Protistology 10 (1), 13–25 (2016)

Protistology

Diversity of symbiotic consortia of prokaryotes in the cells of pelomyxids (Archamoeba, Pelomyxidae)

Ludmila V. Chistyakova[1], Mariia A. Berdieva[2], Alexey Y. Kostygov[3,4] and Alexander O. Frolov[3]

[1] *Core Facility Centre "Culturing of microorganisms", St. Petersburg State University, Botanycheskaya str., St. Petersburg, Russia*
[2] *Institute of Cytology, Russian Academy of Sciences, St. Petersburg, Russia*
[3] *Zoological Institute, Russian Academy of Sciences, St. Petersburg, Russia*
[4] *Life Science Research Centre, Faculty of Science, University of Ostrava, Ostrava, Czech Republic*

Summary

The family Pelomyxidae includes archamoebae of the genera *Mastigella* and *Pelomyxa*. In contrast to other archamoebae, they always contain prokaryotic cytobionts in their cytoplasm. According to the morphological analysis of the cytobionts in 11 pelomyxid species, we classified them into 4 basic types, namely: "Af", "Lt", "S3" and "S4". Being specific for each host species, these cytobionts form constant consortia which can be of two kinds: binary and ternary. Only "Af" cytobionts are common for all pelomyxids. These are slim rod-shaped Gram-positive bacteria autofluorescing in UV. They have electron-dense cytoplasm and distinctive pointed cell ends. We found population of pelomyxae *P. belevskii* (Lt-) that have only "Af" cytobionts in the cytoplasm. Since these bacteria are found in all studied representatives of Pelomyxidae – both in *Mastigella* and *Pelomyxa* – this may indicate the early origin of such symbiosis, presumably associated with the separation of the group from the common stem of archamoebae. The presence of at least two shared cytobionts ("Af" and "Lt") in the prokaryotic consortia of *Mastigella* and *Pelomyxa* serves as an additional evidence in favour of possible phylogenetic proximity of these two archamoebal groups.

Key words: archamoebae, cytobionts, *Mastigella, Pelomyxa*, prokaryotic consortia, phylogeny

Introduction

Archamoebae are free-living and endobiotic amoebozoans inhabiting anaerobic and microaerobic areas. In the course of their evolution, these protists have lost a series of basic eukaryotic organelles such as mitochondria, peroxisomes and Golgi apparatus. Meanwhile, most of them possess more or less developed flagellar apparatus (Brugerolle, 1982; Chávez et al., 1986; Griffin,

1988; Simpson et al., 1997; Walker et al., 2001; Frolov, 2011; Chystyakova et al., 2012; Ptáčková et al., 2013; Zadrobílková et al., 2015a, 2015b). Molecular data that may shed light on the origin and evolution of archamoebae are still limited and contradictory. Nevertheless, multigene and single-gene phylogenetic analyses demonstrate monophyly of Archamoebae (Fiore-Donno et al., 2010; Lahr et al., 2011; Ptáčková et al., 2013; Cavalier-Smith et al., 2015; Zadrobílková et al., 2015a, 2015b).

As demonstrated recently, the family Pelomyxidae includes two genera of Archamoebae: *Mastigella* Frenzel 1897 and *Pelomyxa* Greeff 1874; phylogenetic relationships of these two genera are supported by results of the analysis of 18S rRNA and actin genes (Zadrobílková et al., 2015a). Meanwhile, no apparent synapomorphies in this group can be found (Frolov, 2011; Zadrobílková et al., 2015a). Representatives of the genus *Mastigella* are small to medium-sized amoebae, uninuclear and uniflagellar throughout the major part of the life cycle, using both amoeboid and flagellar types of locomotion. Species of the genus *Pelomyxa* are medium to large sized amoebae, mostly multinuclear and multiflagellar, which use only amoeboid type of movement (Frolov, 2011). In the absence of morphological evidence supporting the concept of the family Pelomyxidae the attention should be paid to the fact that the representatives of both genera, as contrasted to other studied archamoebae, form stable symbiotic associations with prokaryotes (Leiner and Bhowmick, 1967; Chapman-Andresen, 1971; Whatley, 1976; van Bruggen et al., 1983, 1985, 1988; Whatley and Chapman-Andresen, 1990; Cavalier-Smith, 1991; Walker et al., 2001; Frolov, 2011). Despite plenty of confirming articles, numerous contradictions between them hinder estimation of the real diversity and composition of prokaryotic consortia found in Pelomyxidae. Here we performed morphological studies of prokaryotes revealed in ten species of *Pelomyxa* and one species of *Mastigella*. The obtained results allow to conclude about quantitative and qualitative composition of the discovered consortia and open a perspective of their further investigation using molecular methods.

Material and methods

Archamoebae were collected throughout all seasons of years 2011-2015 in water bodies of Pskov region near the village Lyady (58°35'N; 28°55'E), in Sergievka park in St. Petersburg (59°53'31"N; 29°50'10"E), and at the Karelian Isthmus near the village Orekhovo (60°30'38"N; 30°16'08"E). These water bodies are up to 330 km distant from each other; they have no connections and are of different types. The Afanasov pond near the village Lyady is a marshy one surrounded by willow thickets. *Ceratophyllum* pond in Sergievka park is a part of the cultural landscape of Stariy Peterhof. The lake Osinovskoe near Orekhovo is of glacial origin, it has 700 m in diameter and shores covered with coniferous trees.

Accumulation cultures were preserved at 5 °C in plastic vials of 0.3-0.5 L. The selection of cells and preliminary identification of archamoebae was performed under the stereomicroscope Leica M125 in the water from original samples placed in Petri dishes. Morphological studies were completed with the use of the light microscope Leica DM2500 equipped with fluorescence module. Autofluorescence in bacteria was detected using the combined filter BGR-Filter Cube (Leica). Counting of symbionts in archamoebae was performed for 15 specimens of each species (5 from each of the 3 groups of water bodies, see above). The cells of amoebae were disrupted in distilled water with glass homogenizer. The resulting suspension was placed into Goryaev chamber and explored with the use of 40× phase contrast objective. Morphologically similar G+ and G-rod-shaped cytobionts of *P. palustris* were counted together. Their actual numbers were calculated according to the ratio of these bacteria on the Gram-stained glass slides prepared from the same material. All measurements and statistical analyses were performed using Image Tool for Windows v. 3.0 (UTHSCSA). Digital photos were taken with Leica DM 2500 microscope with 14 Mpx USB camera UCMOS14000KPA (TOUPCAM). Material for electron microscopy was prepared as described earlier (Frolov et al., 2005a, 2006) and studied using microscopes TeslaBS500 and Zeiss Libra 120.

Results

Eleven species of Archamoebae were studied: *Mastigella nitens, Pelomyxa belevskii, P. stagnalis, P. secunda, P. flava, P. palustris, P. prima, P. binucleata, P. gruberi, P. paradoxa* and *P. corona* (Fig. 1 A-K). These protists are widely distributed and abundant in water bodies of the Northwest Russia. They can be preserved in accumulation cultures for one year or longer.

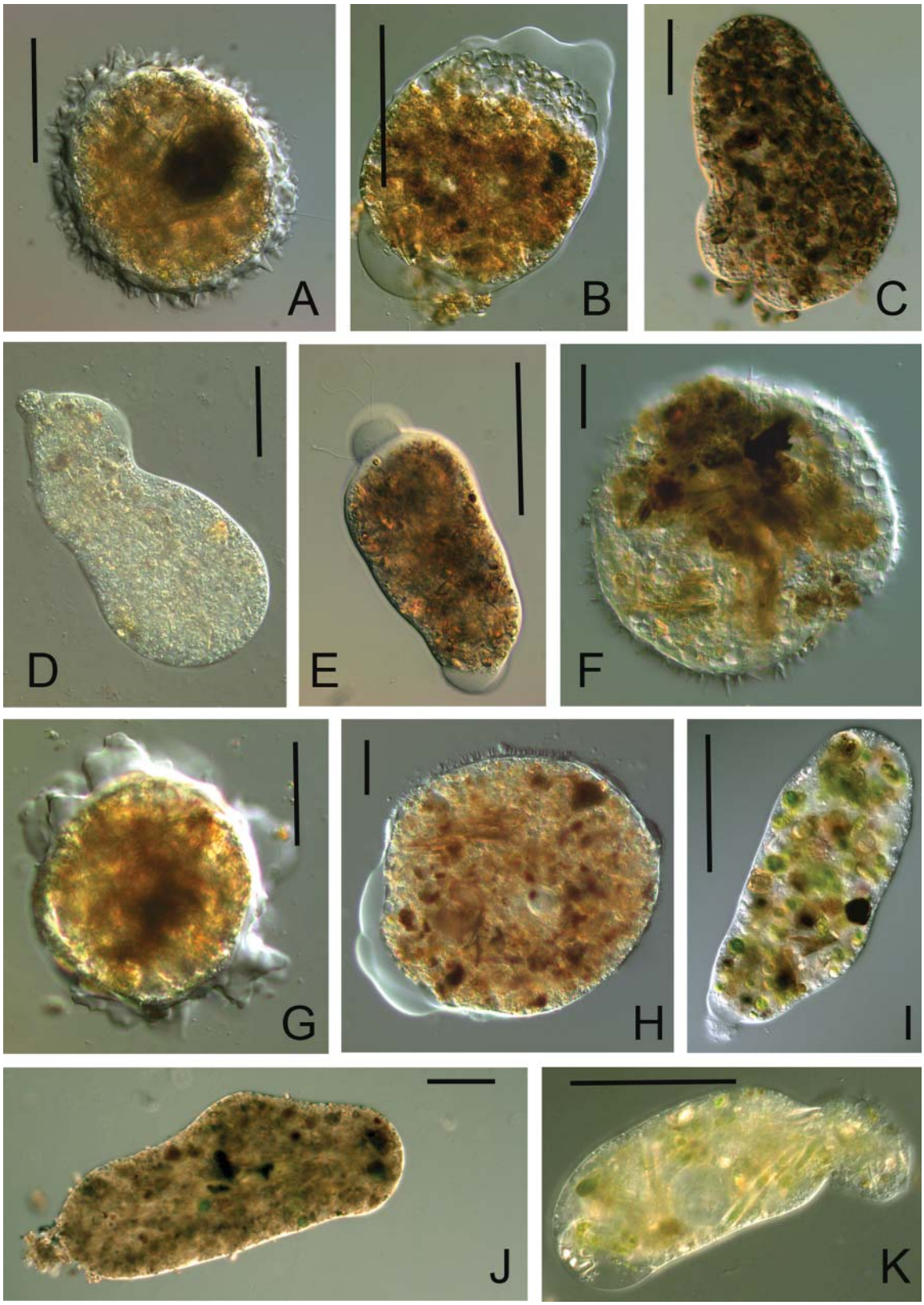

Fig. 1. Morphology of pelomyxids isolated in this study. A, *Mastigella nitens*; B, *Pelomyxa prima*; C, *P. stagnalis*; D, *P. secunda*; E, *P. paradoxa;* F, *Lt- strain of P. belevskii;* G, *P. corona*; H, *P. gruberi*; I, *P. binucleata*; J, *P. palustris*; K, *P. flava*. Scale bars: A – 20 μm, B-K – 100 μm.

Table 1. Morphological characters of prokaryotic cytobionts of pelomyxids.

Main types of cytobionts	Auto fluorescence at 350-420 nm	Gram-staining	Longitudinal ridges of the cell wall	"Cleft" of the cell wall	Length/Width, μm
Af	+	+	–	–	2.0–5.0/0,3–0,4
Lt	–	–	–	+	3.0–5.0/0,7–0,9
Sb3	–	+/–	+	–	1.1–1.5/0.5–0.6
Sb4	–	–	+	–	2.5–5.0/0.5–0.6

The composition of cytobionts in archamoebae cells revealed to be constant and species-specific (Fig. 2 A-P). There were no differences between organisms inspected immediately after sampling and those preserved in accumulation cultures for several months. Intact amoebae demonstrated prokaryotic symbionts in the cytoplasm, where the latter were localized in the space between structural and digestive vacuoles (Fig. 2 A). Sometimes they aggregated around glycogen granules or host nuclei (Fig. 2 B-D). According to the most general morphological characters, such as cell length, width and shape, details of ultrastructure, autofluorescence ability and results of Gram staining, four main types of cytobionts can be distinguished: "Af", "Lt", "S3" and "S4" (Table 1, Figs 2, 4).

"Af" cytobionts

Thin rod-shaped bacteria with slightly tapered ends and dense cytoplasm, frequently forming chains of individuals (Figs 2 E-P; 4 A, C). They are Gram-positive (Fig. 3 A, H-L, N) and exhibit autofluorescence (the name "Af" reflects this fact) at wavelengths 350-420 nm (Fig. 3 B-G). The size of bacteria may vary even within one host cell from 2 to 5 μm in length and 0.3-0.4 μm in diameter. These bacteria are located in the individual vacuoles inside the host cell cytoplasm. The vacuolar membrane fits tightly to the bacterial cell wall, and it is bordered by a coat of microfilaments from the outside (Fig. 4 A, B). In "Af" cytobionts from *P. gruberi* and *P. prima*, such microfilaments were not detected. Multinuclear trophozoites of *P. belevskii, P. stagnalis, P. secunda, P. palustris, P. prima, P. binucleata, P. gruberi* and *P. paradoxa* have these bacteria distributed throughout the entire cytoplasm (Fig. 3 E). However, "Af" cytobionts are localized in uninuclear cells of *P. binucleata, P. gruberi* and *P. corona* as well as in all developmental stages of *Mastigella nitens* around the nuclei (Figs 2 C; 3 F, G; 4 K). In *P. flava*, this type of cytobionts forms separate clusters distributed in the host cell cytoplasm (Fig. 3 D).

We detected "Af" cytobionts in all studied *Pelomyxa* spp. as well as in *Mastigella nitens* (Table 1). Moreover, we discovered a population of *P. belevskii*, strain Lt- (Fig. 1 F) in the Afanasov pond (Pskov region), a significant part of which (47%) was represented by amoebae containing only "Af" cytobionts in the cytoplasm (Figs 2 F; 3 A, B). The number of cytobionts of this type in all studied pelomyxae depended on the developmental stage (which correlated with the size) of host cell (Table 2). For example, there were on average 5×10^4 "Af" cytobionts per cell in the cysts of *P. palustris*, whereas in large and medium size trophozoites of this species the number of these bacteria reached up to 7×10^5. A similar situation was observed in *P. belevskii* and *P. gruberi* (see Table 2). Interestingly, the Lt- strain of *P. belevskii* kept only "Af" cytobionts and contained virtually the same number of them as the "normal" strain with two types of prokaryotic cells in the cytoplasm (Table 2).

"Lt" cytobionts

Large rod-shaped prokaryotes (Fig. 2 E, G–M), being Gram-negative (Fig. 3 H–K, M, N) and not exhibiting autofluorescence in UV (Fig. 3 C). The designation refers to the size of these symbionts ("Lt" stands for "large type"). These cells are 0.7–0.9 μm in diameter and 3–5 μm long, although they can be longer sometimes (up to 7 μm and more), apparently due to unfinished division. They frequently form chains of several individuals (Fig. 3 K). Transverse section profiles of these bacteria demonstrate different shapes: from rounded to almost square ones (Fig. 4 D). Characteristic trait of "Lt" cytobionts is the longitudinal invagination (cleft) of the cell wall (Fig. 4 C, D). Sometimes this cleft extends along almost the whole length of the bacterium and may reach the center of the cell

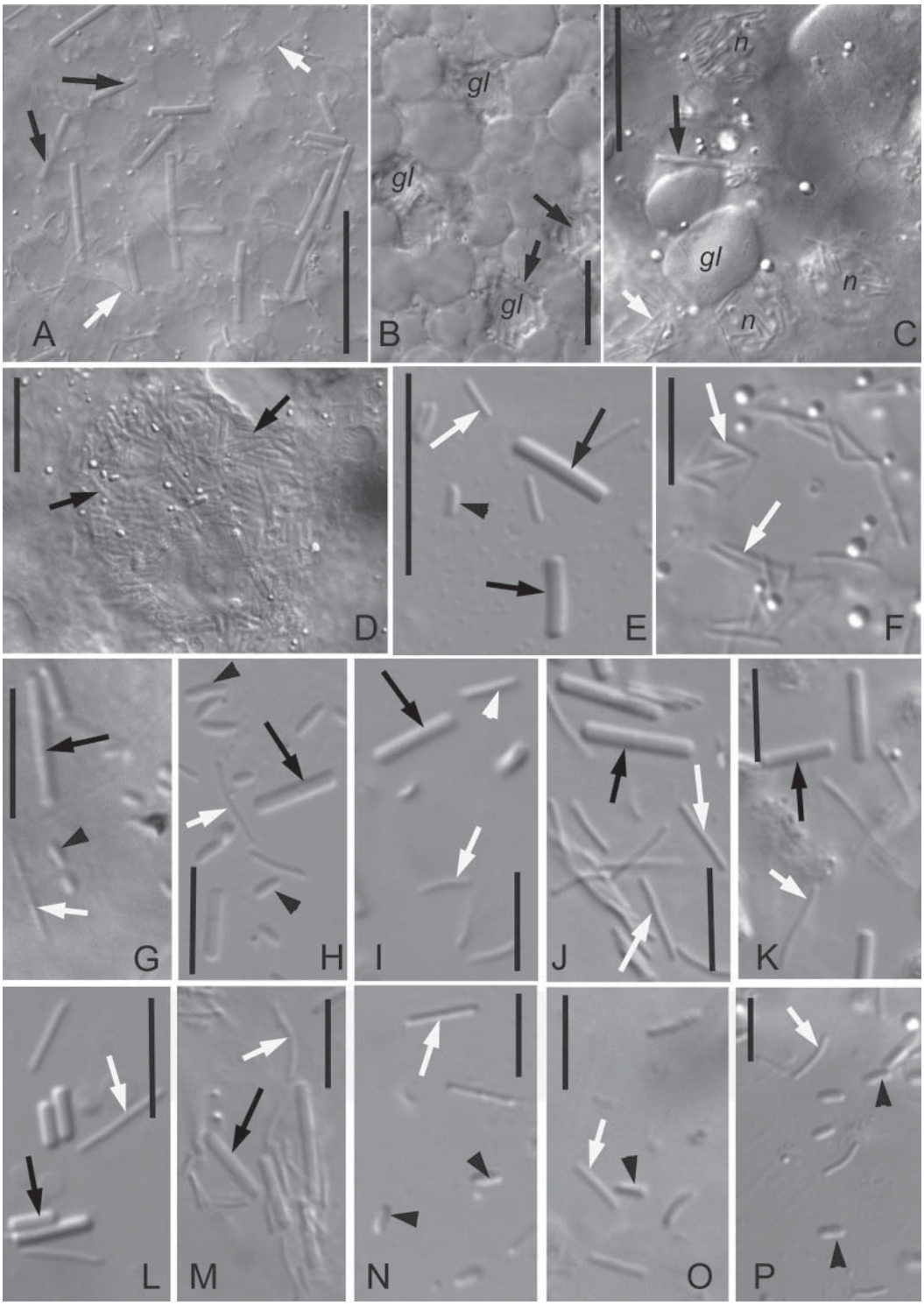

Fig. 2. Prokaryotic cytobionts of pelomyxids (light microscopy, DIC). A, "Af" and "Lt" symbionts in the cytoplasm of *P. belevskii*; B, "Lt" cytobionts around glycogen bodies of *P. palustris*; C, "Af" cells near the surface of *P. corona* nuclei; D, cytobionts near the surface of the nucleus of *Mastigella nitens*; E–P, diversity of prokaryotic consortia of pelomyxids; E, *Mastigella nitens*; F, *P. belevskii* (Lt-); G, *P. binucleata*; H, *P. flava*; I, *P. palustris*; J, *P. prima*; K, *P. gruberi*; L, *P. belevskii*; M, *P. corona*; N, *P. stagnalis*; O, *P. secunda*; P, *P. paradoxa. White arrows* – "Af" cytobionts; *black arrows* – "Lt" cytobionts; *black arrowheads* – "S3" cytobionts; *white arrowheads* – "S4" cytobionts. *Abbreviations*: gl – glycogen granules; n, – nuclei. Scale bars: A, D-F – 10 μm; B, C – 20 μm, G-P – 5 μm.

Table 2. The abundance of prokaryotic cytobionts in the cells of some pelomyxids (number of individuals per host cell).

Main types of cytobionts / Pelomyxids	AF	Lt	S4
Pelomyxa belevskii ~ 300x500 μm	$1.5x10^5 \pm 0.5x10^5$	$0.8x10^5 \pm 0.2x10^5$	–
P. belevskii ~700x800 μm	$3.9x10^5 \pm 0.4x10^5$	$2.1x10^5 \pm 0.2x10^5$	–
P. belevskii (Lt-) ~ 700x800 μm	$3.6x10^5 \pm 0.3x10^5$	–	–
P. flava ~ 200x300 μm	$2.8x10^4 \pm 0.2x10^4$	$1.5x10^4 \pm 0.1x10^4$	–
P. palustris > 2x1 mm	$0.7x10^6 \pm 0.9x10^5$	$1.1x10^6 \pm 0.1x10^6$	$0.1x10^6 \pm 0.9x10^5$
P. palustris cysts	$0.3x10^5 \pm 0.1x10^5$	$0.3x10^5 \pm 0.05x10^5$	$0.3x10^5 \pm 0.1x10^5$
P. gruberi ~ 200x200 μm	$0.6x10^5 \pm 0.2x10^5$	$0.9x10^4 \pm 0.1x10^4$	–
P. gruberi ~ 300x300 μm	$0.9x10^5 \pm 0.1x10^5$	$1.2x10^4 \pm 0.1x10^4$	–

when observed on transverse section (Fig. 4 D). In the host cytoplasm, "Lt" cytobionts are localized in the individual vacuoles. Usually they are situated in the free space between the structural and digestive vacuoles and move together with the cytoplasmic streaming (Fig. 2 A). However, "Lt" cytobionts are frequently concentrated around glycogen granules in *P. palustris* (Fig. 2 B), whereas aggregation around nuclei is typical of the majority of *Pelomyxa* spp. and of *Mastigella nitens* (Figs 2 D; 4 K).

"Lt" symbionts were found in the cells of 7 out 10 studied species of pelomyxae: (*P. palustris, P. belevskii, P. prima, P. binucleata, P. flava, P. gruberi* and *P. corona*) as well as in *Mastigella nitens* (Table 1, Fig. 2 E, G–M). They were absent only in *P. stagnalis, P. secunda, P. paradoxa* and Lt - strain of *P. belevskii* (Fig. 2 F, N–P). As in the case of "Af" bacteria, the number of "Lt" bacteria increased as host cells grew (Table 2). It is noteworthy that "Lt" cytobionts were less abundant than those of "Af" type in the host cells. *P. palustris* was the only exception: all symbionts were found in approximately equal quantities in cysts, whereas in the giant trophozoites the "Lt" bacteria were several-fold more numerous than "Af" ones (Table 2).

"S3" CYTOBIONTS

Small rod-shaped bacteria usually paired in head-to-tail manner (Figs 2 E, G, H, N–P; 4 G–J). They are 0.5–0.6 μm in diameter and 1.1–1.5 μm long (pairs are sized 2.0–2.5 μm). Gram-staining produces variable results, but both cell poles are most frequently stained with gentian and the rest - with safranin (Fig. 3 H, L, M). These symbionts exhibit no autofluorescence at 350–420 nm wavelengths (data not shown). Their cell wall forms numerous longitudinal folds and ridges that prevent adjoining of the symbiontophorous vacuole membrane to the bacterial cell surface as contrasted to the case of "Af" cytobionts (Fig. 4 G–J). The cytoplasm of these bacteria has medium electron density, with more or less conspicuous nucleoid zone (Fig. 4 G–J). The cytobionts are localized in individual vacuoles and do not form separate clusters. When other cytobionts are concentrated near the nuclear envelope, these are found in the common aggregate (Fig. 4 K). "S3" symbionts were found in 5 out 10 studied species of pelomyxae: *P. stagnalis, P. secunda, P. flava, P. binucleata, P. paradoxa*, as well as in *Mastigella nitens* (Fig. 2 E, G, H, N–P). The number of "S3" symbionts was not determined.

"S4" CYTOBIONTS

Thin rod-shaped bacteria with rounded ends, cytoplasm of medium electron density and cell wall forming multiple pointed ridges (Figs 2 I; 4 E, F). These cytobionts are Gram-negative (Fig. 3 N), 0.5–0.6 μm in diameter and 2.5–5.0 μm long. Sometimes "S4" bacteria can form chains composed of several individuals. They are localized in the individual vacuoles and usually surround host nuclei, forming contacts with nuclear envelope. These cytobionts were found only in P. palustris. During the host cell growth from the cyst to the mature trophozoite the quantity of "S4" cytobionts increases, although much slower than those of "Af" and "Lt" bacteria (Table 2).

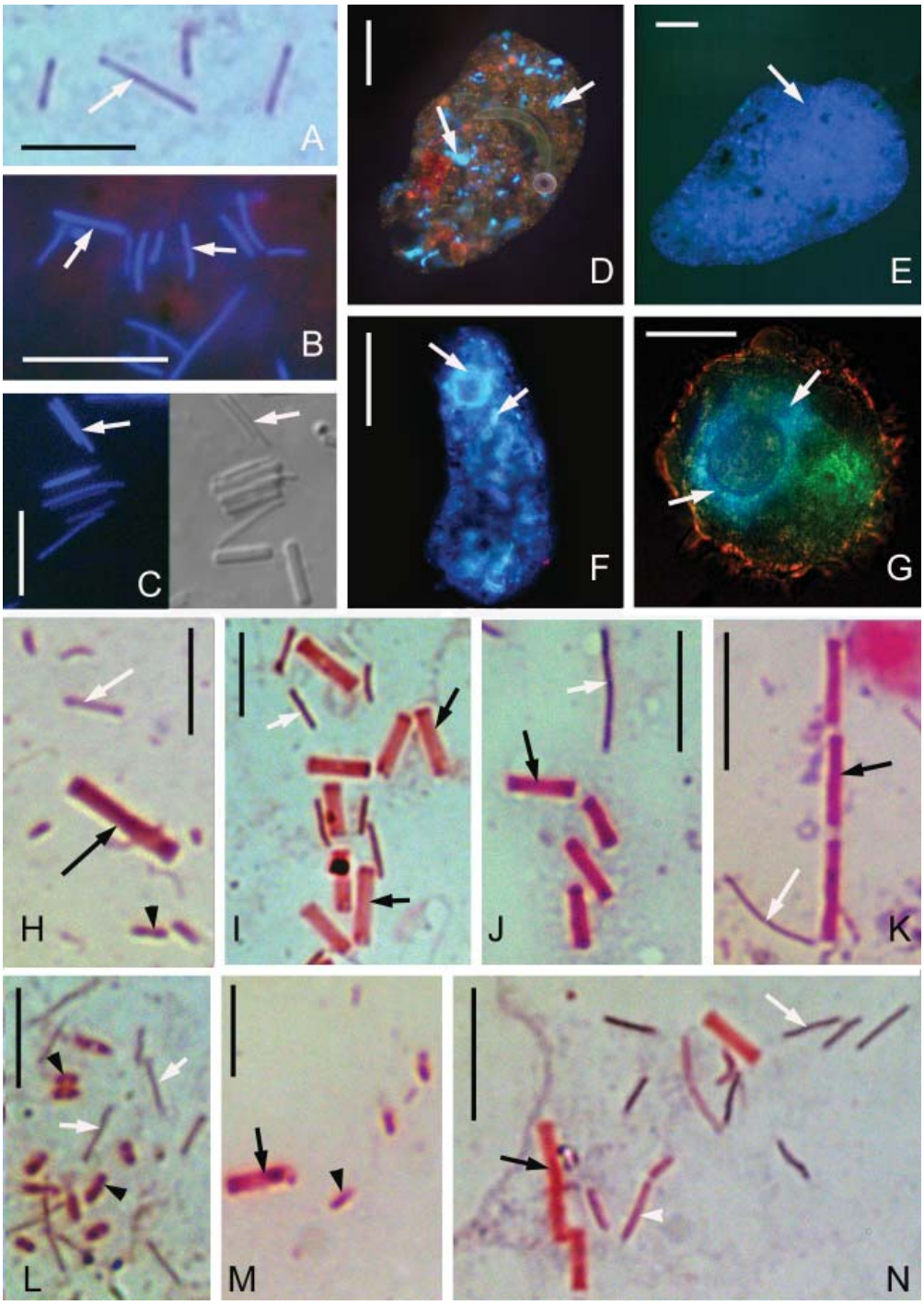

Fig. 3. Prokaryotic cytobionts of pelomyxids. A, H–N, light microscopy, Gram staining; B ,G, autofluorescence in UV; C - autofluorescence in UV and DIC of the same region; A, B, "Af" cytobionts of *P. belevskii*, strain Lt-; C, "Af" cytobionts of *P. prima* (autofluorescence photo and DIC juxtaposed); D, separate clusters of "Af" cytobionts in the cytoplasm of *P. flava*; E, uniform distribution of "Af" cells in the cytoplasm of *P. flava*; B, concentration of some "Af" cytobionts around the nucleus of uninuclear *P. gruberi*; G, concentration of "Af" cytobionts around the nucleus of *Mastigella nitens*; H, ternary consortium of prokaryotic cytobionts in *M. nitens*; I, binary consortium in *P. belevskii*; J, binary consortium in *P. corona*; K, binary consortium in *P. gruberi*; L, binary consortium in *P. stagnalis*; M, cytobionts of *P. flava* ("Af" cells are not shown); N, ternary consortium in *P. palustris*. Labels are the same as on the Fig. 2. Scale bars: A-C – 5 μm, D-F – 50 μm; G – 20 μm; H-N – 5 μm.

The analysis of the diversity of prokaryotic cytobionts in the studied species of archamoebae revealed the presence of strictly constant consortia of microorganisms (Table 3). The composition of each consortium is specific for the archamoebal species and does not depend on such varying parameters as developmental stage of the host, season, locality, and water body type. Our recent discovery of Lt- strain of *P. belevskii* which contains only "Af" cytobionts points to the potential loss of one of the consortium components. However, this does not affect the earlier conclusion as a whole. We can distinguish two main types of the studied associations of archamoebal cytobionts according to their quantitative composition: binary and ternary ones (Table 3). Binary consortia were found in 7 species of pelomyxae. They were composed either of "Af" + "Lt" (*P. belevskii, P. prima, P. gruberi* and *P. corona*) or "Af" + "S3" cytobionts (*P. stagnalis, P. secunda* and *P. paradoxa*). Ternary prokaryotic associations were found in 4 archamoebal species: "Af" + "Lt" + "S4" in *P. palustris* and "Af" + "Lt" + "S3" in *Mastigella nitens, P. binucleata* and *P. flava*.

Discussion

Archamoebae are a very complicated group of amoeboid protists. These organisms are either refractory to laboratory cultivation or uncultivated at all. The majority of them are very difficult to identify because of the absence of significant differential characters. This is one of the reasons for the plenty of contradictory and unconfirmed data in the scientific literature dedicated to the group. Especially it concerns the papers published in the second half of the last century – the period of intensive morphological studies of protists (reviewed in: Goodkov et al., 2004; Frolov, 2011). Application of molecular phylogenetic methods made a breakthrough in the study of the group in the last two decades (Milyutina et al., 2001; Fiore-Donno et al., 2010; Lahr et al., 2011; Ptáčková et al., 2013; Cavalier-Smith et al., 2015; Zadrobílková et al., 2015a, 2015b). However, the researchers have continuously experienced significant difficulties when attempting to interpret the results of their predecessors, who had been working at the time of morphology domination. Our study of the diversity of cytobionts and their associations in the cells of pelomyxids aimed to solve the issues mentioned above. It is evident that the determination of taxonomic composition of prokaryotic consortia is possible only with the use of molecular data. Therefore, we consider our investigation as a preliminary but indispensable stage of such studies.

Our predecessors studying prokaryotic symbionts of pelomyxae believed that *Pelomyxa palustris* Greeff 1874 was the only species in the genus (Leiner and Bhowmick, 1967; Daniels and Breyer, 1967; Chapman-Andresen, 1971; van Bruggen et al., 1983, 1988; Whatley and Chapman-Andresen, 1990). The dissimilarities in morphology and composition of cytobionts of different specimens of "*P. palustris*" were regarded as either individual traits of particular strains of "*P. palustris*", or variability of bacteria, or were ignored at all (Leiner and Bhowmick, 1967; Daniels and Breyer, 1967; Chapman-Andresen, 1971; Whatley, 1976; Whatley and Chapman-Andresen, 1990). However, the studies performed in the 21st century have demonstrated that the genus *Pelomyxa* encompasses more than 10 species of amoebae differing in morphology and composition of symbiotic consortia within them (Frolov et al., 2004, 2005a, 2005b, 2006; Chistyakova and Frolov, 2011; Frolov, 2011; Chistyakova et al., 2014; Berdieva et al., 2015; Zadrobílková et al., 2015a). The diversity of pelomyxae discovered in morphological studies was confirmed by phylogenetic reconstructions using 18S rRNA gene (Ptáčková et al., 2013; Zadrobílková et al., 2015a). Now it is impossible to determine which species were investigated in the works of the second half of the 20th century. Thus, in most cases those results are useless for the analysis of the composition and diversity of symbiotic consortia in pelomyxae. Besides this problem, we revealed a series of significant contradictions. According to different authors, the number of morphologically distinguishable prokaryotic cytobionts in *P. palustris* varies from 1 to 3 (Daniels and Breyer, 1967; Leiner and Bhowmick, 1967; Chapman-Andresen, 1971). The presence of 2 or 3 symbionts in different specimens of "*P. palustris*" is quite understandable since the researchers apparently studied not a single species, as they believed, but various ones. In this aspect, one article in which the authors described 2 types of cytobionts of "*P. palustris*" is very indicative (Daniels and Breyer, 1967). Their images demonstrate nuclei of amoebae, thought to be at different stages of division. As a matter of fact, besides *P. palustris* (Fig. 1, 2 in: Daniels and Breyer, 1967) the authors also used other species of pelomyxae, in particular, *P. stagnalis* (Fig. 16–18 in: Daniels and Breyer, 1967; compare with Fig. 4 in: Chistyakova and Frolov, 2011). Interestingly,

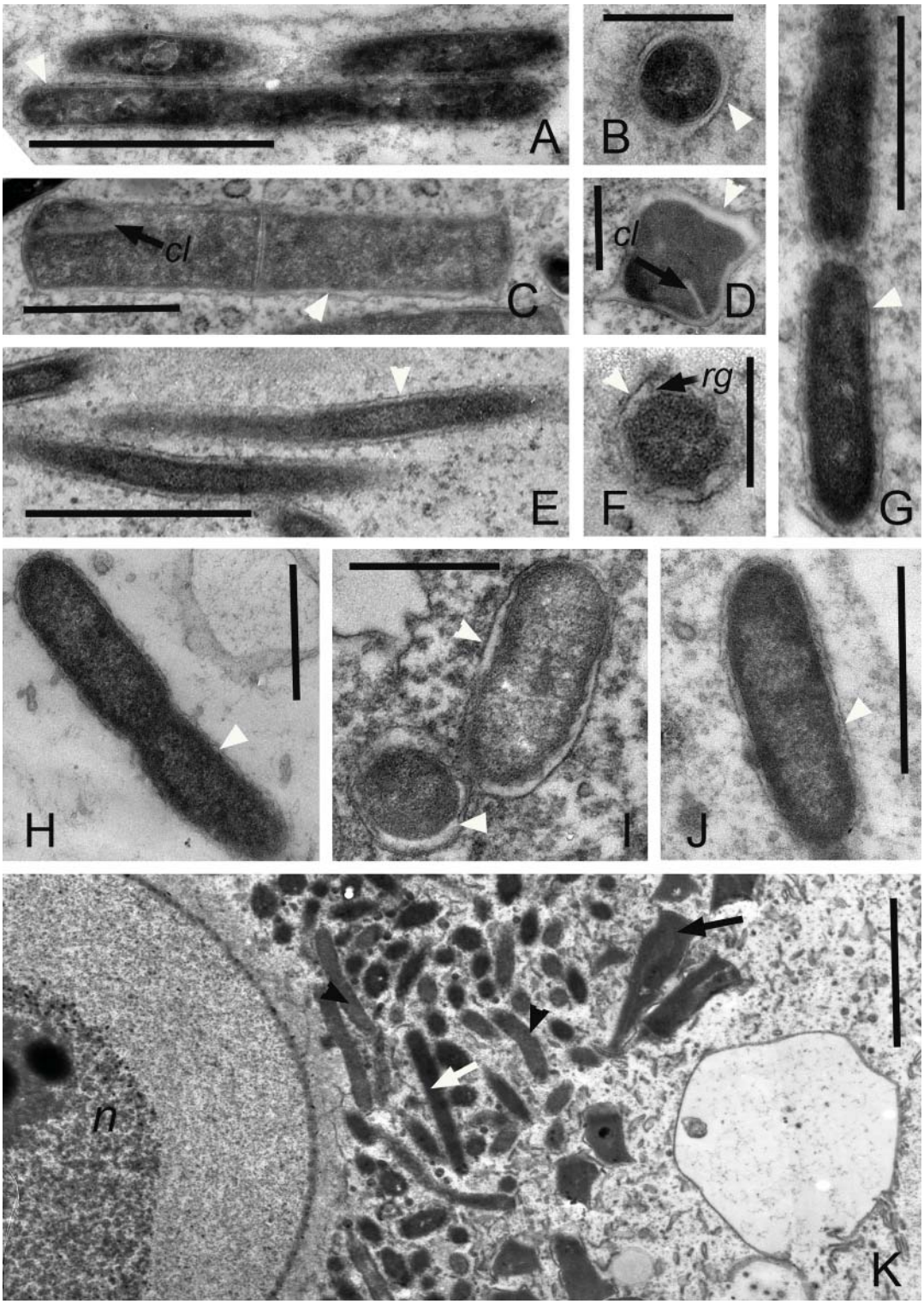

Fig. 4. Ultrastructure of cytobionts of pelomyxids. A, C, "Af" cytobionts of *P. flava*; B, D – "Lt" cytobionts of *P. palustris*; E, F, "S4" cells in *P. palustris*; G, "S3" cytobionts of *Mastigella nitens*; H, "S3" cells in *P. flava*; I, "S3" cytobionts of *P. secunda*; J, "S3" cytobionts of *P. stagnalis*; K, "Af", "Lt" and "S3" cells around the nucleus of *P. binucleata*. *White arrows* – "Af" cytobionts; *black arrows* – "Lt" cytobionts; *black arrowheads* – "S3" cytobionts; *white arrowheads* – membrane of the symbiontophorous vacuole. *Abbreviations*: rg – ridges of the cell wall, cl – invagination of the cell wall. Scale bars: A, C, E – 2 μm; H, I, J, G – 1 μm; K – 5 μm.

Table 3. The composition of the prokaryotic consortia in Pelomyxidae.

Main types of cytobionts / Pelomyxids	Af	Lt	Sb3	Sb4
Binary consortia				
Pelomyxa belevskii	+	+	–	–
P. prima	+	+	–	–
P. corona	+	+	–	–
P. gruberi	+	+	–	–
P. stagnalis	+	–	+	–
P. secunda	+	–	+	–
P. paradoxa	+	–	+	–
Ternary consortia				
P. palustris	+	+	–	+
P. binucleata	+	+	+	–
P. flava	+	+	+	–
Mastigella nitens	+	+	+	–
One cytobiont				
P. belevskii strain Lt(–)	+	–	–	–

the formal resemblance between *P. palustris* and *P. stagnalis* had led to the similar confusion when the first pelomyxan 18S rRNA gene sequence was obtained several decades later (Milyutina et al., 2001). This sequence was revealed to belong to P. stagnalis twelve years later (Ptáčková et al., 2013).

Leiner and Bhowmick (1967) who studied cytobionts on Gram-stained slides made an assumption that *P. palustris* possessed only one species of symbiotic bacteria. They considered "large rods" to be a product of the fusion of "slender" ones (Leiner and Bhowmick, 1967). To the point, we observed such situation on electron microscopy images (Fig. 4 A). Meanwhile, those researchers reported that thin rods were always Gram-positive, whereas large ones might have been positive, negative and variable in relation to Gram staining. Afterwards, the single-symbiont idea was subjected to valid criticism (Chapman-Andresen, 1971) and was not adopted. However, this interpretation of the results of "large" bacteria staining evoked confusion in the subsequent literature, and one can virtually find all three variants from the original paper by Leiner and Bhowmick (1967). These cytobionts were referred to as "large bacterium, usually Gram-negative" (Chapman-Andresen, 1971), «Gram-positive thick-type endosymbiotic bacteria» (van Bruggen et al., 1983, 1985, 1988) and «Gram-variable» (Whatley and Chapman-Andresen, 1990). We accomplished special investigation in order to solve this contradiction and revealed that in all 11 studied species of archamoebae the «large-type» bacteria were Gram-negative (Table 1).

Here we demonstrated the existence of at least 4 types of prokaryotic cytobionts, which are present in the cytoplasm of different pelomyxid species in various combinations. Three of them, namely "Af", "Lt" and "S4" bacteria can be reliably identified by a set of characteristic features.

"Af" cytobionts were found in all pelomyxids studied to date (van Bruggen et al., 1985, 1988; Whatley and Chapman-Andresen, 1990; Frolov et al., 2004, 2005a, 2005b, 2006, 2011; Chistyakova and Frolov, 2011; Chistyakova et al., 2014; Berdieva et al., 2015). These bacteria exhibit autofluorescence and are always Gram-positive. They are of similar sizes, alike in morphology, and have characteristic conical ends. Previously, van Bruggen et al. (1988) who had isolated these cytobionts from *P. palustris* identified them as methanogenic archaea *Methanobacterium formicicum*. Later on, the "Af" cytobionts were described under this name from a number of pelomyxids in a series of publications (Frolov et al., 2005a, 2005b, 2006, 2011; Chistyakova and Frolov, 2011; Frolov, 2011). However, recently the *M. formicicum* genome was sequenced using the strain initially isolated by van Bruggen et al. (Gutiérrez, 2012). This genome did not show any signs of reduction as compared to those of related bona fide free-living methanogens. Given also the fact that this microorganism can be easily maintained *in vitro* it is very likely that *M. formicicum*

is a free-living archaeon rather than a symbiont of pelomyxae (Gutiérrez, 2012). Thus, the question concerning the taxonomical assignment of "Af" cytobionts is still open.

"Lt" cytobionts were described from 7 *Pelomyxa* spp. and 2 *Mastigella* spp. (van Bruggen et al., 1985, 1988; Whatley and Chapman-Andresen, 1990; Frolov et al., 2004, 2005a, 2005b, 2006, 2007, 2011). In all pelomyxids they were of similar size and had a characteristic cell shape. These cytobionts are Gram-negative, do not exhibit autofluorescence and possess a unique feature – a deep longitudinal cleft, which may reach the centre of the bacterial cell.

"S4" cytobionts were found only in *P. palustris* (Whatley and Chapman-Andresen, 1990; Frolov, 2011). They are Gram-negative, do not exhibit autofluorescence and have rounded ends as well as longitudinal folds of the cell surface.

We assume that each of these three groups of cytobionts may be represented in different pelomyxids by several related species of prokaryotes.

The group "S3" includes the smallest bacteria, being members of prokaryotic consortia in 7 of 11 species of pelomyxids studied by us (Chistyakova and Frolov, 2011; Frolov et al., 2011; Chistyakova et al., 2014; Berdieva et al., 2015). Besides the size similarity, they are alike in the mixed type of Gram-staining results ("Gram-variability"). All of them exhibit no autofluorescence and have longitudinal folds of the cell wall. It is very likely that this group is artificial and will be split up during future research. Nevertheless, in the present study it was reasonable to consider these cytobionts as one group in order not to describe too many "unique" consortia. The methods we used in this study restricted our ability to somehow subdivide these bacteria. However, even if the future studies demonstrate that joining these cytobionts together is incorrect, it will not affect our main conclusions, since it will change only the idea about the general diversity of prokaryotic consortia in pelomyxids.

According to our results, the "Af" cytobionts represent the only common component in all types of described consortia (Table 3). Other symbionts of pelomyxids may either substitute ("Af" + "Lt" versus "Af" + "S3") or complement each other ("Af" + "Lt" + "S3" and "Af" + "Lt" + "S4"). Given these facts and the observation that *P. belevskii* Lt- strain exists normally with "Af" cytobionts only, it is logical to assume that the colonization of archamoebal cells by "S3", "S4" and "Lt" prokaryotes is secondary. In other words, these microorganisms apparently have joined an already well-established symbiotic system. The presence of "Af" cytobionts in all studied species of *Mastigella* and *Pelomyxa* points to the early origin of this symbiosis, probably coinciding in time with the rise of the family Pelomyxidae. The sharing of at least two cytobionts (namely "Af" and "Lt") in the prokaryotic consortia of the above two genera is an additional evidence supporting their phylogenetic relatedness (Zadrobílková et al., 2015a).

Acknowledgements

This work was supported by the grant RFBR 15-04-00396_a. Scientific research were performed at the Center for Culturing Collection of Microorganisms and "Chromas" Center of Research park of St. Petersburg State University.

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Address for correspondence: Alexey Y. Kostygov. Angliyskiy prosp. 32, 190121 St. Petersburg, Russia; e-mail: *kostygov@gmail.com*