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Morphology and Life Cycle of Amoeboflagellate *Pharyngomonas* sp. (Heterolobosea, Excavata) from Hypersaline Inland Razval Lake[1]

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Abstract—The morphology, life cycle, feeding and multiplication of amoeboflagellate *Pharyngomonas* sp., strain RL isolated from hypersaline inland Razval Lake was studied. Life cycle includes flagellate, amoeboid, and cyst stages. Flagellated cells have two anterior flagella and two posterior flagella emerging apically from the anterior end of the cell. The nucleus localizes closely to the base of flagella. The longitudinal groove is on the ventral side of the cell. Flagellated cells have a distinct cytopharynx in the anterior part of the ventral groove. The cytopharynx passes into an intracellular channel reaching the posterior end of the cell. Amoeboid cells have two morphotypes—heterolobosean and lobosean. Cysts are spherical, their wall is smooth. Cell division occurs either at flagellated stage or at amoeboid one. Distinctions of *Pharyngomonas* sp., strain RL, from *Pharyngomonas kirbyi* were determined. They include longer flagella and long filamentous branching pseudopodia at the posterior end of amoeboid cells. The morphological distinctions are in agreement with differences in structure of the 18S rRNA gene. The revealed features may be used for differentiation of *Pharyngomonas* species.

Keywords: *Pharyngomonas*, morphology, life cycle, amoeboflagellate, Heterolobosea, Excavata, protists
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INTRODUCTION

Established by Page and Blanton (Page and Blanton, 1985), the class Heterolobosea combined heterolobosean protists known for that moment (mainly amoeboid organisms with pseudopodia of eruptive type) with acrasid cellular slime molds able to produce fruiting bodies. At present the Heterolobosea includes the unicellular and multicellular protists with diverse life cycle, which consists of few stages such as amoeboid cells, flagellates, cysts and fruiting bodies formed as a result of the amoeboid cells aggregation.

Most species represent amoeboflagellates, many of them are able to produce cysts (*Naegleria, Tetramitus, Psalteriomonas* and some acrasids). Also the class Heterolobosea includes some amoebae with unknown flagellate stage (*Vahlkampfia, Sawyeria* and *Marinamoeba*) and a number of flagellates with reduced amoeboid stage (*Percolomonas, Stephanopogon, Pleurostomum*).

From the standpoint of molecular phylogeny heteroloboseans constitute a monophyletic taxon producing in phylogenetic tree the uniform clade inside the supergroup Excavata. The clade is closely related to other excavated protists, in particular Euglenozoa and Jacobida (Nikolaev et al., 2004; Cavalier-Smith and Nikolaev, 2008; Park and Simpson, 2011; Adl et al., 2012). The monophyly of Heterolobosea is confirmed by some common morphological features: producing of eruptive pseudopodia by the amoeboid cells provided that they are being in the life cycle (heterolobosean apomorphy); lack of a typical stacked Golgi apparatus; flattened, often discoidal cristae of mitochondria; closed intranuclear pleuromitosis (according to I.B. Raikov classification).

Composition and level of the taxon Heterolobosea changed several times as a result of repeated taxonomic revisions (Page, 1988; Page and Siemensma, 1991; *The Biology*..., 1991; Cavalier-Smith, 1993; Cavalier-Smith and Nikolaev, 2008; Adl et al., 2012). Originally the class Heterolobosea composed of two orders Schizopyrenida (amoebae of "limax" type, often producing a flagellate stage) and Acrasida (aggregative amoebae forming multicellular sorocarps) (Page and Blanton, 1985). Later it was determined that some flagellates or amoeboflagellates reveal common morphological traits with Heterolobosea and they are phylogenetically related as well. Some of the protists produce a sister lineage to Heterolobosea (*Pharyngomonas*), others form inner clades in phylogenetic tree (*Pleurostomum, Percolomonas, Stephanopogon, Lyromonas, Psalteriomonas*) (Park et al., 2007;

[1] The article was translated by the authors.

Cavalier-Smith and Nikolaev, 2008; Park and Simpson, 2011; Pánek and Čepička, 2012).

At present two taxonomic concepts of Heterolobosea exist (Pánek and Čepička, 2012). The concept *sensu stricto* corresponds to the original definition of Heterolobosea *sensu* Page et Blanton (1985). The concept *sensu lato* emphasizes monophyletic taxa and includes all mentioned genera into a single taxon. According to the recent taxonomic revision all representatives of Heterolobosea *sensu lato* were combined into the phylum Percolozoa, divided into two subphyla and four classes (Cavalier-Smith and Nikolaev, 2008). The monotypical subphylum Pharyngomonada is composed of one class Pharyngomonadea containing the only family Pharyngomonadidae with the genus *Pharyngomonas*.

There are three classes combined in subphylum Tetramitia. They include seven families and unclassified genera at family level: class Percolatea (Percolomonadidae, Stephanopogonidae), class Lyromonadea (Lyromonadidae, Psalteriomonadidae, *Sawyeria*, *Monopylocystis*) and class Heterolobosea (Vahlkampfiidae, Gruberellidae, Acrasidae). Furthermore, several genera have not yet been related to any class or family of the subphylum Tetramitia (*Oramoeba, Fumarolamoeba, Pernina, Tulamoeba, Euplaesiobystra*).

Division into the subphyla is confirmed by morphological features. For instance, representatives of the genus *Pharyngomonas* related to the subphylum Pharyngomonada possess a funnel-shaped cytopharynx opening at the anterior end of a ventral groove and passing into an intracellular channel. In addition they have orthogonally arranged kinetosomes but have not got helix 17_1 in structure of the 18S rRNA (Cavalier-Smith and Nikolaev, 2008). Synapomorphies of Tetramitia include parallel or nearly parallel kinetosomes in the kinetid and specific helix 17_1 in structure of the 18S rRNA (Cavalier-Smith and Nikolaev, 2008).

In last classification of eukaryotes (Adl et al., 2012) Heterolobosea with Euglenozoa, Jacobida and *Tsukubamonas* compose the group Discoba inside the supergroup Excavata. The taxon Heterolobosea in this classification corresponds to the concept *sensu lato*. The taxon is divided into the family Pharyngomonadidae and the subphylum Tetramitia. Their composition is similar to the same taxa in the paper of Cavalier-Smith and Nikolaev (2008).

Heteroloboseans are widespread in fresh and marine habitats, often they are found in soil (Page, 1988; Pánek et al., 2012). It is interesting that the heterolobosean protists are typical for ecosystems with extreme environmental conditions (Pánek and Čepička, 2012). Among representatives of Heterolobosea a number of extremophiles adapted to the marginal levels of different ecological factors (temperature, pH and salinity) was found. At present some extremophilic species are known, such as the thermophiles *Marinamoeba thermophila* (De Jonckheere et al., 2009), *Oramoeba fumarolia* (De Jonckheere et al., 2011a), *Fumarolamoeba ceborucoi* (De Jonckheere et al., 2011b); thermoacidophilic *Tetramitus thermoacidophilus* (Baumgartner et al., 2009); psychrophilic *Vahlkampfia signyensis* (Garstecki et al., 2005); the halophiles *Pleurostomum flabellatum* (Park et al., 2007), *Euplaesiobystra hypersalinica, Tulamoeba peronaphora* (Park et al., 2009), *Pharyngomonas kirbyi* (Park and Simpson, 2011).

Features of Heterolobosea morphology and life cycle have been studied irregularly at present. Ultrastructural features and molecular phylogeny based on the 18S rRNA gene sequences were investigated in detail in the genera *Pleurostomum* (Park et al., 2007), *Euplaesiobystra* and *Tulamoeba* (Park et al., 2009). Morphology and ultrastructure of the flagellated stage in one species of *Pharyngomonas*, *P. kirbyi* was examined (Park and Simpson, 2011). Recently short morphological characteristics of the amoeboid cells and cysts of this species were reported (Harding et al., 2013). Nevertheless the life cycle of the genus *Pharyngomonas* representatives has not yet been characterized quite fully. Morphology of different lifecycle stages at light-microscopic level and their quantitative dynamics has not been described in detail. Exact morphometric signs useful for recognition of the only correctly described in terms of ICZN species *P. kirbyi* from other representatives of the genus are unclear. Therefore the goal of this paper is morphological and morphometric description of life stages and life cycle of a new representative of the genus *Pharyngomonas*, found on the territory of Russia, its comparison with other isolates and taxa of Heterolobosea.

MATERIALS AND METHODS

The flagellated cells of *Pharyngomonas* sp., strain RL were isolated by authors from a superficial layer of the inland Razval Lake brine September 30, 2004. A salinity of the brine sample was 350‰. This lake is of technogenic origin. It is located within Salt-Iletsk town of Orenburg region (51°08′ N, 54°59′ E). It is included in the group of sodium-chloride lakes with different levels of salinity, localized on the surface of salt deposits. Razval Lake is hypersaline according to the Venetian classification. The salinity of the brine superficial layer depends on season and varies from 157 to 350‰. The investigated clone was obtained by the direct isolation of a single cell from the enrichment culture grown in the original medium, containing complex of salts and yeast extract with increased amount of Na, Mg, Cl, salinity—68‰ (Nemtseva et al., 2008). This clone is stored in collections of live protist cultures in the Laboratory of Water Microbiology of the Institute for Cellular and Intracellular Symbiosis, Ural Branch RAS and in the Protozoologists

Group of the Papanin Institute for Biology of Inland Waters RAS.

The protisis were cultivated in Smaltz-Pratt medium, salinity 35‰, with addition bacteria *Pseudomonas fluorescens* Migula, as well in original medium (Nemtseva et al., 2008), without added bacteria at 20–25°C. The life cycle was studied in AS medium (Park et al., 2007), diluted by sterile distilled water up to salinity 122‰.

The microscopes Axioscope A1 and Axiostar Plus (Carl Zeiss, Germany) with a phase-contrast device and objectives with water and oil immersion (×1000) were used for cytological observations. The microscopes were equipped with digital photo cameras Axiocam ERc 5s (Carl Zeiss, Germany) and Power Shot G5 (Canon), and analog video camera AVT HORN MC-1009/S. Video was recorded for more exact identification of found flagellates using VCR Panasonic NV-HS 850 in the mode S-VHS followed by image digitization and saving of the recorded movies in a file of AVI format. Counting and measuring of the cells was performed with a live culture, average cell sizes are given with standard (mean square) deviation.

Drops of the cell suspension were placed on the surface of formvar-coated copper grid for transmission electron microscopy of total specimen. The specimens were fixed in osmium tetroxide vapor for 1–2 min. Then they were coated (set off) with wolfram (Moestrup and Thomsen, 1980) and were studied with JEM-1011 electron microscope (Japan). The cell dimensions slightly decreased after the fixation.

For scanning electron microscopy the flagellated and amoeboid cells on the cover glasses, placed beforehand in the Petri dishes, were fixed with 1.6% glutaraldehyde solution, prepared on the Smaltz-Pratt medium (35‰), for 10 min. The cover glasses with cells were dehydrated in ethanol solutions series with increasing concentration and in anhydrous acetone. Then acetone replaced with carbon dioxide in the HCP-2 critical point unit (Hitachi, Japan), the cells were coated with palladium and gold and viewed with LEO-1420 microscope (Carl Zeiss, Germany).

RESULTS

All experiments were carried out with the clone culture of *Pharyngomonas* sp., strain RL.

The Dynamics of the Culture Growth

The life cycle of amoeboflagellate *Pharyngomonas* sp., strain RL includes flagellated, amoeboid cells, and cysts (Fig. 1). The amoeboid cells appear first after excystation, then flagellates. While culture aging cysts rise and their number progressively increases.

The dynamics of different morphotypes number during culture growth is shown in Fig. 2. After addition of a fresh medium to the old culture, containing cysts, the process of amoeba releasing was observed for the first hours. During the first three days number of vegetative cells represented by amoebae and flagellates increased. Moreover, the flagellates with lobose pseudopodia were present in the culture; perhaps they are transitional forms between amoebae and flagellates (Fig. 6a). During the first two weeks amoebae predominated over flagellates; that seems to be related to consequent transformation of amoeboid cells into flagellates. Number of amoebae reached a maximum on the 10th day, after that decreased smoothly. Flagellates reached a maximal quantity in the end of the second week of their growth and predominated over amoebae until the 20th day, on the background of decrease of vegetative cells number. Producing of cysts began on the 4th day and accelerated significantly after 10th day. After the 15th day number of cysts exceeded number of vegetative cells. On the 30th day the culture entirely composed of cysts; that probably was related to unfavorable changes in growth conditions, such as lack of food resources (bacteria), cumulation of metabolites and increase of medium salinity as a result of evaporation. During the observation an ability of amoeboid cells to transform into flagellates, and vice versa, was established. Cysts in their turn are produced only by amoeboid cells, and only amoebae are released from the cysts.

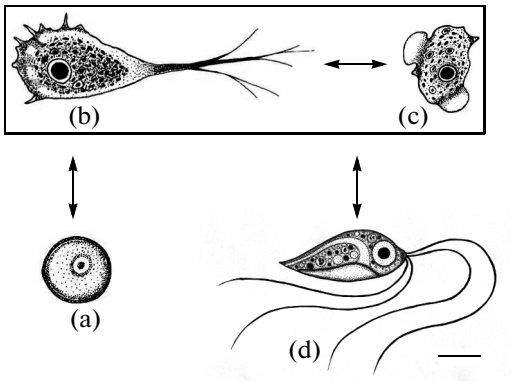

Fig. 1. Scheme of life cycle of *Pharyngomonas* sp., strain RL: (a) cyst, (b) amoeboid cell of lobosean morphotype, (c) amoeboid cell of heterolobosean morphotype, (d) flagellated cell. Scale bar—5 μm.

Morphology of the Flagellated Cells

The body of flagellated cells is spindle-shaped, laterally flattened; dorsal surface of the cell is convex, ventral surface is flattened or sli ghtly concave with a marked longitudinal groove (Figs. 3a, 3b, 3d). In young culture cells are 14–19 μm long, on average 16 μm ($n = 15$), and 4–8 μm wide, on average 6 μm ($n = 15$). In old culture the sizes are more variable; giant cells 20–30 μm long and 4–15 μm wide occur as well as very little cells with length 9–13 μm and width 3–5 μm. The anterior end is rounded, the posterior

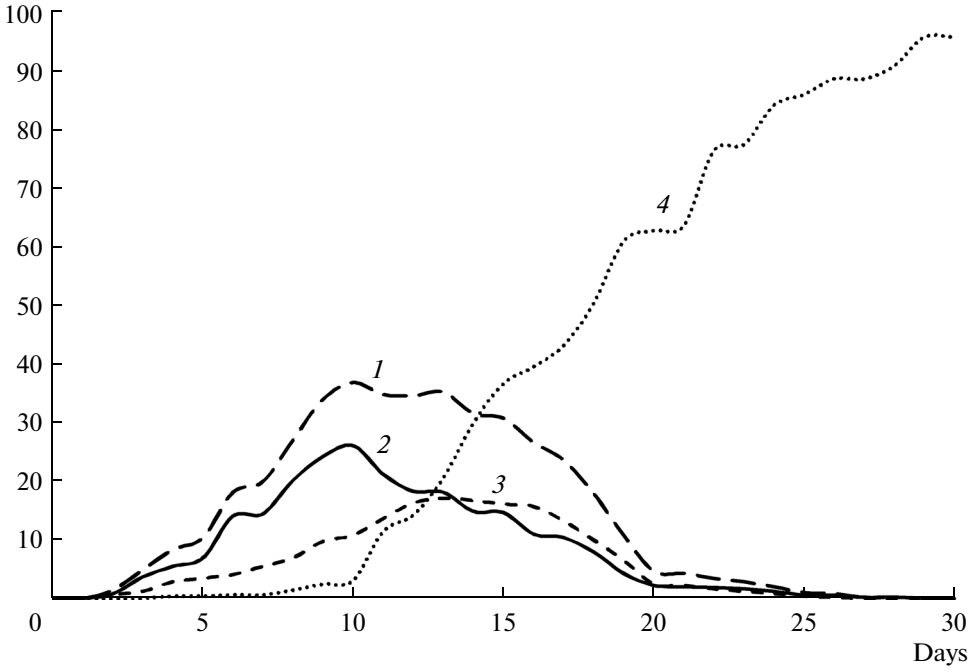

Fig. 2. The dynamics of life forms number (thousands of cells per mL) of *Pharyngomonas* sp., strain RL: (*1*) vegetative cells, (*2*) amoeboid cells, (*3*) flagellated cells, (*4*) cysts.

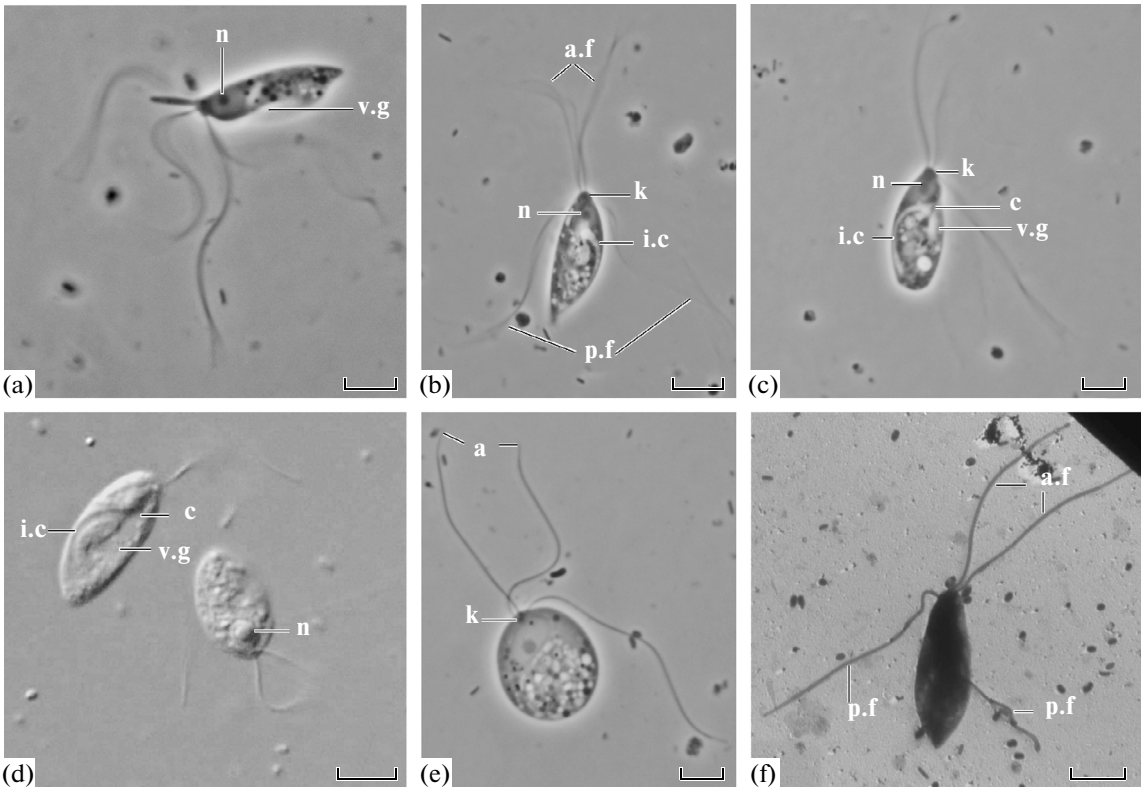

Fig. 3. Morphology of the flagellated cells of *Pharyngomonas* sp., strain RL: (a–e) light microscopy (a, b, c, e) phase contrast; (d) interference contrast); (f) transmission electron microscopy (total specimen). Symbols: a—akronema, v.g—ventral groove, i.c—intracellular channel, p.f—posterior flagellum, k—kinetosome, a.f—anterior flagellum, c—cytopharynx, n—nucleus. Scale bar—5 μm.

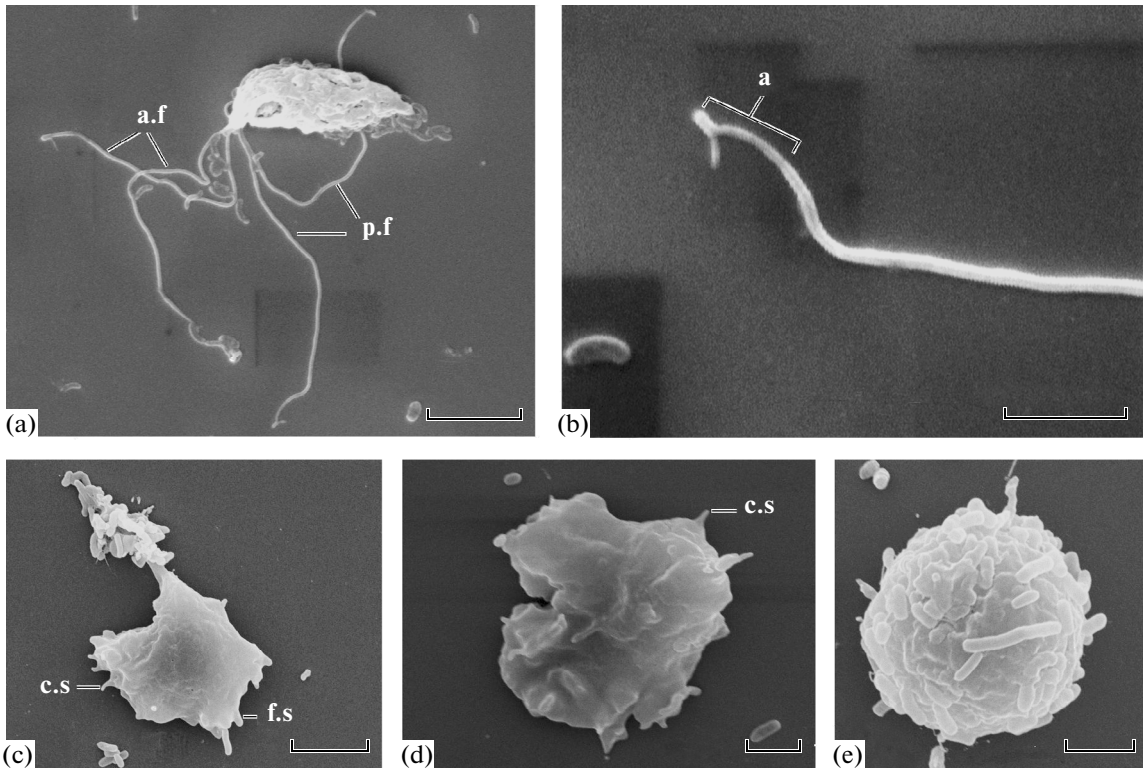

Fig. 4. Morphology of the flagellated cells, the amoeboid cells and the cysts of *Pharyngomonas* sp., strain RL (scanning electron microscopy): (a) flagellated cell; (b) flagellum; (c, d) amoeboid cells; (e) cyst. Symbols: a—akronema, p.f—posterior flagellum, c.s—conical subpseudopodia, f.s—finger-like subpseudopodia. Scale bars (μm): (a, c, e) 5; (b, d) 2.

end is pointed or rarely obtuse (Figs. 3b, 3c). At the posterior end cytoplasmic strands and filaments can be produced; with them the cells attach to a substrate, especially under conditions of increasing salinity.

Four flagella insert apically at the anterior end of the cell (Figs. 3a, 3b, 3f, 4a). The flagella are acronematic (Figs. 3e, 4b), 1.5–2 times over the cell length (Figs. 3a, 3f, 4a). The flagella are differentiated into anterior and posterior pairs, which directed forward and backward, respectively (Figs. 3b, 3f). As a rule, the posterior flagella are longer than anterior; it is seen very well in fixed specimens of flagellates (Fisg. 3f, 4a). Dying cells lose flagella, it can lead to wrong counting of flagella number (Fig. 3e). At the anterior end there are four kinetosomes located close to the point of flagella insertion; they look as rod-shaped light-refractile bodies (Figs. 3d, 3e). The cytoplasm is very vacuolated, contains numerous phagosomes with bacteria and small light-refractile granules (Figs. 3a–3e). There is a roundish vesicular nucleus in the anterior end of the cell; it adjoins closely to kinetosomes (Figs. 3a–3d). A contractile vacuole is absent. In the middle and distal parts of the cell a longitudinal ventral groove is located; the groove is approximately two thirds of the cell length (Figs. 3a, 3c, 3d). In the anterior end of the ventral groove there is a funnel-form cytopharynx (Figs. 3c, 3e), which passes into an intracellular channel. The channel curves and stretches from the cytopharynx to the posterior end of the cell, along the dorsal side of the cell (Figs. 3b–3d). Bacteria and food particles passing through the intracellular channel enter the cytoplasm within formed food vacuoles.

Morphology of the Amoeboid Cells

There are two main morphotypes of amoeboid cells in the culture *Pharyngomonas* sp., strain RL, heterolobosean and lobosean. Cells of lobosean morphotype can be shared into three basic forms: flabellate, ovoid and rectangular (Fig. 5). Flabellate (Figs. 5a, 5e) and ovoid (Fig. 5b) amoebae are the most common; rectangular cells occur rarely (Fig. 5c). Flabellate cells are 10–24 μm long, on average 16 ± 4 μm, 19–28 μm wide, on average 22 ± 8 μm ($n = 17$). Sizes of ovoid cells are 14–32 × 8–26 μm, on average 23 ± 4 × 15 ± 4 μm ($n = 36$); sizes of rectangular cells are 13–21 × 20–28 μm, on average 18 ± 3 × 25 ± 3 μm ($n = 8$). Amoebae of lobosean morphotype are flattened with an undulate or crenulated anterior edge, have marked hyaloplasm, which occupies a front quarter or a third of the cell and extends to lateral surfaces of the cell (Figs. 5a, 5b, 5e, 5i). As a rule, the nucleus of vesicular type, surrounded by granuloplasm with numerous ves-

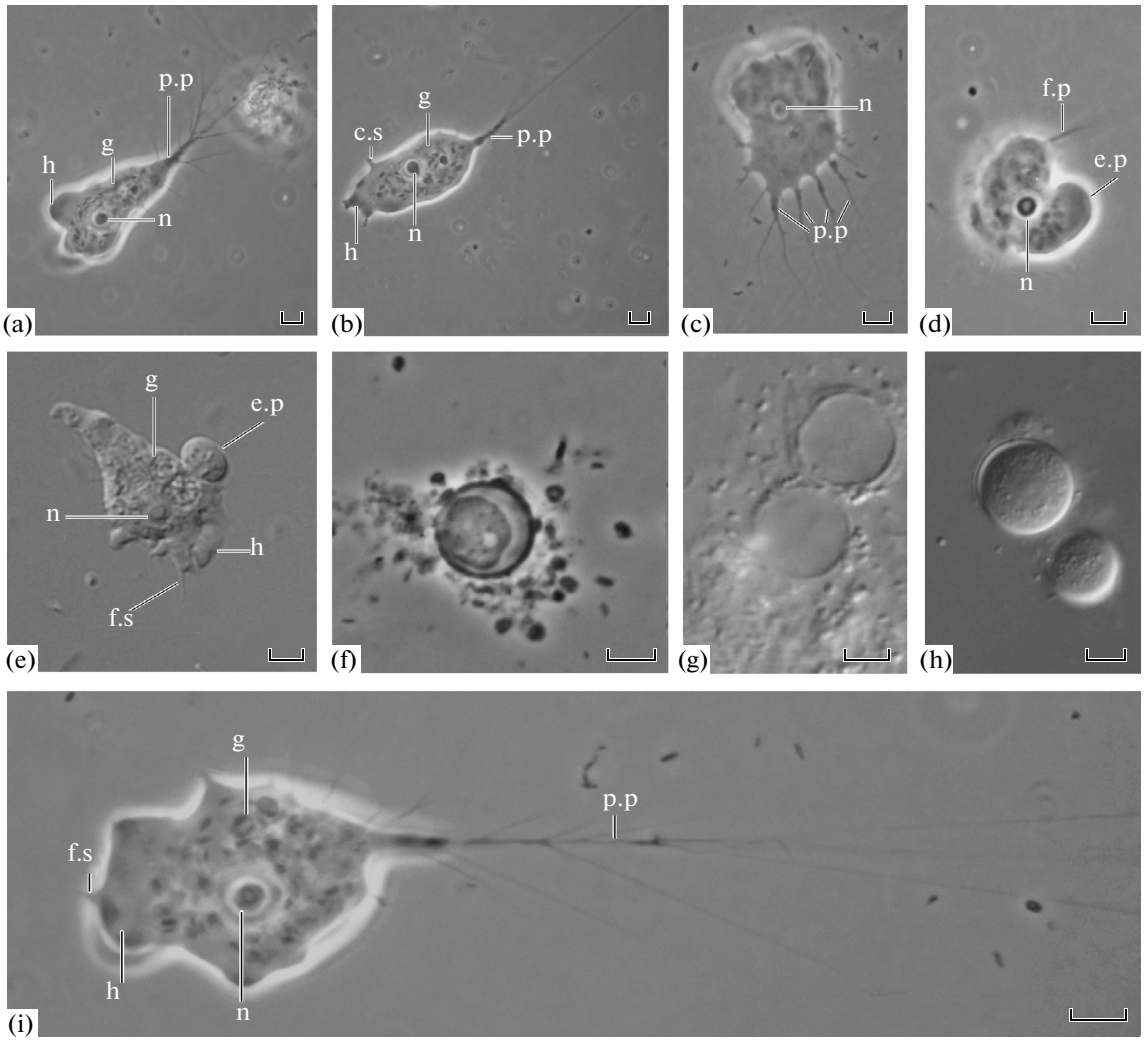

Fig. 5. Morphology of the amoeboid cells ((a, e, i) flabellate form; (b) ovoid form; (c) rectangular form; (d) heterolobosean form) and the cysts (f–h) of *Pharyngomonas* sp., strain RL: (a–h) light microscopy ((a, b, c, d, f, i) phase contrast; (e, g, h) interference contrast). Symbols: h—hyaloplasm, g—granuloplasm, p.p—posterior pseudopodia, c.s—conical subpseudopodia, f.p—filiform pseudopodia, f.s—finger-like subpseudopodia, e.p—eruptive pseudopodia, n—nucleus. Scale bar—5 μm.

icles and phagosomes, is located nearby the cell center (Figs. 5a–5c, 5e, 5i).

During active locomotion amoeboid cells of lobosean morphotype move slowly due to undetectable movement of hyaloplasm and subtle flow of the central part of granuloplasm. The amoebae produce filiform pseudopodia, as well as conical and finger-like subpseudopodia. Short conical and longer finger-like subpseudopodia are formed mainly at the anterior and lateral edges of the cell (Figs. 4c, 4d, 5b, 5e, 5i). At the posterior end the amoebae frequently produce one or few very long and thin branched pseudopodia; length of them is 2–4 times larger than the cell length (Figs. 5a–5c, 5i). Sometimes while moving on the glass amoebae of lobosean morphotype slow down and produce eruptive pseudopodia (Fig. 5e).

Amoebae of heterolobosean morphotype were observed more rarely than lobosean. As a rule, their appearance was registered under conditions of increased illumination under a microscope. Heterolobosean amoebae are similar with amoebae of other genera of Heterolobosea, producing eruptive pseudopodia in the form of hemispheres suddenly appearing at the anterior or lateral edges of the cell (Figs. 5d, 6b). The posterior end is frequently elongated as a "tail", in other case it has short filiform pseudopodia (Fig. 5d).

The surface of the amoeboid cells is smooth without any scales or glikostiles (Figs. 4c, 4d).

Morphology of the Cysts

Cysts are 9–18 μm in diameter on average 12 ± 2 μm (n = 21), with a thick wall without pores, of rounded or slightly ovoid shape (Figs. 4e, 5f–5h). The cysts are resistant to salinity fluctuations, but they are destroyed on fast drying. It was established, the most stimulating effect for encystation of the protist have such factors as

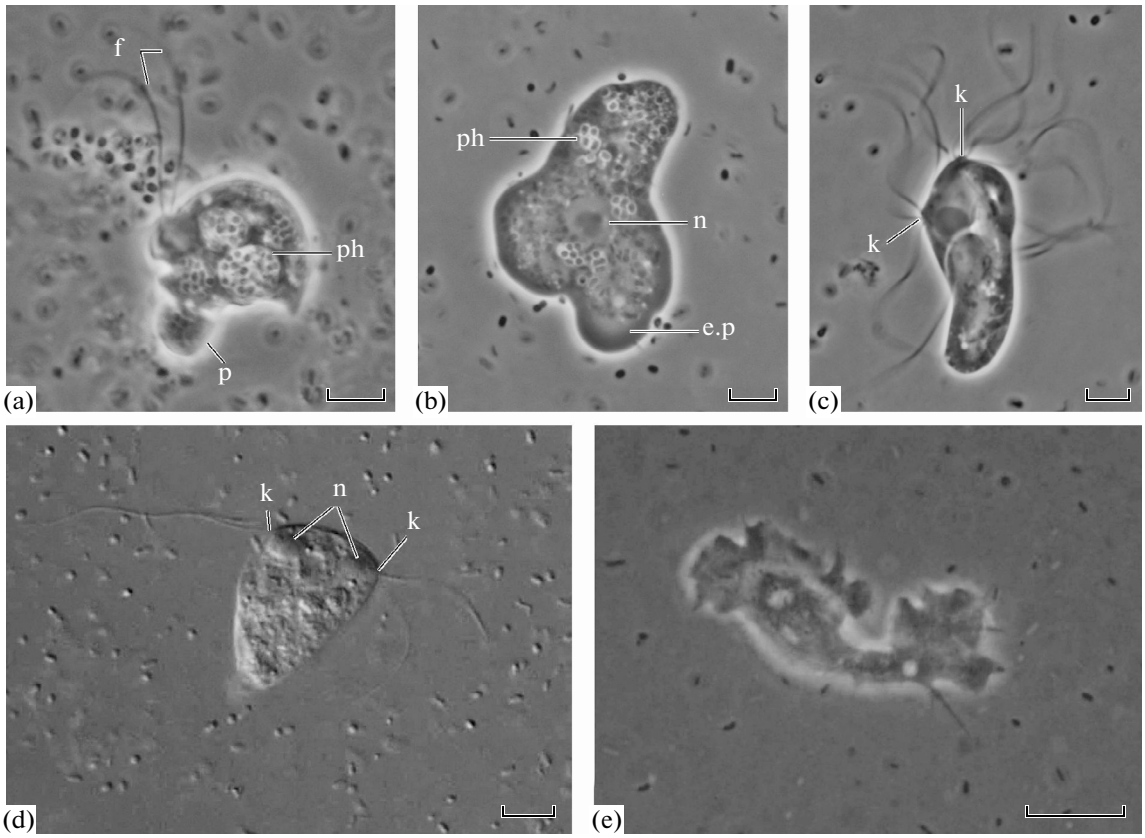

Fig. 6. Morphology of the vegetative cells of *Pharyngomonas* sp., strain RL: (a–e) light microscopy ((a, b, c, e) phase contrast; (d) interference contrast): (a) amoeboflagellate; (b) amoeboid cell with phagosomes; (c, d) cell division of flagellate; (e) cell division of amoeba. Symbols: f—flagellum, k—kinetosome, p—pseudopodia, ph—phagosome, e.p—eruptive pseudopodia, n—nucleus. Scale bar—5 μm.

deficiency of the food resources, salinity increase over 200‰ or salinity decrease below 30‰, and also temperature decrease up to 5–10°C.

Feeding and Reproduction

While cultivation it was established *Pharyngomonas* sp., strain RL, to be predator on bacteria. Due to slow undulating moving of the anterior flagella most flagellated cells swim slowly in a medium with the ventral groove directed down. During swimming of the protist, due to flagella beating, bacteria are directed into the area of ventral groove where they enter the cytopharynx. Some cells move close to the substrate surface and stop for a long period of time for engulfment of bacteria. Amoeboid cells consume bacteria on the surface of substrate, and phagosomes are formed rapidly (Fig. 4b).

The cells of *Pharyngomonas* sp., strain RL multiply by binary division both at flagellated stage (Figs. 6c, 6d) and at amoeboid stage (Fig. 6e). Flagellates reproduce more intensively than amoeboid cells. Cell division of flagellate is longitudinal; it begins from reduplication of flagellar apparatus and nucleus (Figs. 6c, 6d), and then at the anterior end the groove is formed, which separates daughter cells. The cytokinesis ends by separation of the posterior ends of daughter cells.

DISCUSSION

As a result of the research it has been established that life cycle of *Pharyngomonas* sp., strain RL consists of amoeboid cells, flagellates and cysts, which are consequently replace each other. The similar life stages were revealed in *Pharyngomonas kirbyi* (Harding et al., 2013) and amoeboflagellates of other genera in the class Heterolobosea. For instance, capacity to transformation of amoeboid cells into flagellated cells was found for genera *Acrasis*, *Euplaesiobystra, Heteramoeba, Naegleria*, *Oramoeba, Pharyngomonas, Psalteriomonas*, *Stachyamoeba, Tetramitus*, *Tulamoeba*, *Willaertia* (Page, 1967; Patterson et al., 2000; Murase et al., 2010; Panek and Cepicka, 2011; Harding et al., 2013). However feeding and reproduction of flagellated cells was revealed only for some species from four genera of amoeboflagellates such as *Heteramoeba clara*, *Oramoeba fumarolia, Psalteriomonas lanterna*, *P. magna*, *Tetramitus jugosus, T. thorntoni*, *T. rostratus* (Patterson et al., 2000; De Jonckheere, 2011; Panek et al., 2012). Ability of flagellates to feed and reproduce is also a

characteristic of the strains *Pharyngomonas kirbyi* AS12B and SD1A (Park and Simpson, 2011) and the strain *Pharyngomonas* sp. "Summer Lake" (Gunderson, 1981, cited by Harding et al., 2013). In our experiment amoeboid cells prevailed during the first half of the life cycle. For the second half flagellates predominated over amoebae; perhaps it was related to acceleration of the amoebae encystation. It is possible, under other conditions otherwise dynamics of the life stages can be observed, as it was shown for a culture of amoeboflagellate *Oramoeba fumarolia*, in which ratio between flagellates and amoeba depends on bacteria species used as a food source (De Jonckheere, 2011). Thus results of our research correspond to data of other authors and show the genus *Pharyngomonas* includes true amoeboflagellates with two vegetative stages, amoeboid and flagellated cells. Both stages are able to feeding and reproduction; and life cycle of representatives of the genus *Pharyngomonas* is the most complicated as compared with other heteroloboseans, except for acrasids.

Analysis of different scientific papers for a long period of time (Entz, 1904; Namyslowski, 1913; Kirby, 1932; Ruinen, 1938; Pochmann, 1959; Gunderson, 1981; Park and Simpson, 2011) disclosed that representatives of the genus *Pharyngomonas* and organisms very similar to it occur in salt water bodies of every continent, except for Antarctica. On the territory of Russia these amoeboflagellates have been revealed for the first time. Flagellates similar by morphology to *Pharyngomonas* were detected in saline lakes of Orenburg region, such as Lakes Razval, Dunino, Tuzluchnoe (Plotnikov et al., 2011), as well as in salt river of Elton region, the Malaya Samoroda River (Plotnikov and Selivanova, 2014), and of Orenburg region, the Tuzlukkol River (unpublished data of Plotnikov and Selivanova).

The first description of a flagellate similar by morphology to *Pharyngomonas* sp., strain RL was published more than century ago; it was called *Trichomastix salina* sp. n. (Entz, 1904). Length of flagellated cells isolated by G. Entz (Entz, 1904) from a saline spring near Turda town, Romania, was 20–30 μm that corresponds with dimensions of *Pharyngomonas* sp., strain RL. A distinctive feature of the Entz description is misinterpretation of some morphological signs of the cells. For example, kinetosomes at the anterior end of the cell were called "contractile vacuoles"; the intracellular channel with the cytopharynx was described as a right-side ventral groove with an excavation behind the nucleus. It appears that described by the author "anal orifice" at the posterior end of the cell accords with opening of the intracellular channel. Generally, morphology of *Trichomastix salina* cells does not have significant differences from *Pharyngomonas* sp., strain RL. Despite in the Entz article (Entz, 1904) there are no descriptions of cysts and amoeboid cells, good agreement of sizes and morphology between flagellates *Trichomastix salina* and *Pharyngomonas* sp., strain RL suggests their great resemblance, and perhaps close taxonomical relationship.

The flagellate revealed in salt mines of Welizchka, Poland, and described in the paper of B. Namyslowski (Namyslowski, 1913) under the name of *Pharyngomonas* sp. n., is similar by sizes and morphology to *Triflagellum salinum* sp., strain RL. Dimensions of the protist (14 × 4–5 μm) correspond with our isolate. In *Triflagellum salinum* an excavation was described, resembling cytopharinx and localized in the anterior third of the cell on the ventral surface. Amoeboid cells and cysts were not reported. A distinction from our isolate and from *Trichomastix salina* Entz 1904 is presence of three flagella, but it may be due to localization in the ventral groove of one flagellum of the four; this makes the flagellum almost undistinguished. Another possible reason of wrong count is lost of flagella due to slow destruction under cover glass, which was described by us during the observation of the culture *Pharyngomonas* sp., strain RL.

H. Kirby (Kirby, 1932) described a flagellate isolated from saline water body near Marina city, California, USA, and concluded the identity of the protist and *Trichomastix salina* Entz 1904. Due to careful light-microscopical study Kirby (Kirby, 1932) established resemblance of the flagellate with *Tetramitus sulcatus* Stein 1878 and included the species *Trichomastix salina* Entz 1904 into the genus *Tetramitus* with name *Tetramitus salinus* (Entz, 1904). Morphology of the flagellate and sizes of the cell (15–19 × 6–8 μm) corresponds with description of our isolate. In the Kirby paper (Kirby, 1932) in the cells of *Tetramitus salinus* (Entz 1904) cytoplasmic threads or filaments at the anterior end, attaching the cell to a substrate (cover glass), were described for the first time, as well as cytopharynx of conical shape sometimes extending to the middle of the cell. Also it was noticed that some cells have the obtuse posterior end. In the culture of this protist numerous amoebae were revealed, some of them with long filiform pseudopodia at the posterior end; perhaps it was amoeboid stage of the protist. Length of flagella is equal to 1–1.5 length of the cell, shorter than in our strain.

J. Ruinen (Ruinen, 1938) supplemented description of *Tetramitus salinus* (Entz 1904) Kirby 1932, having revealed protists like this in saline water bodies of India, the Islands Madura and Java, Indonesia, Australia. The flagellates studied by Ruinen (Ruinen, 1938) are of less size (8–14 × 4–6 μm), than those described by Entz (Entz, 1904) and Kirby (Kirby, 1932). Also their features are lack of a cytopharynx and a short ventral groove in the anterior part of the cell, never reached to the posterior end. In addition, Ruinen (Ruinen, 1938) found in saline water bodies of Portugal, Madura Island (Indonesia) and Australia a flagellate very similar to *Triflagellum salinum* Namyslowski 1913. The isolate described by Ruinen (Ruinen, 1938) has triangle shape; in the picture there is a cell

with the convex dorsal and flat ventral surfaces and with the obtuse posterior end. The protist has three flagella; pair of them with length equal to length of the cell; and the third flagellum is longer by a third or half of the cell length.

The most detailed description of a flagellate similar in morphology with *Pharyngomonas* sp., strain RL was done by A. Pochmann (Pochmann, 1959) on the basis of light-microscopic study of culture *Choanogaster plattneri* gen. et sp. n., isolated from hypersaline Urmia Lake, Iran. Dimensions of the flagellates cells are 18 × 7–12 μm; but also smaller cells of size up to 12 μm were described. Cells of the isolate have four flagella, forming the anterior and posterior pairs. The anterior flagella have length 18–20 μm, equal to the cell length or slightly longer. The posterior flagella are 1.5–2 times longer than the cell length. Sometimes filiform pseudopodia are produced at the posterior end of the cell; and in the place of flagella insertion dark bodies (kinetosomes) are found. For the first time the author researched morphology of cytopharynx or "oral funnel," ventral groove or "groove" and intracellular channel or "tubular main vacuole;" all the units were united by him into so called "vacuolar system." Pochmann (1959) for the first time noted formation of cysts with diameter 16–18 μm, but did not mention presence of amoeboid cells in the culture. He described finger-like pseudopodia regularly rising at the anterior end, and gave photos of amoeboflagellates.

In the PhD thesis of J.H. Gunderson (Gunderson, 1981; cited by Harding et al., 2013) morphology and ultrastructure of a protist *Tetramitus salinus* Entz 1904, isolated from Summer Lake, Oregon, USA, which likes *Pharyngomonas* sp., strain RL, was described in detail. For the first time all the life stages, flagellated, amoeboid and cyst, were described by Gunderson. Sizes of the flagellates (16.5–26.0 × 10.5–16.5 μm) and the cysts (diameter is about 12 μm) are similar to our isolate. Furthermore, Gunderson (Gunderson, 1981) for the first time noted inability of the flagellated cells to produce cysts and to exit from them, remarking the property only for amoebae; also he described different types of pseudopodia in the amoebae such as lobosean and eruptive.

In 2008 as a result of taxonomical revision the flagellate *Tetramitus salinus* (Entz 1904) Kirby 1932 was renamed to *Pharyngomonas kirbyi* Cavalier-Smith 2008 for more precise recognition of the species and for ordering of taxonomy of phylogenetically close genera *Tetramitus, Percolomonas* and *Pharyngomonas* (Cavalier-Smith and Nikolaev, 2008). In 2011 the data on careful research of morphology and ultrastructure of new halophilic flagellates, isolated from water bodies of the USA and Australia and belonged by authors to the species *Pharyngomonas kirbyi* Cavalier-Smith 2008 (Park and Simpson, 2011) were published. Later in these strains cysts and amoeboid stage were revealed and described (Harding et al., 2013). The described strains AS12B and SD1A of *Pharyngomonas kirbyi* appeared to be similar very much to our isolate by most morphological signs; distinctions concern length of flagella and pseudopodia of amoeboid cells. Thus, length of the anterior flagella in *Pharyngomonas kirbyi* is about equal to size of the cell; the posterior flagella are 1.0–1.5 times longer than the cell (Park and Simpson, 2011); whereas flagella of *Pharyngomonas* sp., strain RL are 1.5–2.0 times longer than the cell. As well, amoeboid cells of our isolate produce at the posterior end thin, long, filiform, often branched pseudopodia; length of those exceeds 2–3 times the cell length. Though this feature was noted by Kirby (Kirby, 1932) in amoeba present in the culture *Tetramitus salinus* (Entz 1904) Kirby 1932, it was not marked in strains AS12B and SD1A *Pharyngomonas kirbyi* (Harding et al., 2013). We suppose, revealed distinctions may serve as diagnostic criteria for differentiation of our isolate from the species *Pharyngomonas kirbyi*.

The cysts produced by amoeboid cells of *Pharyngomonas* sp., strain RL, like cysts of *Tetramitus salinus* (Entz 1904) Kirby 1932 (Gunderson, 1981) and *Pharyngomonas kirbyi* (Harding et al., 2013) by their roundish or slightly ovoid shape and diameter about 12 μm. At the same time cysts of *Choanogaster plattneri* (Pochmann, 1959) have a larger diameter (16–18 μm), than cysts of our isolate (12 ± 2 μm).

Available data on 18S rRNA gene of *Pharyngomonas* sp., strain RL and *Pharyngomonas kirbyi*, in our opinion, are useful for taxonomical differentiation between them. At present 18S rRNA gene has been sequenced in the strains SD1A and AS12B *Pharyngomonas kirbyi*, *Pharyngomonas* sp., strain RL, as well as an organism "*Macropharyngomonas halophila*" nomen nudum, which has not been described yet (nos. of sequences in GenBank HQ898858, HQ898857, JX509943, AF011465, respectively). Comparison of the sequences shows that exon regions of the 18S rRNA gene in the strains SD1A and AS12B *Pharyngomonas kirbyi* are similar by 98%. This circumstance along with identity of the morphology and ultrastructure was a justification for combining of the strains into the same species (Park and Simpson, 2011). "*Macropharyngomonas halophila*" is similar to the strains SD1A and AS12B *Pharyngomonas kirbyi* in sequence of the 18S rRNA gene by 97%. So the organism may be a strain of *Pharyngomonas kirbyi* or a closely related species within the same genus (Park and Simpson, 2011). The strain RL of Pharyngomonas sp. is similar to the strains SD1A and AS12B *Pharyngomonas kirbyi* in sequence of the 18S rRNA gene only by 90.7% (Harding et al., 2013). In addition, in the strain the 18S rRNA gene does not contain introns of I group, which are present in the same gene of the strains SD1A and AS12B *Pharyngomonas kirbyi* in an amount of two and three, respectively (Park and Simpson, 2011; Harding et al., 2013). As well, in the 18S rRNA gene of *Pharyngomonas* sp., strain RL, an

insertion about 75 bp near the 3' end was revealed; the insertion is absent in the gene of the *Pharyngomonas kirbyi* strains (Harding et al., 2013). The mentioned differences in the structure of 18S rRNA gene, in our opinion, prevent referring of *Pharyngomonas* sp., strain RL to the species *Pharyngomonas kirbyi* Cavalier-Smith 2008 sensu Park et Simpson 2011.

Considering the basal place of the genus *Pharyngomonas* in the phylogenetic tree of Heterolobosea (Harding et al., 2013), two types of vegetative cells in the life cycle, flagellates and amoeboid cells, should be considered as ancestral feature remained only in a few of genera and species Heterolobosea. Perhaps this feature has been reduced in most representatives of the class Heterolobosea. Possibly among heterolobosea only protists of the genus *Pharyngomonas* have kept maximally those mechanisms of cellular differentiation into the amoeboid and flagellated types, which were present in protozoan ancestor of Metazoa. It is particularly interesting in view of the hypothesis of L. Seravin and A. Gudkov (Seravin and Gudkov, 2005), suggested that the ancestor of Metazoa was an amoeboflagellate.

CONCLUSIONS

(1) The life cycle of *Pharyngomonas* sp., strain RL includes the life stages of amoeba, flagellated cells and cysts and consists of the following consequent steps: exit of the amoeba from the cysts, reversible transformation of the amoeboid cells into the flagellates (both stages are able to feed and reproduce), encystation of the amoeboid cells.

(2) As a result of careful morphological and morphometric research differences of *Pharyngomonas* sp., strain RL from the only valid species *Pharyngomonas kirbyi* were established. They comprise of a longer length of flagella and thin filiform branched pseudopodia produced at the posterior end of amoeboid cells. The morphological distinctions are in a good agreement with the structure of 18S rRNA gene and may be used for further differentiation of species within the genus *Pharyngomonas*.

(3) Representatives of the genus *Pharyngomonas* and similar protists inhabit saline inner water bodies, but are not found in fresh and marine biotopes; the phenomenon perhaps is related to their halophily.

(4) Cytological, physiological and genetic studies of the genus *Pharyngomonas* are of great value for phylogeny and taxonomy of heterolobosea, as well as for investigations of cellular differentiation mechanisms of amoeboflagellates.

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