



## Unusual archamoeba *Pelomyxa pilosa* sp. nov. (Amoebozoa: Archamoebae: Pelobiontida): a light and electron microscopic study

## Необычная архамёба *Pelomyxa pilosa* sp. nov. (Амoebozoa: Archamoebae: Pelobiontida) – свето- и электронно-микроскопическое исследование

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**Abstract.** This paper presents the results of a study and describes a new species of pelobionts, *Pelomyxa pilosa* sp. nov. (Archamoebae: Pelobiontida). This species was found in the bottom sediments of a pond near St Petersburg (Russia) and was examined using light, immunofluorescence, and transmission electron microscopy. It is placed in the genus *Pelomyxa* Greeff, 1874 based on a combination of morphological features, including the mode of locomotion, the presence of numerous flagella, the organisation of the cytoplasm, and the presence of obligate prokaryotic endocytobionts. The new species can be easily distinguished from congeners by the specific presence of numerous prokaryotic ectobionts on the cell surface and the organisation of the nuclear apparatus. The nucleolar material in this species forms a hollow sphere consisting of tightly adjacent patches of various shapes and sizes, surrounded by granular material.

**Резюме.** В работе представлены результаты исследования и описание нового вида пелобионтов *Pelomyxa pilosa* sp. nov. (Archamoebae: Pelobiontida), обнаруженного в донных отложениях пруда в окрестностях Санкт-Петербурга (Россия). Этот вид был изучен с использованием комплекса методов световой, иммунофлуоресцентной и просвечивающей электронной микроскопии. Он отнесен нами к роду *Pelomyxa* Greeff, 1874 по совокупности морфологических признаков, включающих способ локомоции, наличие жгутиков, особенности организации цитоплазмы и присутствие облигатных прокариотных эндоцитобионтов. Основными отличительными признаками *P. pilosa* sp. nov. являются специфическое наличие на поверхности клетки многочисленных прокариотных эктобионтов и особенности организации ядерного аппарата. Ядрышковый материал имеет вид поллой сферы, внутреннюю поверхность которой образуют плотно прилежащие друг ко другу фрагменты разных форм и размеров, окруженные гранулярным материалом.

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**Key words:** systematics, light microscopy, immunofluorescent staining, tubulin cytoskeleton, ultrastructure, Archamoebae, *Pelomyxa*, new species

**Ключевые слова:** систематика, световая микроскопия, иммунофлуоресцентное окрашивание, микротрубочковый цитоскелет, ультраструктура, Archamoebae, *Pelomyxa*, новый вид

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## Introduction

Free-living representatives of Archamoebae are anaerobic or microaerobic amoeboflagellates and amoeboid protists that possess one or more flagella during at least one stage of the life cycle (Walker et al., 2017; Adl et al., 2019; Chistyakova et al., 2023). Most free-living archamoebae, except the members of the genus *Pelomyxa* Greeff, 1874, have motile flagella used for locomotion. In amoeboflagellates of the genera *Mastigamoeba* Schulze, 1875, *Mastigina* Frenzel, 1897, *Paramastigamoeba* Chistyakova et al., 2023, *Seravinia* Chistyakova et al., 2023, and *Rhizomastix* Alexeieff, 1911, the flagellar apparatus has a morphological connection with the nucleus, forming the so-called karyomastigont, which is absent in *Mastigella* Frenzel, 1897 (Walker et al., 2017; Chistyakova et al., 2023). In contrast, the cells of *Pelomyxa* spp. have numerous immotile or weakly motile flagella, which are not involved in locomotion. These protists possess only amoeboid movement. Moreover, in several species of *Pelomyxa*, flagella were not found (Frolov, 2011; Chistyakova et al., 2014). The obligatory presence of a multinucleate stage in the life cycle is considered another distinctive feature of *Pelomyxa* spp., although multinucleate forms have been observed in *Mastigamoeba* spp. and *Mastigella* spp. (Chavez et al., 1986; Ptáčeková et al., 2013; Zadrobílková et al., 2015). The characteristic features of *Pelomyxa* spp. are a considerable vacuolisation of the cytoplasm (the so-called “foamy cytoplasm”), accumulations of reserved nutrients in the form of “glycogen bodies” or lipid droplets, and the presence of obligate prokaryotic endocytobionts in the cytoplasm (Frolov et al., 2011; Chistyakova et al., 2016; Gutiérrez et al., 2017).

Here, we describe a new species of Archamoebae, which is placed to the genus *Pelomyxa*, based on a combination of morphological characters.

A remarkable feature of the new species is the presence of numerous bacterial ectobionts on the cell surface.

## Material and methods

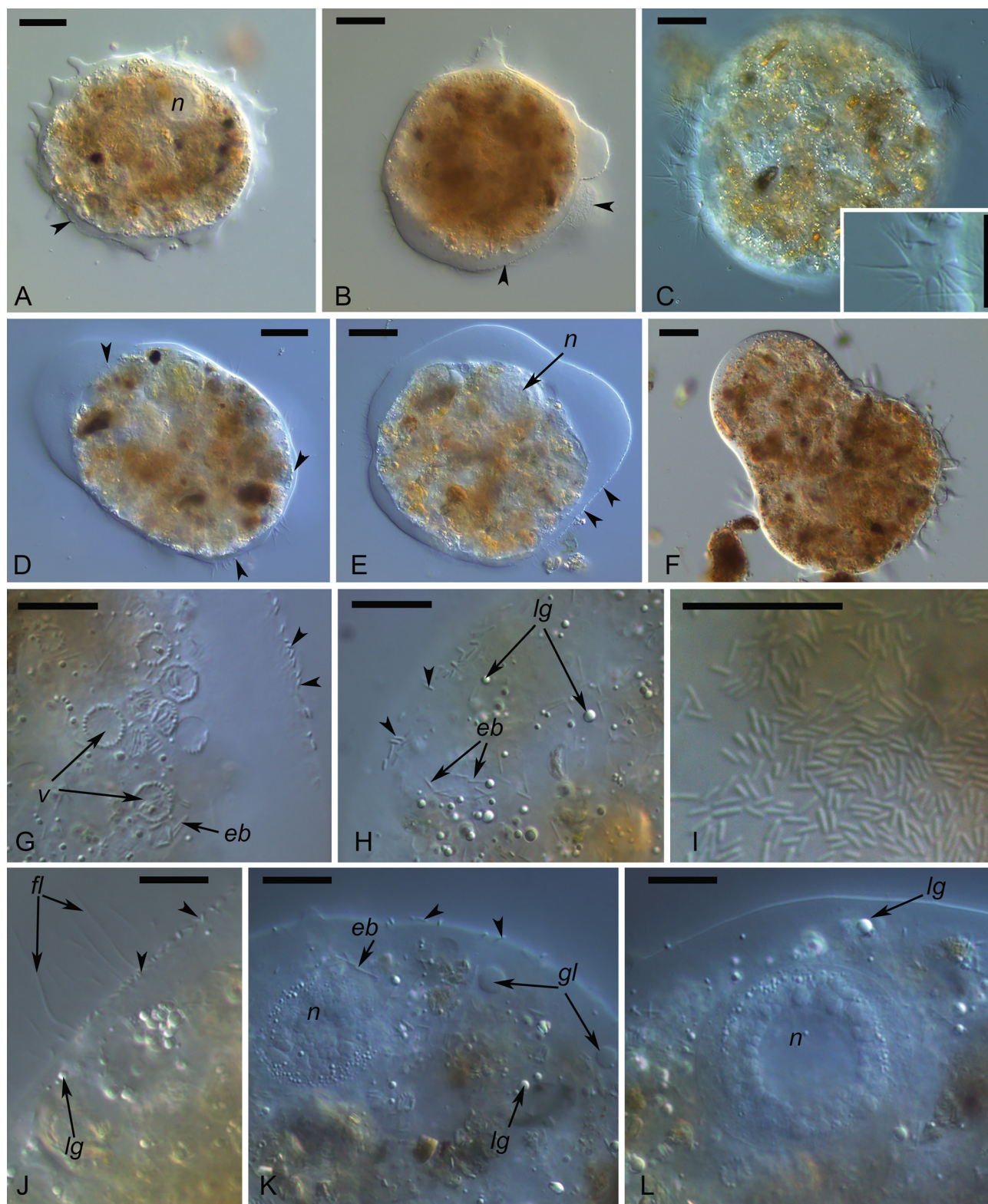
Samples of bottom sediments from a *Ceratophyllum* pond in Sergievka Park (St Petersburg, Russia, 59°53'N 29°50'E) were collected in plastic bottles between 2019 and 2021. Each sample had a volume of one liter, with at least 1/5 of it consisting of detritus. The samples were stored in a refrigerator at 10°C. To search for amoebae, 3–4 ml of bottom sediments (detritus) were transferred to a 90 mm Petri dish, mixed in a 1:1 ratio with pond water, and examined using a Leica M125C stereomicroscope.

Light-microscopic observations and microphotography were performed using a Leica DM2500 microscope equipped with Nomarski contrast, a fluorescence module, and a Leica DFM 495 camera. Filter cube B/G/R (Leica Microsystems, Wetzlar, Germany) was used to observe the autofluorescence of prokaryotic endocytobionts. Immunofluorescent staining of pelomyxae with antibodies to  $\alpha$ -tubulin was carried out as described previously (Chistyakova et al., 2020a). Material for electron microscopy was prepared following Frolov et al. (2005a). Ultrathin sections were examined using a Morgagni 268 electron microscope (FEI, The Netherlands).

## Results

**Light microscopy.** The cells observed in our samples usually did not move and were associated with detritus patches. The amoebae were rounded, 50–150  $\mu$ m in diameter (Fig. 1A–C). A wide rim of hyaloplasm was often visible along the periphery of the cell (Fig. 1B). Sometimes, the pelomyxae formed conical hyaline pseudopodia (Fig. 1A).





**Fig. 1.** Light microscopy (differential interference contrast) of *Pelomyxa pilosa*, **sp. nov.** **A–C**, non-motile cells; **D, E**, beginning of locomotion; **F**, cell during active locomotion; **G, H, J–L**, details of cell structure; **I**, bacteria on the cell surface. Abbreviations: *n* – nucleus; *eb* – prokaryotic endocytobionts in the cytoplasm; *lg* – lipid globules; *gl* – glycogen bodies; *fl* – flagella; *v* – vacuoles with bacteria; arrowheads – bacteria on the cell surface; insert – part of the cell at higher magnification. Scale bars: 20 µm (**A–F**) and 10 µm (**G–L**).

Typically, numerous needle-like or filamentous outgrowths of the hyaloplasm were formed on the cell surface. Sometimes they spread like a fan from small knob-like protuberances of the hyaloplasm (Fig. 1C). Upon transition to active locomotion, the pelomyxae formed a broad leading lobopodium and several lateral finger-shaped pseudopodia in the posterior part of the cell. During this process, needle-like hyaline outgrowths were displaced to the uroidal area of the cell (Fig. 1D–F).

Numerous rod-shaped bacteria were associated with the cell surface. In some areas, the cell body was almost entirely covered with bacteria (Fig. 1B, G–J). In addition, there were many immotile flagella about 10  $\mu\text{m}$  in length (Fig. 1J). Near the cell surface, rounded vacuoles 3–5  $\mu\text{m}$  in diameter were observed. The inner side of the membrane of these vacuoles was lined with a layer of bacteria (Fig. 1G). These vacuoles differed markedly from the digestive ones, which contained detritus particles (Fig. 1A–F). Multiple small refractile bodies, presumably lipid granules (Fig. 1H, J–L), and opaque rounded bodies 2–4  $\mu\text{m}$  in diameter (Fig. 1K) were observed in the cytoplasm.

A single rounded nucleus with a diameter of 30–35  $\mu\text{m}$  occupied a considerable part of the cell volume (Fig. 1A, E). Numerous rounded and oval nuclear bodies were located close to each other and, in optical sections, formed a ring within the nucleus. The larger bodies were located closer to the centre of the nucleus, whereas the smaller ones formed a peripheral layer of this ring (Fig. 1K, L). In three-dimensional space, the layer of nuclear bodies within the nucleus formed a hollow sphere located at a distance from the nuclear envelope.

The cytoplasm contained numerous rod-shaped bacteria (Figs 1G, H, K, 2A), which exhibited autofluorescence when irradiated with 420 nm light (Fig. 2B). In addition to these bacteria, the cytoplasm of the pelomyxae contained unidentified spherical bodies that emitted light in the same spectral region.

**Immunofluorescent microscopy.** Immunofluorescent staining of the cells revealed flagella, with a dense bundle of 5–8  $\mu\text{m}$  long microtubules starting from their base (Fig. 3A, B). In some cases, the bundle was shorter and less dense. In other cases, it was completely absent, and only a fluorescent body (kinetosome), not associated with any microtubule derivatives, was detected at the

base of the flagellum (Fig. 3 – inset). A network of microtubular bundles with various orientations was detected in the cytoplasm of stained cells (Fig. 3B).

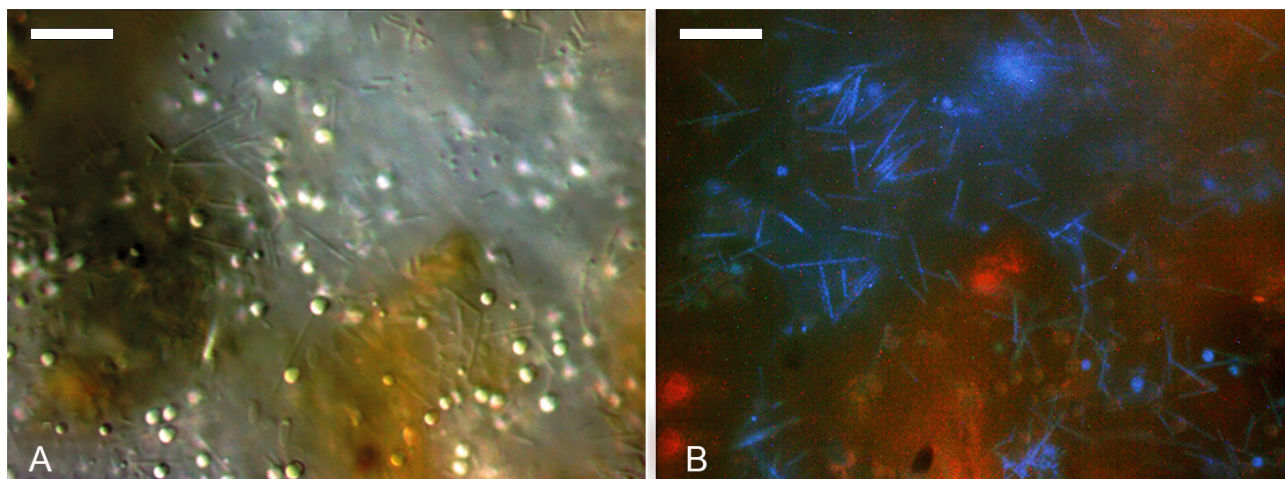
**Transmission electron microscopy.** The surface of the plasma membrane was covered with a 200–400 nm thick layer of filamentous glycocalyx (Fig. 4A). Numerous bacteria, 1.0–1.5  $\mu\text{m}$  long and 0.35–0.40  $\mu\text{m}$  in diameter, with dense cytoplasm were associated with the host cell surface (Fig. 4A, B). The bacterial wall was uneven, and distinct ridges were visible in transverse sections (Fig. 4A).

The cytoplasm of the studied cells contained relatively small rounded vacuoles with bacteria. Morphologically, these bacteria closely resembled those covering the cell surface (Fig. 4C). They were mainly situated near the vacuolar membrane, mirroring the image seen under the light microscope (Fig. 1C).

The cytoplasm contained numerous rounded bodies of medium electron density. These bodies were 1.0–1.5  $\mu\text{m}$  in diameter and had a homogeneous internal structure (Fig. 4D, E). They apparently corresponded to the refractile granules visible at the light-microscopic level (Fig. 1H, J, L). Numerous rod-shaped bacteria, 2.5–3.0  $\mu\text{m}$  in length and 0.25–0.30  $\mu\text{m}$  in diameter, enclosed in individual vacuoles, were seen in the cytoplasm (Fig. 4B, F). The outer surface of the vacuolar membrane was covered with a “brush” of short, thin filaments (Fig. 4B, F). In addition, the cytoplasm contained bodies of medium electron density, measuring 2–4  $\mu\text{m}$  in diameter, with a distinct finely granular structure (Fig. 4G).

The nucleus belonged to the so-called ovular type (Raikov, 1982). In our sections, it was rounded and had a smooth outline, without invaginations or bulges. Numerous pore complexes were visible in the nuclear envelope (Fig. 4H, I). No traces of lamina or any other structure associated with the nuclear envelope could be observed. A layer of electron-dense bodies resembling nucleoli of various sizes and shapes was located inside the nucleus, at a distance from the inner nuclear membrane. Between these bodies, there were areas filled with granular material of moderate electron density (Fig. 4I). Clusters of filamentous structures resembling chromatin were observed closer to the centre of the nucleus (Fig. 4H).





**Fig. 2.** Endocytobionts of *Pelomyxa pilosa*, sp. nov. **A**, differential interference contrast; **B**, autofluorescence when irradiated with  $\lambda$  420 nm light. Scale bars: 10  $\mu$ m (A, B).

A considerable part of the cell body carried flagella. Each flagellum had a single 150–200 nm long kinetosome located at its base (Fig. 4J, K), with a bundle of 10–15 parallel microtubules originating from it (Fig. 4J–L). Organised fascicles of microtubules and groups of variously oriented individual microtubules were observed in the cytoplasm (Fig. 4F, M).

## Discussion

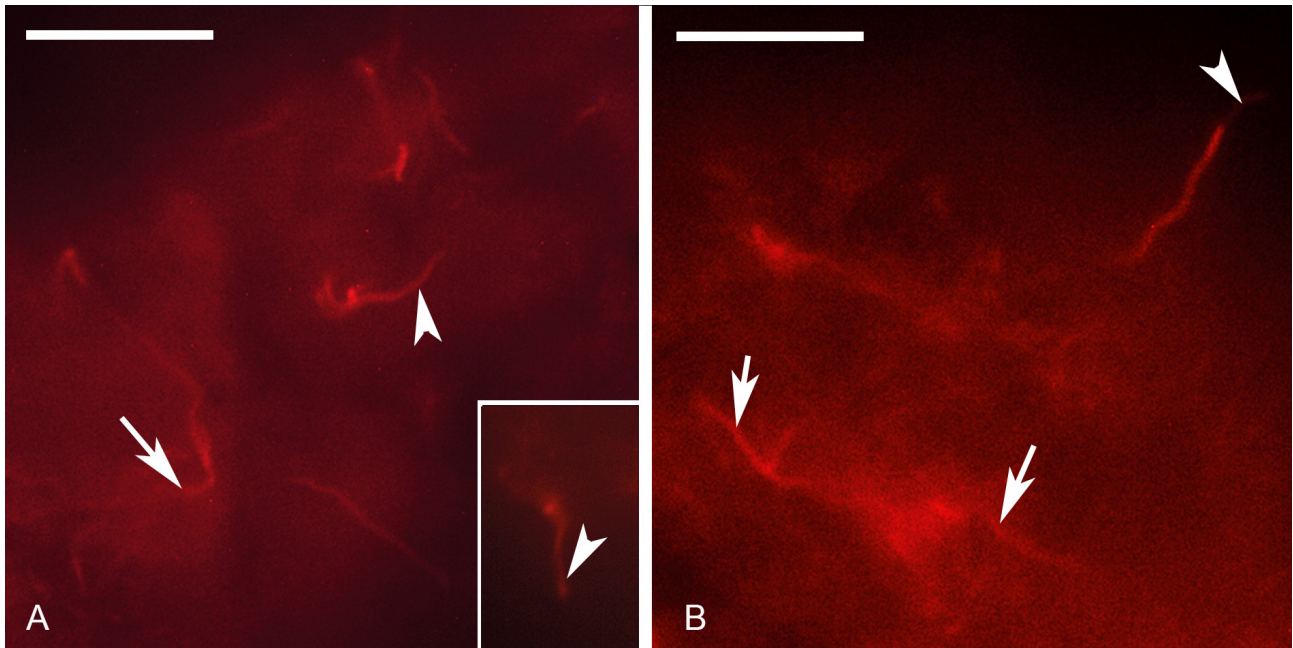
Archamoebae are classified mainly based on the organisation of the flagellar and nuclear apparatus, as well as the mode of locomotion (Chistyakova et al., 2014; Walker et al., 2017). A distinctive feature of most *Pelomyxa* spp. is the presence of numerous (usually immotile) flagella on the cell surface combined with an amoeboid mode of cell locomotion. The species examined and described in this study shows an undeniable morphotypic similarity to representatives of the genus *Pelomyxa*.

The locomotory shape of the studied species is similar to that of *P. prima* (Gruber, 1884), which also forms lateral finger-like pseudopodia. However, it clearly differs from the latter in the structure of the nucleus and the basal apparatus of the flagellum. Nuclei of *P. prima* have a single central spherical nucleolus. At the base of flagella, there is a long kinetosome associated with a cone of radial microtubules, lateral root, and a band of basal microtubules (Frolov et al., 2005a).

The new species has many other cell organisation features typical of the genus *Pelomyxa*. One of them is a strong vacuolisation of the cytoplasm. The cytoplasm of many *Pelomyxa* spp. looks “foamy” under a light microscope due to optically empty (“structural”) vacuoles and numerous digestive vacuoles with fragments of detritus and plant remains (Goodkov et al., 2004; Frolov, 2011).

The finely granular spherical bodies found in the cytoplasm of the organism at the light microscopic level and in TEM studies structurally correspond to glycogen accumulations, present in most known *Pelomyxa* spp. (Chistyakova et al., 2020b). Distinct “glycogen bodies”, similar to those described in our study, are common in *P. palustris* Greeff, 1874, *P. stagnalis* Chistyakova, Frolov, 2010, *P. binucleata* (Gruber, 1884) Penard, 1902, *P. belevskii* Penard, 1893, and *P. corona* Frolov et al., 2004 (Chistyakova et al., 2020b). Reserve nutrients in the form of lipid droplets, observed in our work, are also frequently found in cells of many pelomyxae (Frolov, 2011).

All representatives of the genus *Pelomyxa* lack mitochondria and form symbiotic associations with prokaryotes. The cytoplasm of different *Pelomyxa* species contains one to three distinct bacteria, and the composition of prokaryotic endocytobionts is considered to be species-specific (Chistyakova et al., 2016). In most *Pelomyxa* species, rod-shaped endocytobionts with an electron-dense content show characteristic autofluorescence when irradiated with 420 nm light.



**Fig. 3.** *Pelomyxa pilosa*, sp. nov., immunofluorescent staining for  $\alpha$ -tubulins. Abbreviations: arrowheads – flagella; arrows – bundles of the microtubules. Scale bars: 10  $\mu$ m.

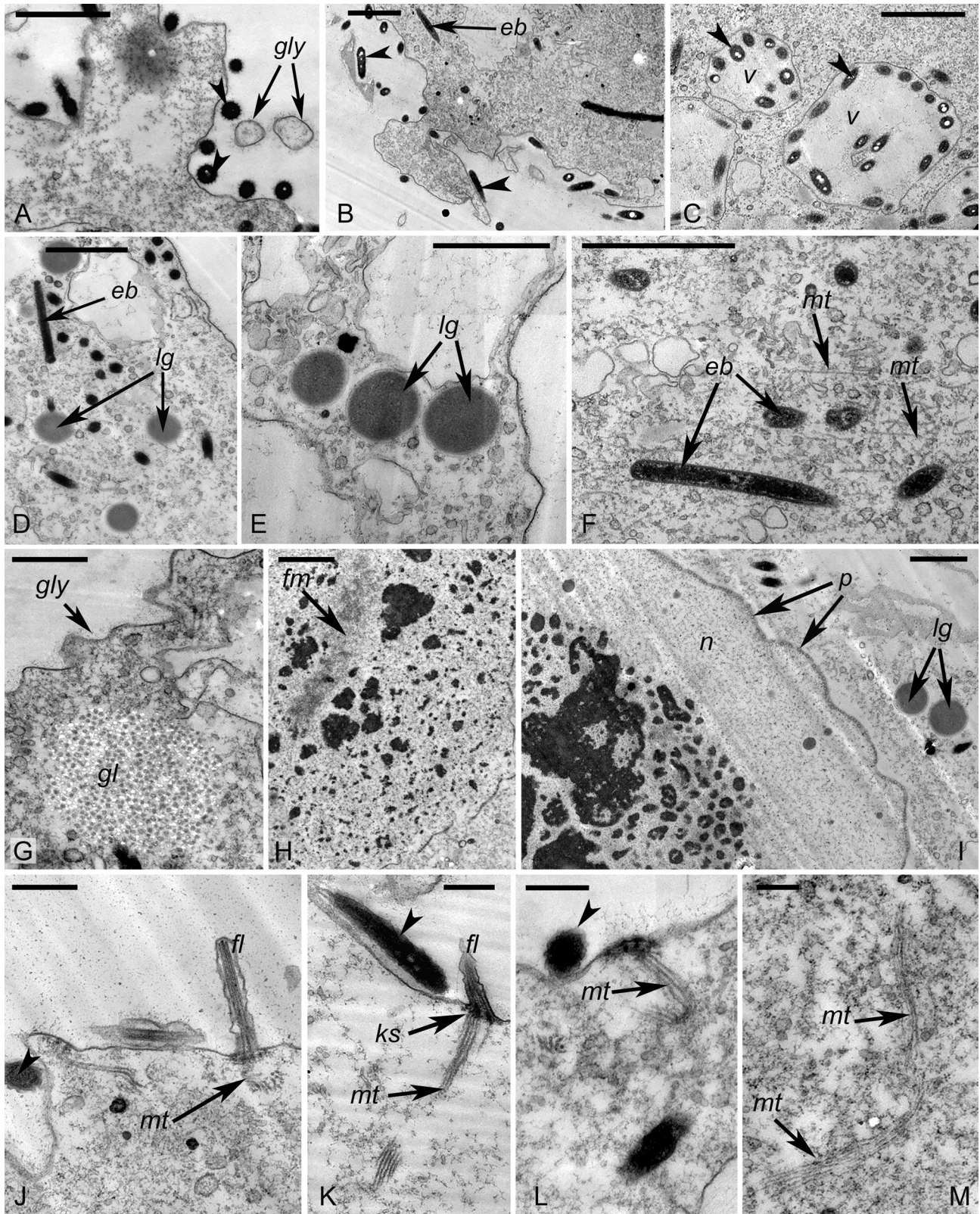
In *P. palustris*, such bacteria have been identified as *Rhodococcus erythropolis* (Gutiérrez et al., 2017). In the cytoplasm of pelomyxae, these bacteria are enclosed in symbiontophorous vacuoles, whose membrane has a rim of thin, short filaments (Chistyakova et al., 2016). Prokaryotic endocytobionts with the same morphotype and location in the cytoplasm were also present in the species examined in this study.

Pelomyxae, as well as other archamoebae, are characterised by the presence of a single kinetosome at the base of the flagellum, with a complex of radial microtubules forming a cone or a bundle associated with its lateral surface (Chistiakova et al., 2013). In addition, the flagellar root apparatus in pelomyxae may include basal microtubules and a lateral rootlet, thus forming a species-specific set of microtubule derivatives associated with the kinetosome (Walker et al., 2017). In the species examined, a single short kinetosome is located at the base of the flagellum, and the bundle of associated microtubules probably corresponds to the radial microtubules in other *Pelomyxa* spp. A similar organisation of the basal apparatus of the flagellum is known in *P. palustris*, *P. binucleata*, *P. belevskii*, and *P. stagnalis* (Goodkov, 1989; Frolov et al., 2005b; Chistyakova & Frolov, 2011; Chistyakova

et al., 2020a). It should be noted that, although some flagella of *Pelomyxa pilosa* sp. nov. possess a relatively well-developed basal apparatus, others have only kinetosomes without additional microtubular derivatives. This phenomenon, observed in many other *Pelomyxa* spp., can be explained by a partial reduction of the flagellar system resulting from the loss of its functional significance (Chistyakova et al., 2020a).

Pelomyxae are usually multinucleate (Frolov et al., 2011; Walker et al., 2017). However, the number of nuclei in a cell varies greatly in different *Pelomyxa* spp. For instance, it can exceed a thousand in large individuals of *P. palustris* (Greeff, 1874). The life cycle of *P. flava* Frolov et al., 2010 involves the alternation of binucleate forms and forms with four nuclei, while that of *P. binucleata* involves the alternation of uninucleate and binucleate forms (Frolov et al., 2005b, 2007, 2011). The relative duration of the uninucleate and multinucleate stages in the life cycle varies in different species. For example, uninucleate forms predominate in the life cycle of *P. paradoxa* Penard, 1902 and *P. gruberi* Frolov et al., 2006 (Frolov et al., 2006; Chistyakova et al., 2014). The succession of life cycle stages in some *Pelomyxa* spp. has been shown to depend on the season (Frolov et al., 2006, 2007).





**Fig. 4.** Transmission electron microscopy of *Pelomyxa pilosa*, **sp. nov.** Abbreviations: *n* – nucleus; *eb* – prokaryotic endocytobionts; *lg* – lipid globules; *gl* – glycogen bodies; *fl* – flagella; *v* – vacuoles with bacteria; *gly* – glycocalyx; *fm* – filamentous material; *mt* – microtubules; *p* – pore complexes; arrowheads – bacteria on the cell surface and in vacuoles. Scale bars: 2 μm (A–J) and 500 nm (K–M).



Therefore, although we observed only uninucleate cells in *P. pilosa* sp. nov., it may have forms with more nuclei, e.g., in winter.

The structure of the nucleus is one of the most important characters used in the identification of *Pelomyxa* spp., which are very diverse in this aspect (Chistyakova et al., 2013). An increased complexity of the nuclear envelope is often observed, including the formation of an additional layer of small vesicles on its outer side (Goodkov, 1989; Chistyakova & Frolov, 2011). Among the representatives of the genus, there are different variants of the organisation of the nucleolar material. For instance, *P. prima* and *P. gruberi* are characterised by a spherical central nucleolus (Frolov et al., 2005a, 2006). In *P. stagnalis* and *P. binucleata*, the nucleolus is also central but can be formed by several irregularly shaped fragments (Frolov et al., 2005b; Chistyakova & Frolov, 2011). In *P. palustris*, *P. belevskii*, *P. tarda* Gruber, 1887, *P. flava*, and *P. schiedti* Schaeffer, 1918, small nucleoli are located along the periphery of the nucleus directly under the nuclear membrane (Frolov et al., 2007, 2011; Ptáčková et al., 2013; Zadrobílková et al., 2015; Chistyakova et al., 2021). The nucleolar material in *P. paradoxa*, *P. secunda* (Gruber, 1884) Penard, 1902, and *P. corona* is located along the periphery of the nucleus but is arranged in larger fragments (Frolov et al., 2004; Chistyakova et al., 2014; Berdieva et al., 2015). Finally, in *P. doughnuta* Chistyakova et al., 2022, fragments of nucleolar material are located both along the periphery and in the central part of the nucleus (Chistyakova et al., 2022). In contrast to the above, in *P. pilosa* sp. nov., the nucleolar material is arranged in a hollow sphere. The inner part of the sphere is mostly formed by larger fragments of the nucleolar material tightly attached to each other. Smaller fragments form the outer layer of this sphere. This pattern of nuclear organisation clearly differs from that in all studied species of *Pelomyxa*.

We conclude that the species examined in this study is not conspecific with any known representative of the genus *Pelomyxa*. Therefore, we describe a new species and name it *Pelomyxa pilosa* sp. nov. The primary distinctive characteristics of the new species are the structure of the nuclear apparatus and the presence of prokaryotic ectobionts.

The presence of numerous bacteria associated with the cell surface is a striking feature of the

new species and deserves special discussion. All these bacteria are morphologically identical. We never found *Pelomyxa pilosa* sp. nov. without them. This observation suggests an ectosymbiotic relationship between the species examined and these bacteria. So far, only one species of archamoebae, *Mastigamoeba aspera* Schulze, 1875, has been shown to possess obligate prokaryotic ectobionts (Chistyakova et al., 2012, 2023). However, in *P. pilosa* sp. nov., bacteria morphologically indistinguishable from those on the cell surface were also invariably detected in cytoplasmic vacuoles. Nothing of the kind has been observed in *M. aspera*. We hypothesise that the association between the cells of *P. pilosa* sp. nov. and its bacteria has arisen relatively recently and is still unstable. A consequence of this instability is the excessive multiplication of the prokaryotic ectobionts and their phagocytosis by the new species. It should also be noted that the cell coats of *M. aspera* and *P. pilosa* sp. nov. are markedly different. In the former species, prokaryotic ectobionts are immersed in a thick layer of amorphous glycocalyx (Chistyakova et al., 2012, 2023), while in the latter, they are located on top of the relatively thin glycocalyx layer.

The presence of bacteria associated with the cell surface in *M. aspera* has been regarded as its distinctive feature, enabling easy identification of this species in samples from natural water bodies (Page, 1970). Researchers conducting faunistic studies should now bear in mind that there is at least one more species of mononucleate pelobionts with ectobiotic prokaryotes.

## Taxonomic summary

Phylum **Amoebozoa** Lühse 1913

Class **Archamoebae** Cavalier-Smith 1983

Order **Pelobiontida** Page 1976

Family **Pelomyxidae** Schulze, 1877

Genus ***Pelomyxa*** Greeff, 1874

***Pelomyxa pilosa* sp. nov.**

(Figs 1–4)

*Hapantotype*. A block containing cells fixed with glutaraldehyde-osmic solution and embedded in Epon-Araldite is deposited in the collection of prepa-



rations of the Laboratory of Cytology of Unicellular Organisms, the Institute of Cytology of the Russian Academy of Sciences (St Petersburg), under accession number 1073. Type locality: *Ceratophyllum* Pond, Sergievka Park, St Petersburg, Russia, 59°53'N, 29°50'E.

**Diagnosis.** Non-moving cells rounded, 50–100 µm in diameter, with small knobs of hyaloplasm on cell surface producing numerous needle-like outgrowths. Moving cells forming a broad frontal lobopodium and small lateral finger-like pseudopodia. Glycocalyx 200–400 nm thick, with filaments perpendicular to the surface of plasma membrane. Symbiontophorous vacuoles in cytoplasm with rod-shaped prokaryotic endocytobionts capable of autofluorescence when irradiated with 420 nm light. Single nucleus of ovoid type, 30–35 µm in diameter, with a large conglomerate of nuclear bodies of different sizes and shapes in central part. Numerous immotile flagella. Short kinetosome with a bundle of 10–15 parallel microtubules. Numerous ectobiotic prokaryotes on cell surface.

**Differential diagnosis.** *Pelomyxa pilosa* sp. nov. differs from all previously known congeners in obligate association with prokaryotic ectobionts and unique nucleus structure: round and oval nuclear bodies closely adjacent to each other, together forming a hollow sphere. Space between these bodies filled with granular material.

**Etymology.** The specific name *pilosa* is a Latin adjective meaning “hairy” or “shaggy”. It reflects the presence of numerous flagella and thin outgrowths of cytoplasm on the cell surface.

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