Protistology

A forgotten *Pelomyxa* species: redescription of *Pelomyxa tarda* Gruber, 1887 (Archamoebae, Pelobiontida)

Ludmila Chistyakova[1], Mariia Berdieva[2], Andrew Goodkov[2] and Alexander Frolov[1]

[1] *Zoological Institute RAS, 199034 St. Petersburg, Russia*
[2] *Institute of Cytology RAS, 194064 St. Petersburg, Russia*

Summary

A pelobiont *Pelomyxa tarda* Gruber, 1887 was isolated from a natural habitat for the first time since its original description. We examined it with the use of light, immunofluorescent and electron microscopy. *P. tarda* can be reliably distinguished from the other *Pelomyxa* spp. based on a number of characters, in particular, the morphology of the nuclei and the organization of reserve glycogen. An updated diagnosis of the species *Pelomyxa tarda* Gruber, 1887 is proposed.

Introduction

Representatives of the genus *Pelomyxa* (Archamoebae) inhabit freshwater oxygen-poor water bodies. These amoeboid protists have numerous nuclei at least at some life-cycle stages and a well-developed microtubular cytoskeleton; most of them have some immobile flagella randomly distributed over the cell surface (Frolov, 2011; Ptáčková et al., 2013; Walker et al., 2017; Adl et al., 2019). Pelomyxae are considered to lack several cellular compartments generally characteristic of protists, such as mitochondria and Golgi dictyosomes, although recent studies assume the presence of mitochondrion-related organelles in these organisms (Záhonová et al., 2021). Their cytoplasm is rich in various prokaryotic endocytobionts (Chistyakova et al., 2016; Gutierrez et al., 2017).

Most *Pelomyxa* spp. were described in the late 19th and the early 20th century, often based on one or a few individuals, and many of the species remain known only from the first descriptions (Leidy, 1879; Gruber, 1884, 1887; Bourne, 1891; Blochmann, 1894; Penard, 1902, 1904; Schaeffer, 1918; etc.). It was thought for some time that most pelomyxae were not independent species but stages of a complex life cycle of one polymorphic species *Pelomyxa palustris* Greeff, 1874 (Whatley, Chapman-Andresen, 1990; Brugerolle, Patterson, 2000; Goodkov et al., 2004), but later the monotypy of *Pelomyxa* genus was disproved. Recent studies have revealed a fairly diverse fauna of these organisms. There are currently 12 species in the genus (including *P. tarda*), most of them re-isolated based on first descriptions and redescribed at the modern level (Frolov et al., 2004, 2005, 2006, 2010; Chistyakova, Frolov, 2010;

Frolov, 2011; Berdieva et al., 2015).

The development of standard laboratory cultivation techniques has come up against virtually insurmountable obstacles in the case of most pelomyxae species. The only exception is a very small species, *Pelomyxa schiedti*, one of whose isolates is still successfully maintained in the laboratory culture (Zadrobilková et al., 2015; Záhonová et al., 2021). In turn, the cells of pelomyxae isolated from nature are packed with the remains of various eukaryotes. For these reasons, it is difficult, though not impossible, to apply molecular genetic methods to the study of these protists. Nevertheless, the molecular data available for some pelomyxae support the validity of the species identified based on morphological characters (Ptáčková et al., 2013; Zadrobilková et al., 2015).

Pelomyxa spp. are currently distinguished by a complex of features such as the morphology of the nuclei, the external appearance of the locomotor forms, the structural organization of the cytoplasm, the organization of microtubular cytoskeleton and the flagellar apparatus (if any), the absence or presence of reserve glycogen and its organization in the cytoplasm as well as the species composition of the prokaryotic symbiome (Frolov, 2011; Berdieva et al., 2015, Chistyakova et al., 2020a, 2020b).

It should be noted that some of the first descriptions of pelomyxae were so thorough and so well-illustrated that a reliable redescription of these "overlooked" organisms is still possible. In this work, we isolated and studied one such "forgotten" species, *Pelomyxa tarda* Gruber, 1887, using the methods of light, electron and immunofluorescent microscopy as well as cytochemistry.

Material and methods

Pelomyxa tarda were found in the samples of bottom sediments collected in the summer and the autumn of 2019-2020 in Afanas'ev Pond (Lyady Village, Pskov Region, Russia), in a small bogged basin formed by a broadening of a stream flowing into the Plyussa River near the Lyady Village (Pskov Region, Russia, approximately 58°35′ N and 28°55′ E) and in the Ceratophyllum Pond (Sergievka Park, St. Petersburg, Russia, 59°53′N, 29°50′E). The volume of each sample was 1 l, with the amount of detritus making up at least 1/5 of the sample. The samples were stored in the fridge at 10 °C.

So far, numerous attempts to develop a technique of laboratory cultivation of pelomyxae have failed.

Therefore, the protists for the study were taken directly from the samples. To obtain the pelomyxae, 3−4 ml of the bottom sediments (detritus) were placed into the Petri dish with a diameter of 90 mm, diluted with pond water 1:1 and viewed under a stereomicroscope Leica 125C (Leica-Microsystems, Wetzlar, Germany). Light-optical observations and microphotographing were made with the use of Leica DM2500 microscope equipped with Nomarski contrast, a fluorescent module and a Leica DFM 495 camera (Leica-Microsystems, Wetzlar, Germany).

Detection of glycogen-like polysaccharides in the cells and immunofluorescent staining of pelomyxae using antibodies against α-tubulin were performed as described before (Chistyakova et al., 2020a, 2020b). For the study of the organization of microtubular cytoskeleton, we mostly fixed the cells that were actively moving on the substrate. Material for electron microscopy was prepared as described earlier (Frolov et al., 2005, 2006) and studied using microscope Morgagni 268 (FEI, Netherlands).

Results

Pelomyxa tarda found in the samples were rounded or slightly elongated and generally moved little (Fig. 1, A, B). They were 400−800 μm in diameter, the largest specimens reaching 1 mm. Much of the cell surface was usually covered with short hyaline villi, and a narrow zone of transparent hyaloplasm was often prominent under the plasma membrane (Fig. 1, A, D, *arrowheads*). During the transition to directed locomotion, the pelomyxae became pear-shaped, broadened anteriorly, and slightly narrowed posteriorly, where a uroid zone, with a rather broad border of hyaloplasm and numerous, often branching villi, was usually present (Fig. 1, D, E). During prolonged active movement, the pelomyxae were generally cylindrical, and detritus particles adhering to the uroid were often visible at the posterior end of the cell (Fig. 1, C). Smaller pelomyxae isolated from spring and summer samples were more active than larger pelomyxae usually found in the samples collected in late autumn. The latter may reach 700−800 μm in diameter and were extremely reluctant to start directed locomotion.

The cytoplasm of the pelomyxae contained numerous food vacuoles mostly filled with plant residues. As a result, *P. tarda* cells were usually yellow-green or brown (Fig. 1, A−C). The space between the food vacuoles was occupied by rounded struc-
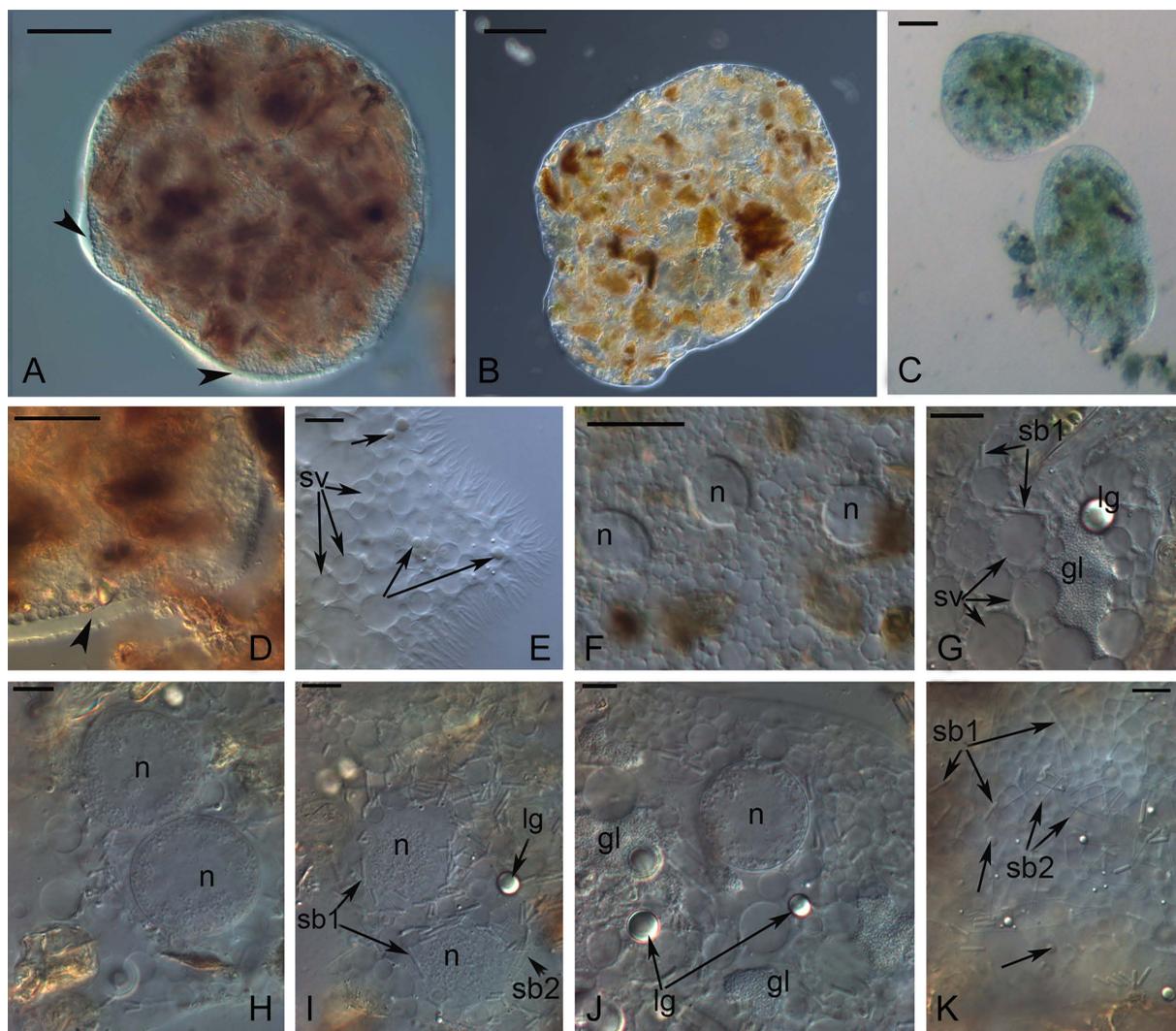
Fig. 1. *Pelomyxa tarda*, light microscopy (DIC). A–C – General view of the cells; D–K – details of the cell morphology. *Abbreviations*: gl – glycogen accumulations, lg – lipid globules, n – nucleus, sb1, sb2 – prokaryotic endocytobionts, sv – structural vacuoles; *arrowheads* – hyaloplasmic rim at the cell periphery, *arrows* – yellow spherical bodies. Scale bars: A–C – 100 µm; D, F – 50 µm, E, H, J, I, K – 10 µm.

tural vacuoles 3–6 µm in diameter (Fig. 1, E, G). Irregularly-shaped accumulations of finely-grained material were found between the structural vacuoles; the number and the size of such accumulations in the cells of the pelomyxae increased markedly in late autumn (Fig. 1, G, J). A specific PAS reaction for glycogen revealed stained aggregations similar to these accumulations in shape and size (Fig. 2, A, B). These aggregations were detected particularly well at the cell periphery, where they were not masked by various food inclusions (Fig. 2, A, B)

A colouration of *P. tarda* may also be associated with numerous yellow bodies, which were distributed throughout the cytoplasm fairly evenly; when exa-mined under a light microscope, they appear to lie within vacuoles (Fig. 1, E, K, *arrows*). In addition, shiny rounded bodies of presumably lipid nature were found in the cytoplasm (Fig. 1, G, I, J).

The cells of *P. tarda* contained numerous rod--shaped prokaryotic endocytobionts of two mor-photypes (Fig. 1, G, I, K; Fig. 3, A). Some of them were up to 3 µm long and no more than 1 µm wide. Others were thin rod-shaped bacteria, reaching a length of 10 µm and more, which were capable of autofluorescence when exposed to light with a wavelength of 420 nm (Fig. 3, B). The bacteria were usually distributed throughout the cytoplasm uniformly but on rare occasions their accumulations were observed around the nuclei (Fig. 1, I).

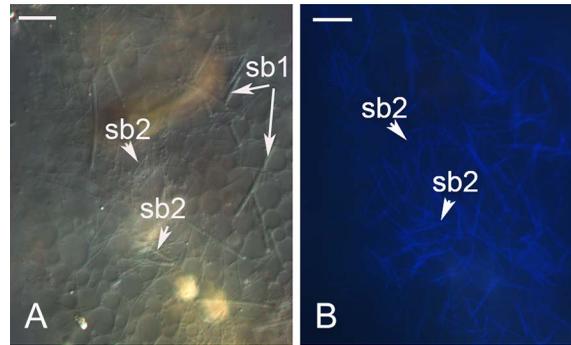The cells of *P. tarda* usually contained no more
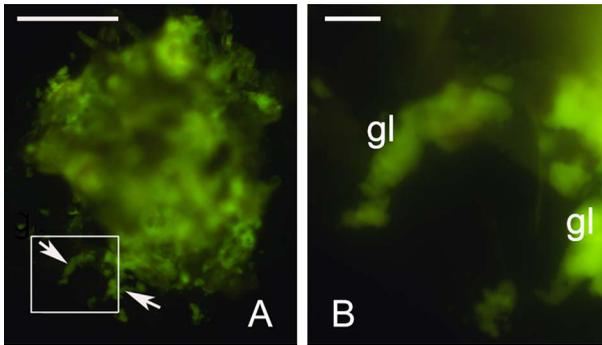
Fig. 2. *P. tarda*, specific PAS-reaction for glycogen detection. A — Whole stained cell; B — the enlarged area highlighted in Fig. 2, A. *Arrows* and gl — glycogen accumulations. Scale bars: A — 100 μm, B — 10 μm.

Fig. 3. *P. tarda*, endocytobionts. A — DIC; B — autofluorescence of sb2, light irradiation with λ 420 nm. *Abbreviations*: sb1, sb2 — prokaryotic endocytobionts. Scale bars: 10 μm.

than ten nuclei. They were rounded, 30−35 μm in diameter (Fig. 1, F, H). Accumulations of finely-grained material can be seen in the nucleoplasm at the nuclear periphery (Fig. 1, H−J).

Immunofluorescent localization of α-tubulin revealed an intense labelling around the nuclei of *P. tarda* (Fig. 4, A−C). Mostly, no other distinguishable elements of the microtubular cytoskeleton were observed in these pelomyxae, except for short bundles discerned sometimes in some optical slices (Fig. 4, B).

A layer of filamentous glycocalyx, 80−90 nm thick, could be seen on the surface of the plasma membrane of *P. tarda* at the electron micrographs (Fig. 5, A, B). The short hyaline villi branching from the cell surface were also prominent in peripheral zone (Fig. 5, C). An area of homogeneous hyaloplasm was visible along almost the entire cell periphery (Fig. 5, B, D). As a rule, it was separated from the granuloplasm by a layer of microfilaments, usually arranged chaotically but sometimes assembled in rather dense bundles (Fig. 5, B). In addition, a zone filled with densely packed structural vacuoles was often formed at the boundary between the hyalo- and granuloplasm (Fig. 5, D, *asterisk*). The granuloplasm contained numerous food vacuoles, empty structural vacuoles, and RER vesicles (Fig. 5, A, D, E, G). In addition, vacuoles 1 to 2 μm in diameter, filled with granular material, were present in the cytoplasm (Fig. 5, G). Rounded homogeneous bodies enclosed in an envelope − presumably, lipid storage − were also found in the cells (Fig. 5, G). At some electron micrographs accumulations of finely-grained material forming characteristic rosette-like structures could be seen

in the cytoplasm (Fig. 5, F). We assume that they correspond to the glycogen bodies detected at the light-optical level.

Prokaryotic endobionts of *P. tarda* lied in individual symbiontophoric vacuoles, the membrane of the vacuole usually tightly adjoining the bacterial cell wall. Endobionts of the first morphotype, thick short rods, were characterized by a long slit-like invagination of the cell wall, which could reach the middle of the bacterium, and by a medium-density bacterioplasm (Fig. 5, E, F, H). On ultrathin sections, these endobionts were often seen dividing, forming chains of two or more bacteria (Fig. 5, E). Bacteria of the second morphotype were thin long rods with a very dense bacterioplasm; a layer of microfilaments was usually associated with the membrane of their symbiontophoric vacuoles (Fig. 5, E, F).

In the nuclei of *P. tarda*, numerous accumulations of electron-dense material and distinct bodies mainly located immediately below the nuclear envelope could be seen at electron micrographs (Fig. 6, A, B). Short microtubules, with a length of about 100 nm, radiate from the nuclear envelope into the cytoplasm at a regular distance from each other (Fig. 6, B, C, D). In addition, microtubules lying along the nuclear membrane in close proximity to the nucleus are sometimes seen (Fig. 6, C, D).

Discussion

In his description of *Pelomyxa tarda*, A. Gruber (1887) notes that these organisms are 400 μm to 1 mm in size and that their cytoplasm is brown. During locomotion, they become oval or pear-

Fig. 4. *P. tarda,* immunofluorescent staining for α-tubulines (A−C). *Abbreviation*: n − nucleus; *arrowhead −* short bundle of microtubules. Scale bars: A − 50 μm, B, C − 10 μm.

shaped with a pronounced zone of short villi at the posterior part of the cell (Fig. 7, A). Food vacuoles containing pieces of detritus and plant debris are numerous in the cytoplasm, and rods about 10 μm long, similar to those found in *Pelomyxa palustris*, are occasionally seen (Fig. 7, A) (Gruber, 1887). Accumulations of irregularly-shaped finely-grained material considered by A. Gruber as food particles can be clearly seen in his illustrations of *P. tarda* cells (Fig. 7, A, B). According to A. Gruber (1887), the number of nuclei in *P. tarda* does not exceed eight, and their diameter is about 30 μm. The nucleoli are rather numerous and distributed irregularly (Fig. 7, B); a layer of chromatin surrounds a lighter central zone of the nucleus.

The light-microscopic characters of the pelomyxae isolated by us from the water bodies of the Pskov Region and St. Petersburg agree with those given in the original description of *P. tarda* (Gruber 1887). The descriptions of size and shape of the cell, the number and organization of the nuclei and cytoplasmic inclusions match. The rods observed by Gruber (1887) are identified as symbiotic bacteria. Accumulations of finely-grained material in the cytoplasm appear to be glycogen storage. Therefore, we identify the species used in this study as *P. tarda* Gruber 1887.

It is curious that for some reason *P. tarda* fell off the radar of the researchers undertaking a taxonomic revision of the genus *Pelomyxa* in the second half of 20th century (Page, 1976, 1981; Chapman-Andresen, 1978, 1982; Whatley and Chapman-

Andresen, 1990). As a result, *P. tarda* formally retained the status of a species, while all the other pelomyxae known at that time were made synonymous with *P. palustris*. At present, the genus *Pelomyxa* comprises 12 species (including *P. tarda*), some new and some re-isolated based on original descriptions (Chistyakova et al., 2013, Berdieva et al., 2015).

P. tarda differs from the other *Pelomyxa* spp. known to date primarily in the structure of the nuclear apparatus and the shape of the cells during directed locomotion. In addition, its characteristic feature is the presence of yellow rounded inclusions in the cytoplasm. The nature of these inclusions remains unclear. Filamentous glycocalyx, characteristic of *P. tarda*, has also been found in *P. palustris* and *P. gruberi* (Frolov et al., 2006, 2007). In many pelomyxae, a boundary layer formed by microfilaments (as in *P. tarda*) or by the channels and vesicles of the endoplasmic reticulum is detected between the ecto- and the endoplasm (Frolov et al., 2004, 2005, 2006, 2011). However, the tightly adjoining structural vacuoles underlying the ectoplasm are found only in *P. tarda*.

Based on the results of light-microscopic and electron-microscopic observations as well as the results of fluorescent staining with the use of specific PAS reaction, we conclude that the accumulations of finely-grained material in the cytoplasm of *P. tarda* consist of glycogen. Recent studies have shown that only some *Pelomyxa* spp. can accumulate glycogen in their cells (Chistyakova et al., 2020a).
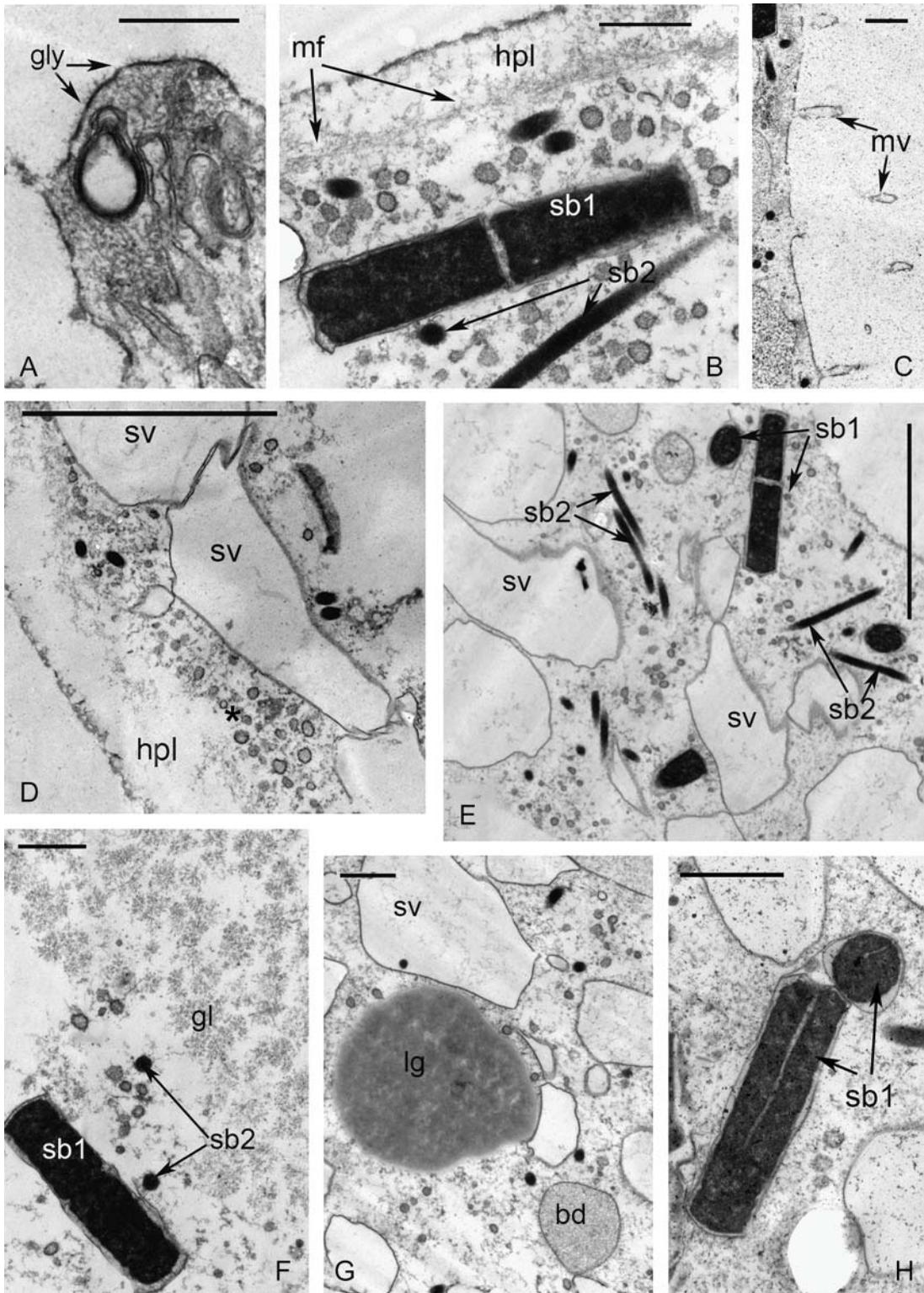
Fig. 5. *P. tarda*, transmission electron microscopy: peripheral zone and cytoplasmic inclusions (A–H). *Abbreviations*: bd – vacuoles with granular content, gl – glycogen accumulations, gly – glycocalix, hpl – hyaloplasm, lg – lipid globules, mf – microfilaments, mv – microvilli, sb1, sb2 – prokaryotic endocytobionts, sv – structural vacuoles; *asterisk* – zone filled with densely packed structural vacuoles. Scale bars: B, E – 5 μm, others – 1 μm.
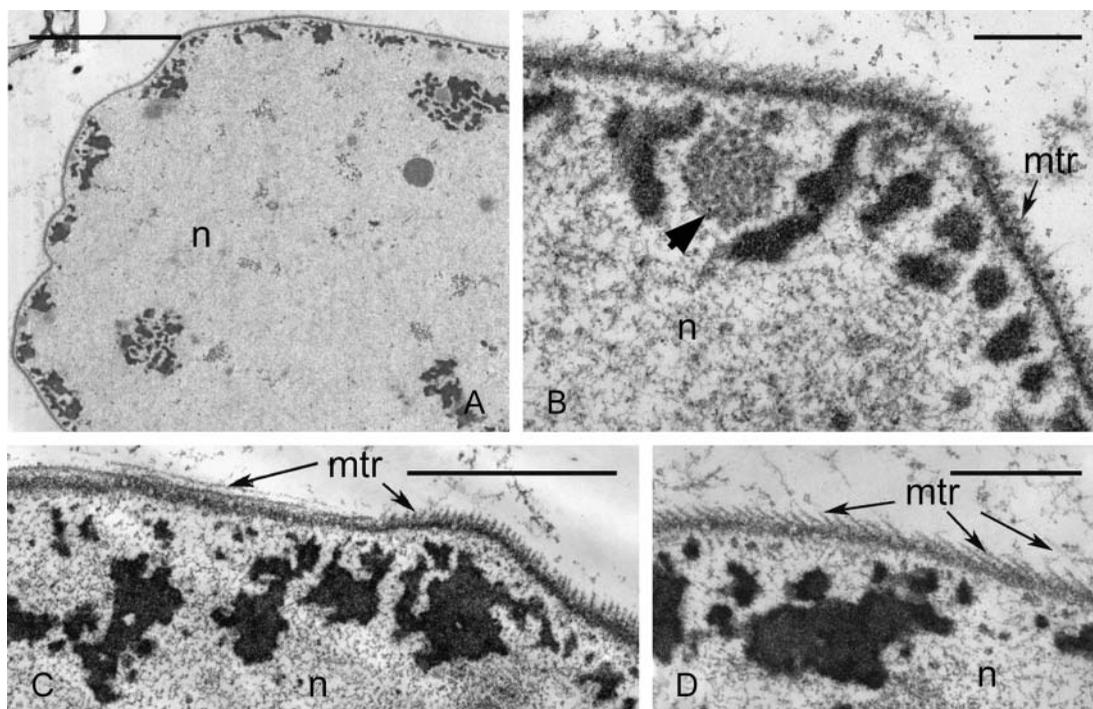
Fig. 6. *P. tarda*, transmission electron microscopy: nuclear apparatus (A−D). *Abbreviations*: mtr − microtubules, n − nucleus; *arrowhead* − nuclear body. Scale bars: A − 5 µm, B − 0,5 µm, C − 2 µm, D − 1 µm.
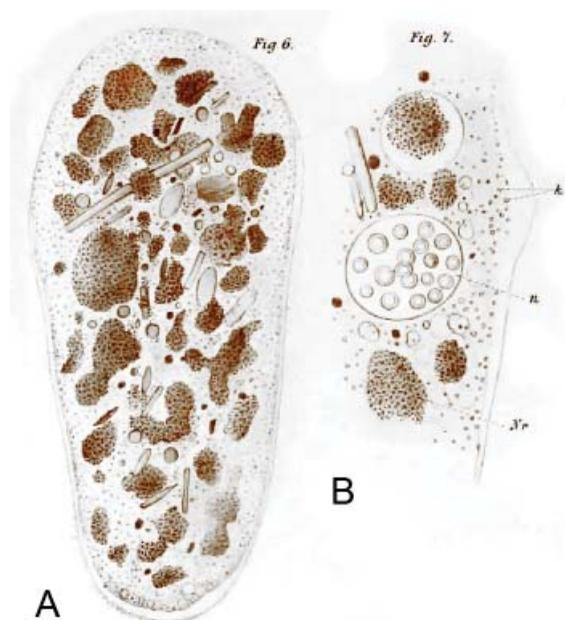
However, in most of them, glycogen forms dense morphologically distinct bodies clearly visible under the light microscope. In *P. secunda*, glycogen is distributed in the cytoplasm rather diffusely, does not form dense accumulations, and cannot be detected at the light-microscopic level without the use of specific staining (Berdieva et al., 2015). Thus, *P. tarda* differs from all the currently known *Pelomyxa* spp. in the organization of glycogen reserves. An increase in glycogen reserves in various pelomyxae is usually observed in late autumn (Chistyakova et al., 2020a), and *P. tarda* is no exception.

All members of the genus *Pelomyxa* typically contain numerous prokaryotic endocytobionts of several different morphotypes (Chistyakova et al, 2016). All pelomyxae without exception harbour the so-called af-symbionts: thin rod-shaped bacteria, with a dense bacterioplasm, which are capable of autofluorescence when exposed to light with a wave length of 420 nm. At the same time, af-symbionts from different host species may vary considerably in size. The second most frequent morphotype is represented by large-type symbionts: thick rods with a characteristic deep invagination of the cell wall. These bacteria have been found in more than half of the known *Pelomyxa* spp. The structure of large-type symbionts from different hosts is much more similar than that of af-symbionts. The taxonomic affiliation of *P. palustris* endobionts has been determined by the 16S RNA analysis: large-type symbionts are methanogenic archaea from the genus *Methanothrix*, while af-symbionts are bacteria from the genus *Rhodococcus* (Gutierrez et al., 2017). The species of prokaryotic endobionts in other pelomyxae has not been identified yet, although it is assumed that at least large-type symbionts from different hosts are conspecific (Chistyakova et al., 2016).

A comparative analysis shows that the symbiotic bacteria of *P. tarda* belong to the af-morphotype and the large-type morphotype. An occasional concentration of large-type symbionts around the nuclei, previously observed in different species of pelomyxae, was also registered in *P. tarda* (Chistyakova et al., 2016).

All *Pelomyxa* spp. possess a developed microtubular cytoskeleton, which may include the following elements: flagellar apparatus, cytoplasmic microtubules and perinuclear microtubules (Chistyakova et al., 2020b). A characteristic feature of most *Pelomyxa* spp. is the presence of numerous non-functioning flagella, which were not found only in *P. corona* and *P. secunda* (Frolov et al., 2004; Berdieva et al., 2015; Chistyakova et al, 2020b). At the same time, the flagellar rootlet appa-

nuclear envelope is usually detected (Chistyakova et al., 2020b).

Taxonomic summary

Taking into account the newly obtained data presented in this work, we suggest the following updated diagnosis of *Pelomyxa tarda* Gruber, 1887.

Diagnosis. Cells cylindrical or pear-shaped during locomotion, with pronounced zone of hyaline villi at posterior end. Food vacuoles mostly filled with plant residues, accounting for brown or yellow-green colouration of cells. Glycocalyx of filamentous type. Reserve glycogen represented by irregularly-shaped accumulations of finely-grained material. Two morphological types of prokaryotic endocytobionts present in cytoplasm. Nuclei 2−10 in number, 30−35 μm in diameter. Accumulations of irregularly-shaped finely-grained material present along nuclear periphery. Short microtubules, about 100 nm in length, radiating from nuclear envelope into cytoplasm at equal distance from each other.

Differential diagnosis. *P. tarda* differs from the other known species of the genus *Pelomyxa* by the structure of the nuclei and the organization of reserve glycogen.

Acknowledgments

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Fig. 7. *P. tarda*, figures made by A. Gruber (1887). A − General view of the cell, B − enlarged area, nucleus and "food particles". *Abbreviations*: k − "Körnchen" (granules), n − "Kern" (nucleus), Nr − "Nahrung" (food). From: Gruber, 1887.

ratus may be well-developed (e.g., in *P. flava, P. prima, P. gruberi, P. paradoxa*) or almost entirely reduced (e.g., in *P. stagnalis, P. palustris*) (Frolov et al., 2005, 2006, 2007, 2011; Chistyakova et al., 2010, 2014). In *P. tarda*, no flagella were detected. Electron micrographs of *P. tarda* cells revealed only short, spaced equally apart microtubules radiating from the nuclear envelope, which apparently serves as a MTOC. The immunofluorescence staining confirmed this pattern of organization of microtubules, although their length has not allowed to recognize them as separate linear structures. The localization of microtubules in the perinuclear zone has been previously noted in several *Pelomyxa* spp, for instance, in *P. secunda* (Berdieva et al., 2015). However, in this species, in contrast to *P. tarda*, short microtubules are arranged around the nucleus not in one but in two or even three layers (Berdieva et al., 2015). Short regularly arranged microtubules radiating from the nuclear envelope were also found in *P. flava* (Frolov et al., 2011). However, in this organism, a layer of ER vesicles is located outside the layer of microtubules. Perinuclear microtubules have also been found in *P. prima, P. gruberi* and *P. belevskii,* but in these species they are much longer, are arranged chaotically around the nuclei, and no direct connection between the microtubules and the

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