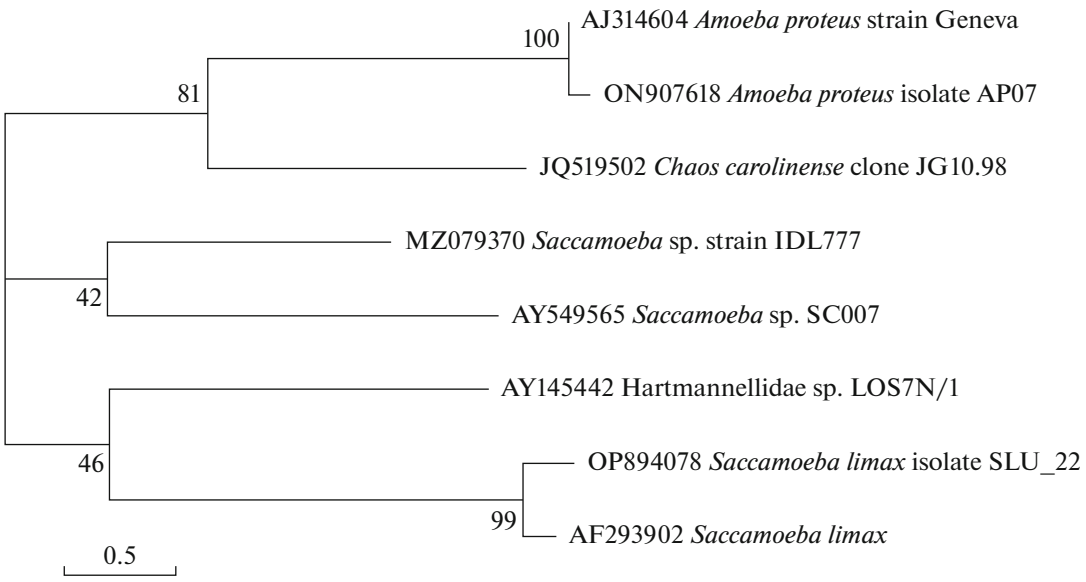

Fig. 1. Fragment of the phylogenetic tree based on the 18S rRNA gene sequences for the members of the genera *Amoeba* and *Saccamoeba*. The scale shows the equivalence of the distance between the sequences.

Goodkov, 1988, *Stenamoeba* Smirnov et al., 2007, *Thecamoeba* Fromentel, 1874, *Acanthamoeba* Volkonsky, 1931, *Vahlkampfia* Chatton and Lalung-Bonnaire, 1912, and *Willaertia* de Jonckheere et al., 1984, we performed a phylogenetic analysis, using the MEGA 10.0 software program (Kumar et al., 2018). The phylogenetic analysis was based on both the sequences of our study and those of other naked amoebae species available under the GenBank database. The phylogenetic analysis was performed using the method of maximum likelihood with the Neighbor-Joining algorithm of the MEGA 10.0 software. The validity of the constructed dendrograms was evaluated using the bootstrapping technique (1000).

RESULTS

As a result of the studies conducted from 2013 through 2022, we isolated 44 species of naked amoeba from freshwater bodies and 23 species from soils and identified 24 species using molecular genetic methods. The 18S rRNA gene sequences of naked amoebae isolated from natural biotopes are given in Table 1. All sequences obtained during our studies were compared with the related sequences of naked amoebae for SSU rDNA from GenBank (Figs. 1–5).

The phylogenetic analysis shows that the sequence of *Amoeba proteus* isolate AP07 (ON907618, Stokhid R.) is reliably grouped with that of the *Amoeba proteus* sp. strain Geneva deposited with the GenBank database (AJ314604), which was found in bodies of water in Geneva (Switzerland). *Chaos carolinense* clone JG10.98 (JQ519502) is its sister species. Another group of sequences includes unidentified species of the genus *Saccamoeba* (*Saccamoeba* sp. strain IDL777 (MZ079370, Teteriv R.) and *Saccamoeba* sp. SC007 (AY549565)) with a low bootstrap result. *Saccamoeba limax* isolate SLU_22 (OP894078), which we isolated from Stokhid R. in Volyns'ka oblast, is well grouped with *Saccamoeba limax*, the identified DNA sequence of which has the GenBank accession number AF293902 with a comparably high result of bootstrap analysis. *Hartmannellidae* sp. LOS7N/1 (AY145442) belonging to the family Hartmannellidae Volkonsky, 1931, is a sister species relative to the above indicated group of amoebae with a low result of bootstrap analysis. The phylogenetic tree (Fig. 1) represents species of naked amoebae isolated from freshwater bodies.

Nine different sequences of naked amoebae from the genera *Thecamoeba* and *Stenamoeba,* which were obtained during our studies, were compared with five related GenBank sequences for the SSU rDNA gene (Fig. 2). The analysis of some fragment from the phylogenetic tree shows three groups of *Thecamoeba*-like amoebae and one group of *Stenamoeba*-like amoebae. The first group includes sequences of such species as *Thecamoeba* sp. ATCC PRA-35 (EF455775), *Thecamoeba striata* isolate THS19 (OQ134482) from Kamianka R. near the city of Zhytomyr, and *Thecamoeba striata* isolate THS20 (OQ134483) from Lake Geneva, Switzerland. The remaining species are reliably grouped with the above indicated species. *Thecamoeba* sp. THS203 (MZ079371) that we found in Teteriv R. near the city of Zhytomyr is a sister species relative to the indicated group. The second group includes sequences of such species as *Thecamoeba similis* isolate Prut river (OL604177) and *Thecamoeba similis* isolate Baggersee Innsbruck (OL604178), which we sampled in Prut R. near the city of Chernivtsi and Lake Baggersee, Austria, respectively. The

Table 1. 18S rRNA gene sequences of naked amoebae isolated from natural biotopes

Amoeba species	Sampling locality/biotope	Identified DNA sequences in GenBank
Amoeba proteus isolate AP07	Stokhid R., Volyn oblast, Ukraine/top layer of bottom soil and a small amount of near-bottom water	ON907618
Saccamoeba limax isolate SLU_22	Stokhid R., Kovel raion, Volyn oblast, Ukraine/top layer of bottom soil and a small amount of near-bottom water	OP894078
Saccamoeba limax isolate SL_Uk19	Lake Geneva, Switzerland/top layer of bottom soil and a small amount of near-bottom water	OQ520144
Saccamoeba sp. strain IDL777	Teteriv R., city of Zhytomyr, Ukraine/top layer of bottom soil and a small amount of near-bottom water	MZ079370
Thecamoeba striata isolate *THS19*	Kamianka R., city of Zhytomyr, Ukraine/top layer of bottom soil and a small amount of near-bottom water	OQ134482
Thecamoeba striata isolate THS20	Lake Geneva, Switzerland/top layer of bottom soil and a small amount of near-bottom water	OQ134483
Thecamoeba similis isolate Prut river	Prut R., city of Chernivtsi, Chernivtsi oblast, Ukraine/top layer of bottom soil and a small amount of near-bottom water	OL604177
Thecamoeba similis isolate Baggersee Innsbruck (Baggersee Rossau)	Lake Baggersee Innsbruck (Baggersee Rossau), Austria/top layer of bottom soil and a small amount of near-bottom water	OL604178
Thecamoeba quadrilineata isolate THQD2	R. Dnipro, Zaporizhzha oblast, Ukraine/top layer of bottom soil and a small amount of near-bottom water	ON398269
Thecamoeba quadrilineata isolate THQA1	Reservoirs near town of Vocklabruck/top layer of bottom soil and a small amount of near-bottom water;	ON398268
Thecamoeba sp. strain THS203	Teteriv R., city of Zhytomyr, Ukraine/top layer of bottom soil and a small amount of near-bottom water	MZ079371
Stenamoeba stenopodia isolate UKSS7	Huiva R., city of Zhytomyr, Ukraine/top layer of bottom soil and a small amount of near-bottom water	OP375108
Stenamoeba stenopodia isolate POLSS7	Varta R., near the city of Poznan', Poland/top layer of bottom soil and a small amount of near-bottom water	OP419588
Korotnevella stella isolate KSD2	R. Dnipro, Kherson oblast, Ukraine/top layer of bottom soil and a small amount of near-bottom water	ON398267
Korotnevella stella isolate KSA1	Lake Baggersee Innsbruck (Baggersee Rossau), Austria/top layer of bottom soil and a small amount of near-bottom water	ON398266
Vexillifera bacillipedes isolate river Dnepr	R. Dnipro, Zaporizhzha oblast, Ukraine/top layer of bottom soil and a small amount of near-bottom water	OK649262
Vannella lata isolate Kamenka river	Kamianka R., city of Zhytomyr, Ukraine/top layer of bottom soil and a small amount of near-bottom water	OL305063
Vannella lata isolate Varta river	Varta R., near the city of Poznan', Poland/top layer of bottom soil and a small amount of near-bottom water	OL305064
Vannella sp. strain VLS303	Teteriv R., city of Zhytomyr, Ukraine/top layer of bottom soil and a small amount of near-bottom water	MZ079372
Vannella simplex isolate Black Sea	Black Sea, Odessa, Ukraine/top layer of bottom soil and a small amount of near-bottom water	OM403052
Vannella simplex isolate Mediterranean Sea	Mediterranean Sea, city of Side, Turkey/top layer of bottom soil and a small amount of near-bottom water	OM403053
Ripella sp. strain RPL100	Huiva R., city of Zhytomyr, Ukraine/top layer of bottom soil and a small amount of near-bottom water	MZ079369
Mayorella vespertilioides isolate MV_7	Kamianka R., city of Zhytomyr, Ukraine/top layer of bottom soil and a small amount of near-bottom water	OP739500
Mayorella sp. isolate MY_7	Floodplain reservoir, Vinnytsia oblast, Ukraine/top layer of bottom soil and a small amount of near-bottom water	OP729930
Acanthamoeba sp. strain ATM123	Huiva R., city of Zhytomyr, Ukraine/top layer of bottom soil and a small amount of near-bottom water	MZ079366

Table 1. (Contd.)

Amoeba species	Sampling locality/biotope	Identified DNA sequences in GenBank
Acanthamoeba sp. isolate river Elbe	Elbe R., near Usti nad Labem, Czech Republic/top layer of bottom soil and a small amount of near-bottom water	OK649261
Acanthamoeba polyphaga isolate AcPoly01	moss, Zhytomyr oblast, Ukraine/top layer of bottom soil and a small amount of near-bottom water	ON908497
Acanthamoeba polyphaga isolate AcPoly15	moss, Poland	ON908496
Acanthamoeba griffini isolate Black sea	Black Sea, Odessa, Ukraine/top layer of bottom soil and a small amount of near-bottom water	OM522832
Acanthamoeba griffini isolate Mediterranean Sea	Mediterranean Sea, city of Side, Turkey/top layer of bottom soil and a small amount of near-bottom water	OM522833
Cochliopodium actinophorum strain COP101	Kamianka R., city of Zhytomyr, Ukraine/top layer of bottom soil and a small amount of near-bottom water	MZ079367
Cochliopodium minus isolate river Stokhid	Stokhid R., Kovel raion, Volyn oblast, Ukraine/top layer of bottom soil and a small amount of near-bottom water	OK649264
Cochliopodium sp. strain COP102	Teteriv R., city of Zhytomyr, Ukraine/top layer of bottom soil and a small amount of near-bottom water	MZ079368
Vahlkampfia avara isolate VA7	Kamianka R., city of Zhytomyr, Ukraine/top layer of bottom soil and a small amount of near-bottom water	OP179657
Willaertia magna isolate river Teterev	Teteriv R., city of Zhytomyr, Ukraine/top layer of bottom soil and a small amount of near-bottom water	OK649263

sequence of *Thecamoeba similis* strain UKNCC: CCAP1583 is in sister relations with the identified DNA sequence from the GenBank database (accession number JQ271722). According to the phylogenetic analysis, the first two species are 99% similar, while the third species is 58% similar. The third group includes sequences of such naked amoebae as *Thecamoeba quadrilineata* isolate THQD2 (ON398269) and *Thecamoeba quadrilineata* isolate THQA1 (ON398268), which we found in the Dnipro R. of Zaporizhzha oblast and in floodwater ponds near the town of Vöcklabruck, Austria, respectively. The latter are grouped with low values of bootstrap analysis. *Thecamoeba quadrilineata* is a sister species and its GenBank DNA sequence identifier is DQ122381. This species has a high value of bootstrap analysis within this group. The fourth group comprised sequences for the members of the genus *Stenamoeba* (OP375108, AY294144, OP419588), which are reliably grouped between one another (Fig. 2). All naked amoeba species represented on the phylogenetic tree are well-known from freshwater bodies.

Figure 3 shows that all sequences of naked amoebae from the genus *Korotnevella* (AY183893, OM407395, ON398266, AY686573, ON398267) are grouped into the same community with high values of bootstrap analysis (90–98%) between one another. The *Vexillifera*-like amoebae well-known from Dnipro R. of Zaporizhzha oblast (OK649262) and water bodies of Czechia (HQ687484), which comparably reliably grouped between one another, forms a sister group relative to *Korotnevella* amoebae. The second group comprises sequences of marine and freshwater naked amoebae from the genus *Vannella*. *Vannella lata* isolate Kamenka river (OL305063) from Kamianka R. near the city of Zhytomyr is 62% similar to *Vannella lata* the DNA sequence number of which in GenBank is AF464917, and these amoebae are reliably grouped with *Vannella lata* isolate Varta river (OL305064) sampled by us from R. Varta near the city of Poznan', Poland (98%). These freshwater amoebae form a sister group with marine species (such as *Vanella simplex* isolate Black Sea (OM403052), *Vanella simplex* isolate Mediterranean Sea (OM403053), and *Vanella simplex* (AF464914)).

Acanthamoeba griffini isolate Mediterranean Sea (OM522833) is grouped with *Acanthamoeba griffini* isolate B18 (GU553135) from hot springs with a comparably high bootstrap result (86%). The *Acanthamoeba griffini* isolate Black Sea (OM522832) is similar to the above indicated isolates by 66% (Fig. 4). *Acanthamoeba polyphaga* isolate PA29 (MF399035 from waste waters of Spain) (similarity attains 43%) is a sister species relative to the indicated amoeba group. The *Acanthamoeba* isolate river Elbe (OK649261) sequence of the amoeba sampled by us from Elbe River (Czechia) is grouped with a low bootstrap result with *Acanthamoeba polyphaga* Panola Mountain (AF019052). The sequences of *Acanthamoeba polyphaga* isolate AcPoly01 (ON908497) from mosses in Ukraine and *Acanthamoeba polyphaga* isolate AcPoly15 (ON908496) found in the mosses of Poland form

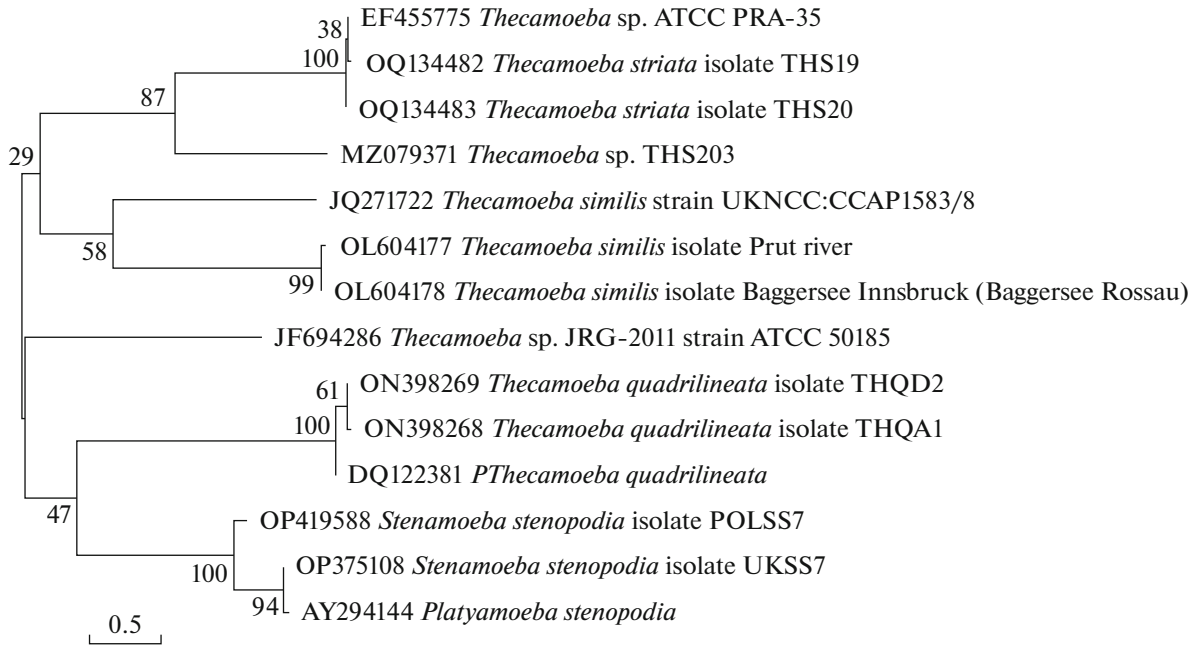

Fig. 2. Fragment of the phylogenetic tree based on the 18S rRNA gene sequences for the members of the genera *Thecamoeba* and *Stenamoeba*. The scale shows the equivalence of the distance between the sequences.

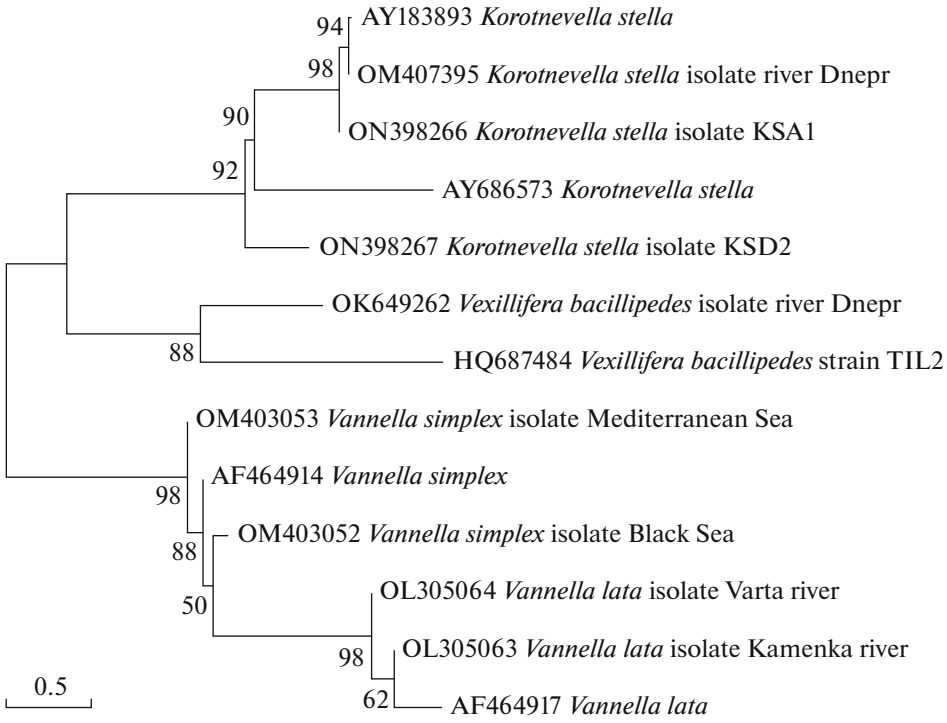

Fig. 3. Fragment of the phylogenetic tree based on the 18S rRNA gene sequences for the members of the genera *Korotnevella*, *Vexillifera*, *Vannella*. The scale shows the equivalence of the distance between the sequences.

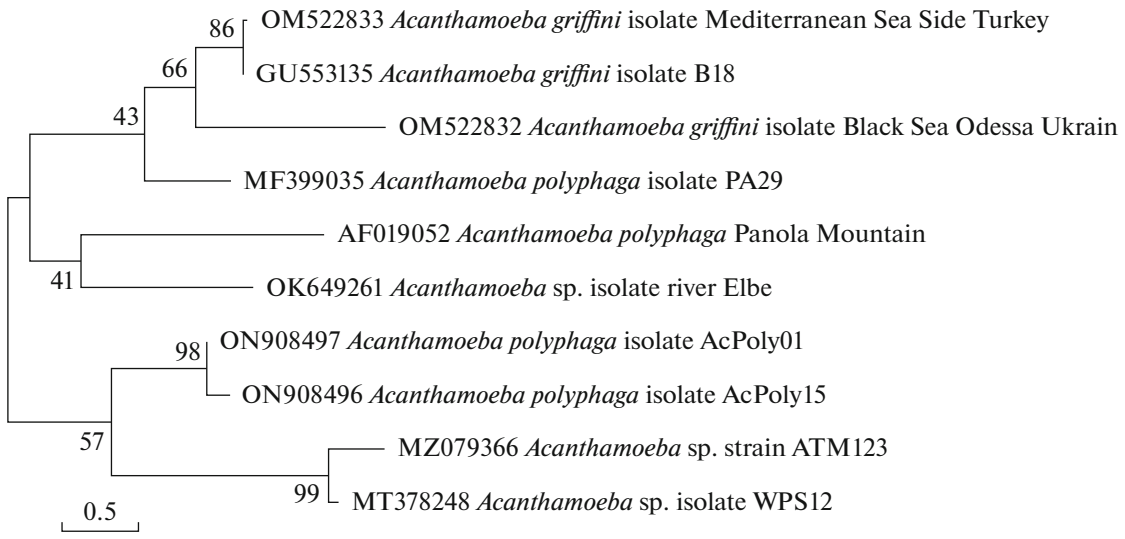

Fig. 4. Fragment of the phylogenetic tree based on the 18S rRNA gene sequences for the members of the genus *Acanthamoeba*. The scale shows the equivalence of the distance between the sequences.

a particular group. The validity of the group attains 98%. The sequences of *Acanthamoeba* sp. strain ATM123 and *Acanthamoeba* sp. isolate WPS12 (MZ079366 and MT378248) form a sister group (Fig. 4).

Members of the genus *Cochliopodium* form some heterologous group on the phylogenetic tree, depicting different species (*Cochliopodium* sp. ATCC 30936 (KC747718), *Cochliopodium minus* strain SUM3P (JQ271675), *Cochliopodium minus* isolate river Stokhid (OK649264), *Cochliopodium actinophorum* strain COP101 (MZ079367)). These species are grouped with a reliable support with one another (from 87 to 95%) (Fig. 5). *Cochliopodium minus* isolate river Stokhid (OK649264) sampled by us from Stokhid R. of Volyns'ka oblast, is relatively significantly grouped with (*Cochliopodium* sp. ATCC 30936 (KC747718) and *Cochliopodium minus* strain SUM3P (JQ271675). *Cochliopodium actinophorum* strain COP101 (MZ079367) from Kamianka R. (Ukraine) is a sister strain relative to the group of the above indicated amoebae (95%). As regards to heterolobose amoebae, representatives of the genus *Willaertia* are grouped with those of the genus *Vahlkampfia* (Fig. 5). All species of naked amoebae on the phylogenetic tree are of the freshwater type.

We have constructed a phylogenetic tree for different species of naked amoebae isolated from natural biotopes (Fig. 6). *Willaerta magna* isolate river Teterev (OK649263), a heterolobose amoeba, was chosen as an outgroup. As Fig. 6 shows, *Amoeba proteus* isolate AP07 (ON907618) and *Chaos carolinense* clone JG10.98 (JQ519502) form a group with a comparably high value of bootstrap analysis (88%), and *Saccamoeba limax* isolate SLU_22 (OP894078) is a sister species with low significance (33%). These protists belong to polytactic and monopodial morphotypes and are included into the Tubilinea group. The remaining species include naked amoebae with cylindrical, sometimes flattened, pseudopods, monoaxial streaming of the cytoplasm, without glycostyles and squamae (Dykova et al., 2008; Cavalier-Smith et al., 2016; Anderson, 2018). This group included freshwater naked amoebae. The phylogenetic tree further group amoebae from the Discosea group—the flattened amoebae forming no tubular pseudopodia, with polyaxial streaming of the cytoplasm are glycostyles and organic squamae (Dykova et al., 2008; Corsaro et al., 2013; Cavalier-Smith et al., 2016; Anderson, 2018). This clade embraces the species well-known from freshwater, marine bodies, and terrestrial biotopes. Some particular group includes amoebae with the lens-like morphotype from the genus *Cochliopodium*. *Cochliopodium minus* strain SUM3P (JQ271675) from water bodies of Czechia is reliably grouped with *Cochliopodium minus* isolate river Stokhid (OK649264) from Stokhid R. of Volyns'ka oblast, while *Cochliopodium actinophorum* strain COP101 (MZ079367) from Kamianka R. near the city of Zhytomyr is its sister species (the similarity attains 71%). *Mayorella vespertilioides* isolate MV_7 (OP739500) from Kamianka R. (Zhytomyr) forms a separate branch. The species belongs to the mayorellian morphotype. The freshwater fan-shaped amoebae (*Vannella lata* isolate Kamenka river (OL305063) + *Vannella lata* isolate Varta river (OL305064)) and marine amoebae (*Vannella simplex* isolate Mediterranean Sea (OM403053) + (*Vannella simplex* isolate Black Sea (OM403052)) are included on the phylogenetic tree in different groups with sister relationships with one another. *Korotnevella stella* isolate KSD2 (ON398267) from Dnipro R. is reliably grouped with *Korotnevella*

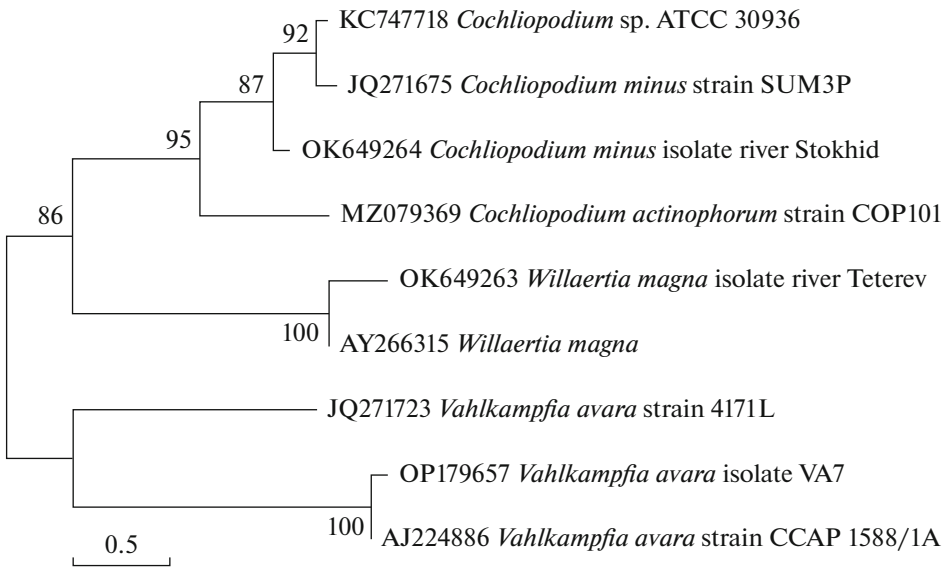

Fig. 5. Fragment of the phylogenetic tree based on the 18S rRNA gene sequences for the members of the genera *Cochliopodium*, *Vahlkampfia*, and *Willaertia*. The scale shows the equivalence of the distance between the sequences.

stella isolate KSA1 (ON398266) from Lake Baggersee, Austria. The *Vexillifera bacillipedes* isolate river Dnepr (OK649262) from Dnipro R. (similarity attains 47%) is a sister species. The indicated species belong to a dactylopodial morphotype. The members of the genus *Stenamoeba* is well grouped with amoebae from the genus *Thecamoeba* (85–99%).

A reliable group of marine *Acanthamoeba* members (OM522832 and OM522833), which were sampled by us from the Black and Mediterranean seas, are grouped with low similarity (58%) with *Thecamoeba quadrilineata* isolate THQD2 (ON398269) and *Thecamoeba quadrilineata* isolate THQD1 (ON398268), which were isolated by us from Dnipro R. of Zaporizhzha oblast and near Vöcklabruck, Austria, respectively.

DISCUSSION

Isolating a sufficient number of naked amoebae from natural biotopes and their morphological and molecular genetic characterization remain the main purpose necessary for clarifying the taxonomic and phylogenetic position for the majority of these protist species. We have sequenced the 18S rRNA gene for 24 species of naked amoebae. Based on the obtained sequences of the 18S rRNA gene and the sequences of the related amoeba species, which are deposited in GenBank, we have analyzed phylogenetic relationships among different species sampled from freshwater and marine bodies and terrestrial biotopes.

According to the literature, although the naked amoeba species of the family Amoebidae Ehrenberg, 1838, are considered the most widespread group of protists, individual findings of the species are also recorded among the genera *Amoeba* Bory de Saint-Vincent, 1822, *Polychaos* Schaeffer, 1926, *Deuteramoeba* Page, 1987, *Chaos* Linnaeus, 1767, *Trichamoeba* Frometal, 1874, *Hydramoeba* Reynolds and Loope, 1928, and *Parachaos* Willumsen, Siemensma, and Suhr-Jessen, 1987 (Willumsen, 1982; Page and Robson, 1983; Bolivar et al., 2001; Mrva, 2010/2011). This regularity is also observed in our studies, and *Amoeba proteus* isolate AP07 was recorded several times in our samples from Stokhid R. (Volyns'ka oblast, Ukraine). The number of phylogenetic trees based on the 18S rRNA gene sequences for members of the family Amoebidae is not large. The species from the genera *Amoeba* (including *A. proteus*) and *Chaos* were first sequenced by I. Bolivar (Bolivar et al., 2001), and the sequences were used for constructing phylogenetic trees, which showed that the genera formed a stable clade. Multigene data on *A. proteus* were given in Lahr et al. (2013). Other members of the family were absent on the tree. Representatives of the genera *Amoeba* and *Chaos* on the phylogenetic trees constructed for the 18S rRNA gene group with the members of the genera *Saccamoeba* and *Hartmannella* with high values of bootstrap analysis (Bolivar et al., 2001; Corsaro et al., 2010; Dykova et al., 2008). These trees represent amoeba species sampled from different localities. Our phylogenetic analysis based on the 18S rRNA gene placed *Amoeba proteus* isolate AP07 (AJ314604) and *Amoeba proteus* strain Geneva (ON907618) within a sister group with *Chaos carolinense* JG 10.98 (JQ519502), which confirmed its position reported earlier in (Bolivar et al., 2001). Our data also confirm relatedness between the two families Amoebidae and Hartmannellidae (*Saccamoeba* and

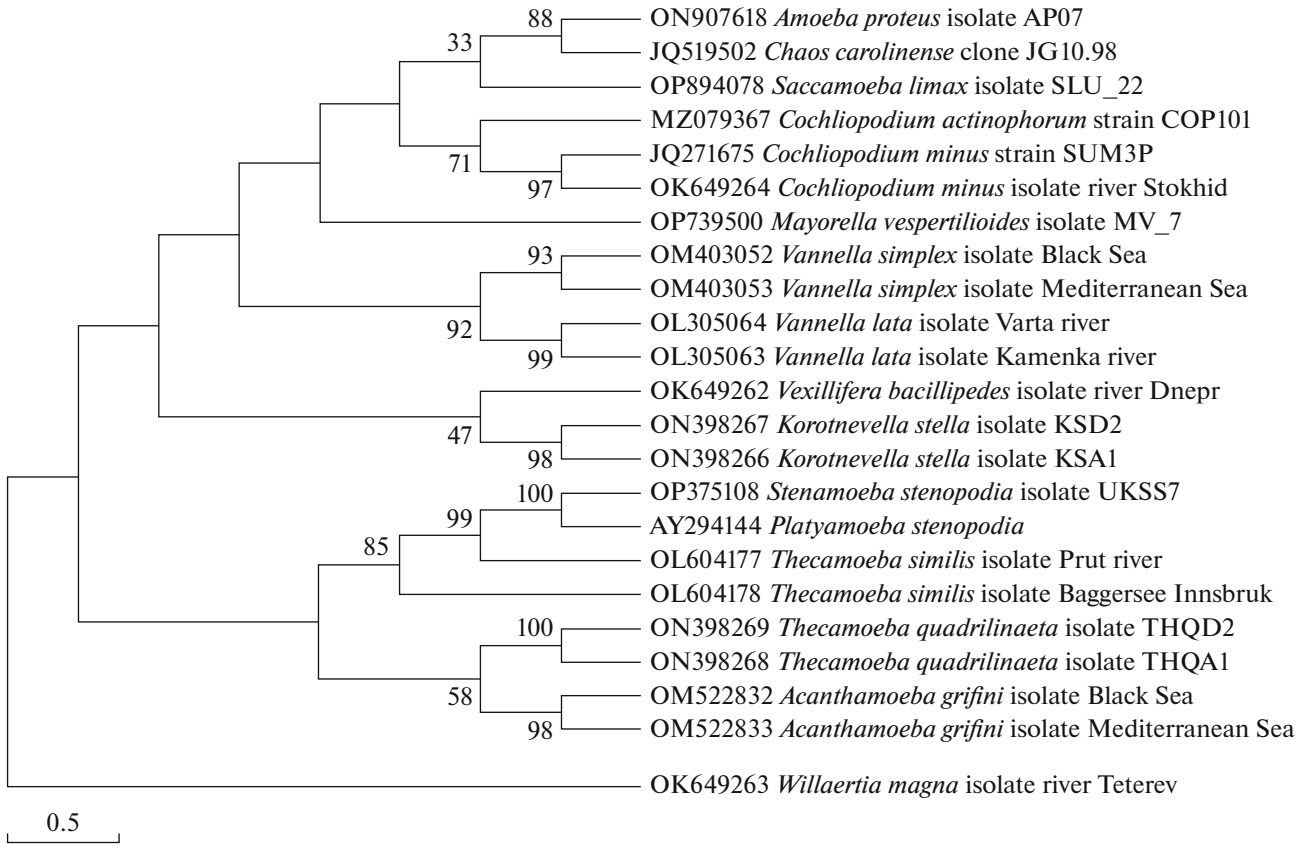

Fig. 6. Phylogenetic tree based on the 18S rRNA gene sequences for the members of the Tubulinea and Discosea groups. The outgroup is the heterolobose amoeba *W. magna*. The scale shows the equivalence of the distance between the sequences.

Hartmannella), although with low bootstrap values (Fig. 1).

The family Thecamoebidae embraces morphologically heterogeneous amoebae. The genus *Thecamoeba* is known to form a stable clade on phylogenetic trees constructed for the 18S rRNA gene (Pawlowski and Burki, 2009). *S. stenopodia* (earlier *Platyamoeba stenopodia* Page, 1969) is grouped with *Thecamoeba/Sappinia* (Brown et al., 2007; Pawlowski and Burki, 2009). This is also confirmed by our studies. Figure 2 shows that some groups comprise different species of *Thecamoeba*-like and *Stenamoeba*-like amoebae (striate and lingulate morphotypes, respectively) sampled from different freshwater bodies.

Vannella + *Korotnevella* + *Vexillifera* are usually a relatively stable clade whose members can be grouped on the phylogenetic trees with high values of bootstrap analysis (Cavalier-Smith et al., 2004; Teckle et al., 2008). *Vannella*-like amoebae are considered to be the most widespread among all naked amoebae, which are included on phylogenetic trees based on the 18S rRNA gene into separate groups (from freshwater and marine bodies) with high values of bootstrap analysis (Dykova et al., 2005). According to our data, amoebae of the genera *Korotnevella* and *Vexillifera* (belong to the dactylopodial morphotype) are separately united on the phylogenetic tree (Fig. 3) with high bootstrap support values (90–98 and 88%, respectively). The first group includes freshwater amoeba species from the genus *Korotnevella*, whereas the second group represents amoebae from the genus *Vexillifera*. As regards to shaped amoebae, members of the genus *Vannella* form two separate relatively stable groups of naked amoebae on the phylogenetic tree: from bodies of fresh and saline water bodies (Fig. 3).

The joint Acanthapodida + Balamuthia clade, which is strongly supported by all types of analyses, was included into the Variosea group; however, this relatedness has not been confirmed on different trees based on SSU rDNA (Cavalier-Smith et al., 2004; Pawlowski and Burki, 2009). As Fig. 4 shows, acanthopodial amoebae form separate stable groups of species from marine, freshwater bodies, and terrestrial biotopes. However, these groups of amoebae are unstable relative one another on the phylogenetic tree.

As regards to the genus *Cochliopodium*, representatives of the taxon form on the phylogenetic tree a relatively particular branch having no clear relationships with any stable clade of amoeboids (Teckle et al., 2008; Cole et al., 2010). Figure 5 shows a stable group of lens-like freshwater amoebae from the genus *Cochliopodium*. *Willaertia* + *Vahlkampfia* + *Naegleria*,

belonging to heterolobose amoebae, are phylogenetically well grouped and form individual clades on phylogenetic trees (Brown et al., 1999; Bass et al., 2016). According to our data, freshwater species of the genus *Willaertia* are reliably grouped on the phylogenetic tree, whereas those from the genus *Vahlkampfia* are grouped individually.

CONCLUSIONS

Our studies show that the diversity of naked amoebae isolated from aquatic and terrestrial biotopes is understudied. The largest number of species has been isolated from freshwater bodies. The study of this group of protists using the 18S rRNA gene sequences and the morphological data enabled us to identify a large number of species. Based on morphological and molecular investigations and on the identified DNA sequences from the GenBank database, we identified 24 species of naked amoebae. These species are as follows: *Amoeba proteus* isolate AP07 (ON907618), *Saccamoeba limax* isolate SLU_22 (OP894078), *Saccamoeba limax* isolate SL_Uk19 (OQ520144), *Saccamoeba* sp. strain IDL777 (MZ079370), *Thecamoeba striata* isolate THS19 (OQ134482), *Thecamoeba striata* isolate THS20 (OQ134483), *Thecamoeba similis* isolate Prut river (OL604177), *Thecamoeba similis* isolate Baggersee Innsbruck (Baggersee Rossau) (OL604178), *Thecamoeba quadrilineata* isolate THQD2 (ON398269), *Thecamoeba quadrilineata* isolate THQA1 (ON398268), *Thecamoeba* sp. strain THS203 (MZ079371), *Stenamoeba stenopodia* isolate UKSS7 (OP375108), *Stenamoeba stenopodia* isolate POLSS7 (OP419588), *Korotnevella stella* isolate KSD2 (ON398267), *Korotnevella stella* isolate KSA1 (ON398266), *Vexillifera bacillipedes* isolate river Dnepr (OK649262), *Vannella lata* isolate Kamenka river (OL305063), *Vannella lata* isolate Varta river (OL305064), *Vannella* sp. strain VLS303 (MZ079372), *Vannella simplex* isolate Black Sea (OM403052), *Vannella simplex* isolate Mediterranean Sea (OM403053), *Ripella* sp. strain RPL100 (MZ079369), *Mayorella vespertilioides* isolate MV_7 (OP739500), *Mayorella* sp. isolate MY_7 (OP729930), *Acanthamoeba* sp. strain ATM123 (MZ079366), *Acanthamoeba* sp. isolate river Elbe (OK649261), *Acanthamoeba polyphaga* isolate AcPoly01 (ON908497), *Acanthamoeba polyphaga* isolate AcPoly15 (ON908496), *Acanthamoeba griffini* isolate Black sea (OM522832), *Acanthamoeba griffini* isolate Mediterranean Sea (OM522833), *Cochliopodium actinophorum* strain COP101 (MZ079367), *Cochliopodium minus* isolate river Stokhid (OK649264), *Cochliopodium* sp. strain COP102 (MZ079368), *Vahlkampfia avara* isolate VA7 (OP179657), *Willaertia magna* isolate river Teterev (OK649263). We used the 18S rRNA sequences of these organisms to determine phylogenetic relationships between different species of naked amoebae sampled from freshwater and marine bodies and terrestrial biotopes. They are grouped separately on phylogenetic trees, being sister species relative to one another with low bootstrap values, which confirms low validity for distance between separate groups of naked amoebae. Fan-shaped amoebae isolated from marine bodies, such as *Vannella simplex* isolate Mediterranean Sea (OM403053), *Vannella simplex* (AF464914), and *Vannella simplex* isolate Black Sea (OM403052), form an individual group on the phylogenetic tree; they are grouped with freshwater *Vannella*-like amoebae with low bootstrap values. *Acanthamoeba griffini* isolate Mediterranean Sea (OM522833), *Acanthamoeba griffini* isolate B18 (GU553135), and *Acanthamoeba griffini* isolate Black Sea (OM522832) known from marine bodies are united into a separate group and grouped with freshwater Acanthamoebae with low bootstrap values. Amoebae sampled from terrestrial biotopes (Epiphytic mosses), such as *Acanthamoeba polyphaga* isolate AcPoly01 (ON908497) and *Acanthamoeba polyphaga* isolate AcPoly15 (ON908496), are reliably grouped on the phylogenetic tree with one another, being sister species relative to freshwater members of the genus *Acanthamoeba* with low bootstrap values. Our investigations have shown that, in general, naked amoebae on phylogenetic trees correspond to the earlier established systems, using which both morphological and genetic traits of these protists were considered.

FUNDING

This study has not been supported by any particular grant from any financial organizations in the state, commercial, or noncommercial sectors.

COMPLIANCE WITH ETHICAL STANDARDS

The author declares no conflicts of interest. This article does not contain any studies with the use of humans and animals as objects.

REFERENCES

Amaral Zettler, L.A., Nerad, T.A., O'Kelly, C.J., Peglar, M.T., Gillevet, pp.M., Silberman, J.D., and Sogin, M.L., A Molecular reassessment of the leptomyxid amoebae, *Protist*, 2000, vol. 151, pp. 275–282. https://doi.org/10.1078/1434-4610-00025

Anderson, O.R., A Half-century of research on free-living amoebae (1965–2017): Review of biogeographic, ecological and physiological studies, *Acta Protozool.*, 2018, vol. 57, no. 1, pp. 1–28. https://doi.org/10.4467/16890027AP.18.001.8395

Bass, D., Silberman, J.D., Brown, M.W., Tice, A.K., Jousset, A., Geisen, S., and Hartikainen, H., Coprophilic amoebae and flagellates, including *Guttulinopsis, Rosculus* and *Helkesimastix*, characterise a divergent and diverse rhizarian radiation and contribute to a large diversity of faecal-associated protists, *Environ. Microbiol.*, 2016, vol. 18, pp. 1604–1619. https://doi.org/10.1111/1462-2920.13235

Bolivar, I., Fahrni, J.F., Smirnov, A., and Pawlowski, J., SSU rRNA-based phylogenetic position of the genera *Amoeba* and *Chaos* (Lobosea, Gymnamoebia): the origin of gymnamoebae revisited, *Mol. Biol. Evol.*, 2001, vol. 18, pp. 2306–2314. https://doi.org/10.1093/oxfordjournals.molbev.a003777

Brown, S. and Jonckheere, J.F., A reevaluation of the amoeba genus *Vahlkampfia* based on SSUrDNA sequences, *Eur. J. Protistol.*, 1999, vol. 35, no. 1, pp. 49–54. https://doi.org/10.1016/S0932-4739(99)80021-2

Brown, M.W., Spiegel, F.W., and Silberman, J.D., Amoeba at attention: phylogenetic affinity of *Sappinia pedata, J. Eukaryotic Microbiol.*, 2007, vol. 54, pp. 511–519. https://doi.org/10.1111/j.1550-7408.2007.00292.x

Bütschli, O., Protozoa mit einem beitrag: Palaeontologische entwicklung der Rhizopoda, in *Die Klassen und Ordnungen des Thierreichs, Wissenschaftlich Dargestellt in Wort und Bild*, vol. 1: *Abtheilung: Sarkodina und Sporozoa*, Schwager, C. and Bronn, H.G., Eds., Leipzig: Winter'sche Verlagshandlung, 1880–1882, pp. I–XVIII.

Calkins, G.N., *The Protozoa*, New York: Columbia Press/Macmillan, 1901.

Cavalier-Smith, T., A revised six-kingdom system of life, *Biol. Rev.*, 1998, vol. 73, pp. 203–266. https://doi.org/10.1017/s0006323198005167

Cavalier-Smith, T., Chao, E.E.-Y., and Oates, B., Molecular phylogeny of Amoebozoa and the evolutionary significance of the unikont *Phalansterium, Eur. J. Protistol.*, 2004, vol. 40, pp. 21–48. https://doi.org/10.1016/j.ejop.2003.10.001

Cavalier-Smith, T., Megaphylogeny, cell body plans, adaptive zones: causes and timing of eukaryote basal radiations, *J. Eukaryotic Microbiol.*, 2009, vol. 56, pp. 26–33. https://doi.org/10.1111/j.1550-7408.2008.00373.x

Cavalier-Smith, T., Chao, E.E., and Lewis, R., 187-gene phylogeny of protozoan phylum Amoebozoa reveals a new class (Cutosea) of deep-branching, ultrastructurally unique, enveloped marine Lobosa and clarifies amoeba evolution, *Mol. Phylogenet. Evol.*, 2016, vol. 99, pp. 275–296. https://doi.org/10.1016/j.ympev.2016.03.023

Cole, J., Anderson, O.R., Tekle, Y.I., Grant, J., Katz, L.A., and Nerad, T., A description of a new "Amoebozoan" isolated from the American lobster, *Homarus americanus, J. Eukaryotic Microbiol.*, 2010, vol. 57, pp. 40–47.

Corsaro, D. and Venditti, D., Molecular phylogenetics evidence for a novel lineage of amoebae within discosea (Amoebozoa: Lobosa), *Acta Protozool.*, 2013, vol. 52, pp. 309–316.

Corsaro, D., Michel, R., Walochnikc, J., Muller, K.-D., and Greuba, G., *Saccamoeba lacustris*, sp. nov. (Amoebozoa: Lobosea: Hartmannellidae), a new lobose amoeba, parasitized by the novel chlamydia "*Candidatus* Metachlamydia lacustris" (Chlamydiae: Parachlamydiaceae), *Eur. J. Protistol.*, 2010, vol. 46, pp. 86–95.

Delage, Y. and Hérouard, E., *Traité de Zoologie Concrète*, vol. 1: *La Cellule et les Protozoaires*, Paris: Schleicher Frères, 1896.

Dykova, I., Bohacova, L., Fiala, I., Machackova, B., Peckova, H., and Dvorakova, H., Amoebae of the genera *Vannella* Bovee, 1965 and *Platyamoeba* Page, 1969 isolated from fish and their phylogeny inferred from SSU rRNA gene and ITS sequences, *Eur. J. Protistol.*, 2005, vol. 41, pp. 219–230.

Dykova, I., Kostka, M., and Peckova, H., Morphology and SSU rDNA-based phylogeny of a new strain of *Saccamoeba* sp. (*Saccamoeba* Frenzel, 1892, Amoebozoa), *Acta Protozool.*, 2008, vol. 47, no. 4, pp. 397–405.

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K., MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms, *Mol. Biol. Evol.*, 2018, vol. 35, no. 6, pp. 1547–1549. https://doi.org/10.1093/molbev/msy096

Lahr, D.J.G., Grant, J.R., and Katz, L.A., Multigene phylogenetic reconstruction of the Tubulinea (Amoebozoa) corroborates four of the six major lineages, while additionally revealing that shell composition does not predict phylogeny in the Arcellinida, *Protist*, 2013, vol. 164, no. 3, pp. 323–339. https://doi.org/10.1016/j.protis.2013.02.003

Lahr, D.J., Grant, J., Molestina, R., Katz, L.A., and Anderson, O.R., *Sapocribrum chincoteaguense* n. gen. n. sp.: A small, scale-bearing amoebozoan with flabellinid affinities, *J. Eukaryotic Microbiol.*, 2015, vol. 4, pp. 444–453. https://doi.org/10.1111/jeu.12199

Leidy, J., *Freshwater rhizopods of North America. United States Geological Survey of the Territories Report*, 1879, vol. 12, pp. 1–324.

Maniatis, T., Fritsch, E.F., and Sambrook, J., *Molecular Cloning, a Laboratory Manual*, New York: Cold Spring Harbor, 1982.

Medlin, L., Elwood, H.J., Stickel, S., and Sogin, M.L., The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions, *Gene*, 1988, vol. 71, pp. 491–499.

Mrva, M., Morphological studies on two rare soil amoebae *Deuteramoeba algonquinensis* and *D. mycophaga* (Gymnamoebia, Amoebidae), *Protist*, 2010, vol. 6, pp. 284–289.

Page, F.C., *Marine Gymnamoebae*, Cambridge: Institute Terrestrial Ecology, 1983.

Page, F.C., *A New Key to Freshwater and Soil Gymnamoebea*, Ambleside: Freshwater Biological Association, 1988.

Page, F.C. and Robson, E.A., Fine structure and taxonomic position of *Hydramoeba hydroxena* (Entz, 1912), *Protistologica*, 1983, vol. 19, pp. 41–50.

Page, F.C. and Siemensma, F.J., Nackte Rhizopoda und Heliozoea, in *Protozoenfauna*, Stuttgart: Gustav Fischer Verlag, 1991, pp. 3–170.

Pawlowski, J. and Burki, F., Untangling the phylogeny of amoeboid protists, *J. Eukaryotic Microbiol.*, 2009, vol. 56, pp. 16–25. https://doi.org/10.1111/j.1550-7408.2008.00379.x

Peglar, M.T., Amaral Zettler, L.A., Anderson, O.R., Nerad, T.A., Gillevet, P.M., Mullen, T.E., Frasca, Jr.S., Silberman, J.D., O'Kelly, C.J., and Sogin, M.L., Two new small subunit ribosomal RNA gene lineages with in the subclass Gymnamoebia, *J.*

Eukaryotic Microbiol., 2003, vol. 50, pp. 224–232. https://doi.org/10.1111/j.1550-7408.2003.tb00122.x

Penard, E., *Faune Rhizopodique du Bassin du Leman*, Geneve: Henry Kundig, 1902.

Sims, G.P., Rogerson, A., and Aitken, R., Primary and secondary structure of the small-subunit ribosomal RNA of the naked, marine amoeba *Vannella anglica*: Phylogenetic implications, *J. Mol. Evol.*, 2018, vol. 48, pp. 740–749. https://doi.org/10.1007/pl00006518

Tekle, Y.I., Grant, J., Anderson, O.R., Nerad, T.A., Cole, J.C., Patterson, D.J., and Katz, L.A., Phylogenetic placement of diverse amoebae inferred from multigene analyses and assessment of clade stability within "Amoebozoa" upon removal of varying rate classes of SSU-rDNA, *Mol. Phylogenet. Evol.*, 2008, vol. 47, pp. 339–352. https://doi.org/10.1016/j.ympev.2007.11.015

Willumsen, N.B.S., *Chaos zoochlorellae* sp. nov. (Gymnamoebia, Amoebidae) from a Danis freshwater pond, *J. Nat. Hist.*, 1982, vol. 16, pp. 803–813.

Translated by N. Tarasyuk