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Light- and electron-microscopic study of *Pelomyxa binucleata* (Gruber, 1884) (Peloflagellatea, Pelobiontida)

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Summary

Morphology of a pelobiont *Pelomyxa binucleata* (Gruber, 1884) has been studied using light and electron microscopy. The organisation of *P. binucleata* has been shown to differ from that of *P. palustris, P. prima* and *P. corona*. The cell surface of *P. binucleata* is represented by the plasma membrane with a thin but distinct layer of non-structured glycocalyx. The ectoplasm, containing a network of fine fibrils, is separated from the endoplasm with a boundary layer of cisterns and reticulum channels. The flagella are poorly motile. The axoneme has a non-stable set of microtubules. A short kinetosome is about 200 nm long; the transition zone, also about 200 nm long, is situated above the cell border and contains a transition cylinder. Microtubular rootlet and microtubular cone are absent. From the kinetosome, almost in parallel to its longitudinal axis, 35-40 microtubules arranged in 3-4 rows start. Right below the kinetosome base they bend at a right angle to its longitudinal axis and pass further as a bundle in the ectoplasm below the cell surface. These microtubules do not penetrate into the endoplasm. The system of structural vacuoles is poorly developed. Glycogen bodies are rounded, smooth and surrounded with flattened reticulum cisterns. The set of endocytobionts includes two kinds of rod-like bacteria: thick bacteria with a poorly developed cleft and thin Gram-positive ones. The length of both kinds of bacteria may be 30 μm and more. The nuclear envelope bears a multilaminar layer on its outer surface. The nucleolus is fragmented and situated at the centre of the nucleus. The species composition of the genus *Pelomyxa* Greeff, 1874 is discussed in the context of revision of outdated ideas about its monotypy. Morphology of its representatives is considered in comparative aspect.

Key words: pelobionts, Peloflagellatea, Pelobiontida, *Pelomyxa binucleata, Pelomyxa palustris,* systematics, ultrastructure

Introduction

The present work continues our discussion "by correspondence" (Goodkov et al., 2004; Frolov et al., 2004; Frolov et al., 2005) with the followers of the concept postulating monotypy of the genus *Pelomyxa* Greeff 1874 (Griffin, 1988; Whatley and Chapman-Andresen, 1990; Brugerolle and Patterson, 2000). The bone of contention is, in fact, the only question: whether *Pelomyxa palustris* Greeff 1874 is a nominal species or a group of species erroneously united into a single one as polymorphic stages of its life cycle (Whatley and Chapman-Andresen, 1990).

The question under discussion is an old one. Throughout the 150-year-old history of study of *Pelomyxa*-like amoebae (hereafter, pelomyxoids), descriptions of new species of these organisms were more often than not followed by their reduction into synonyms of *P. palustris* (see Goodkov et al., 2004). This phenomenon culminated in the late XX century in a paper by Whatley and Chapman-Andresen (1990). Proposing a scheme of the *P. palustris* life cycle, the authors reserved in it a place for all pelomyxoid species, including those still "open" at that time. Though the description of the *P. palustris* life cycle was a significant breakthrough in studying the biology of this species, the above paper (Whatley and Chapman-Andresen, 1990) in fact effectively prevented further investigations of the pelomixoid amoebae fauna even at the light microscopic level. Basically, the authors arrive at four conclusions: 1. The cell size of pelomyxoids cannot be used as a diagnostic character, since the range of this character's variation in *P. palustris* at different life cycle stages (100 μm - 5 mm) includes, by default, all the variants possible. 2. The colour of pelomyxoids cannot be used as a diagnostic character, since it is determined by the qualitative composition of the food particles engulfed. 3. The number of nuclei cannot be used as a diagnostic character, since it changes regularly throughout the life cycle from one to two, then to dozens, hundreds and so on. 4. The size and morphology of the nuclei are constant in an individual but can change in the course of the life cycle and therefore cannot be used as a diagnostic character, either.

In our opinion, the organism the present work is focussed on occupies one of the key positions in this discussion. *Pelomyxa binucleata* (Gruber, 1884) was described by Gruber as *Amoeba binucleata* Gruber 1884 on the basis of all the four debatable groups of characters mentioned above (the size, the colour, the number and structure of nuclei). Transferred into the genus *Pelomyxa* by Penard (1902), this organism was investigated by Page (1981) who used Penard's stained slides. Noteworthy, on completion of his research, Page failed to reach a conclusion: "Again, one cannot say definitely whether *P. binucleata* is a distinct species or fits into the cycle of phenotypic change in *P. palustris*..." (Page, 1981, p. 30). A year later, however, C. Chapman-Andresen, a well-known researcher of *P. palustris*, summing up her investigations, confidently gave an answer to this question: "These observations on the origin and development of "*P. binucleata*" clearly show that this type of *Pelomyxa* is not a distinct species, but a stage in the life cycle of *P. palustris*" (Chapman-Andresen, 1982, p. 500). This idea was further developed in the above-mentioned paper (Whatley and Chapman-Andresen, 1990) and currently dominates the modern literature on the subject (Brugerolle and Patterson, 2000).

Material and methods

Organisms that we identified as *P. binucleata* are widely distributed in water bodies of North-western Russia. *Pelomyxa* amoebae used in the present investigation were obtained from samples of silt sediment of a small water body (S ~ 10 m^2) in a raised *Sphagnum* bog near the Sosnovo Village (Leningrad District, Russia; 60°30' N, 30°30' E). All attempts to establish *P. binucleata* in culture failed. However, the amoebae survived in samples for up to 9 months in 100-500 ml hermetically closed vessels at a temperature of app. 10° C. Live amoebae were investigated in closed microaquaria with a volume of 2.5 ml^3, connected by a running system with a 0.5 l vessel filled with water and silt from their habitat. The system was maintained at 10°C, being transferred to room temperature only for the observations.

Investigations were conducted with the use of Ergoval and Leika microscopes equipped with visualisation systems on the basis of Panasonic 650 CCTV and PC PIII. All measurements were made on live individuals with the image analysis system IT v.3.0 (UTHSCSA).

For electron microscopy amoebae were fixed with a cocktail of 5% glutaraldehyde and 0.5% OsO_4 on 0.1 M cacodylate buffer. Fixation was performed on melting ice in the dark for 4 hours, with the complete replacement of the fixator 15 min after the beginning of the fixation. Then the amoebae were washed for 15 min in 0.1M cacodylate buffer and postfixed with 2% OsO4 on 0.1 M cacodylate buffer in the dark on melting ice (1 h). After a transition through a graded ascending alcohol series the material was embedded in Epon-Araldite mixture. In order to facilitate the preparation of ultrathin sections the objects embedded in the resin were treated with 10% solution of hydrofluoric acid (HF). Ultrathin sections were cut with a Reichert ultratome and viewed in the Tesla BS-300 electron microscope.

Results

Light microscopy

According to our observations, *Pelomyxa binucleata* (Gruber, 1884) is one of the most common *Pelomyxa* species in water bodies of North-western Russia. This organism may be found in almost all kinds of stagnant and slowly flowing waters with sufficient littoral silt sediments. These amoebae are especially numerous in places with a rich aquatic vegetation at small depths (10-60 cm). *P. binucleata* are found all year round, being as common in samples from frozen water bodies in the middle and the end of winter as in summer and autumn samples. The abundance of *P. binucleata* does not usually exceed 10-15 individuals per 1 cm^3 of silt. Adult amoebae are the most numerous. They demonstrate three morphological forms (Fig. 1 A-C), that reflect the characteristic behavioral features of individuals and may substitute each other at random.

The amoebae with a broadly oval body shape (Fig. 1 A) are, as a rule, rather active but almost never relocate in space. The cell length of this form varies from 200 to 350 μm . They often form a strong hyaline lobopodium and start moving in its direction, but almost at once the zone of hyaloplasm shifts to the lateral cell surface, and the vector of movement changes, and so on. Very often an amoeba remains at the same place for a long time. This form of *P. binucleata* has a pronounced hyaline bulbous uroid with conical hyaline villi (Fig. 1 A). Besides, such amoebae may form small lateral hyaline pseudopodia (as a rule, conical ones). Numerous flagella are scattered all over the cell surface except the frontal lobopodium (Fig. 1 H). They are often found in groups of 5-6 flagella and are always present in the uroid zone.

Starting movement in a definite direction, *P. binucleata* individuals acquire a cigar-shaped form (Fig. 1 B). Such cells reach 400-450 μm in length. Only a narrow zone of hyaloplasm is formed during movement on the anterior body end. There are no lateral pseudopodia. The uroid is small and often oriented towards the substrate during movement (Fig. 1 B).

The third type of cells is characterised by a spherical shape (Fig. 1 C). The cell diameter varies from 150 to 300 μm. The uroid is not expressed but there is a zone of hyaline villi on the body surface, among which numerous flagella are visible. Cells of this form have a pronounced layer of peripheral hyaloplasm. Short hyaline pseudopodia of various shape are generated spontaneously all over the surface.

Young uninucleate individuals, whose size varies from 100 to 150 μm , usually have an oval or ovoid body (Fig. 1 D, E). Cytoplasmic flows are active. The cells are sometimes surrounded with a border of short hyaline pseudopodia, and sometimes form a pronounced lobopodium (Fig. 1 D). Instead of a distinct uroid, there is the zone of hyaline villi, similar to that noted in rounded adult forms.

Cell organisation of *P. binucleata* at the light microscopic level is very characteristic, so that it is very easy to tell this species apart from other pelomyxoids. Bright and contrasting colouring is very typical of *P. binucleata* individuals, regardless of the biotope of provenance. This is associated with a large number of food inclusions, mostly various unicellular chlorophyll-containing microorganisms (Fig. 1 F, G). Besides, the cytoplasm of *P. binucleata* often contains bacterial colonies of different shades of red (Fig. 1 G). The analysis of local samples from different biotopes has demonstrated that the set of food inclusions in *P. binucleata* is almost independent of its habitat, allowing an unmistakable identification of this species among other pelomyxae. A telling example: in Osinovskoye Lake (70 km to the North of St. Petersburg) littoral detritus is mostly made up of decomposing remains of woody plants; the silt is black, contains much sand and smells lastingly of hydrogen sulphide. Several *Pelomyxa* species dwell there, including large grey *P. palustris*. The cytoplasm of pelomyxoids from this biotope contains numerous sand grains, diatom frustules, spores of coniferous plants and various fragments of vascular plants. Chlorophyll-containing microorganisms are present in their cytoplasm in limited numbers. *P. binucleata* is very rarely found in this biotope: 1-2 individuals per 10 ml of samples. However, the individuals found have a typical and characteristic set of unicellular chlorophyll-containing microorganisms, distinguishing them from all other pelomyxoids, including small uni- and binucleate products of plasmotomy in *P. palustris*.

Another feature of *P. binucleata* cells is a very small number of structural vacuoles in the cytoplasm. It is due to this fact that the cytoplasm of these amoebae appears transparent under a light microscope and all their cytoplasmic inclusions, very contrasting. Singular vacuoles and their groups do occur in the cytoplasm, moving freely with its flows, but great care should be taken in drawing homologies between them and the structural vacuoles filling the whole cell volume in other pelomyxoids.

The cytoplasm of *P. binucleata* always contains numerous small refractive granules with a diameter of about 3 μm (Figs 1 G; 2 A). In adult individuals of *P. binucleata,* glycogen bodies are always present in large numbers (Fig. 2 B). They are spherical, smooth and reach 10-15 μm in diameter.

A characteristic feature of *P. binucleata* is the presence of very long rod-like prokaryotic endocytobionts of two types (Fig. 2 A). The length of both bacterial species varies in a broad range: from 3 μm to

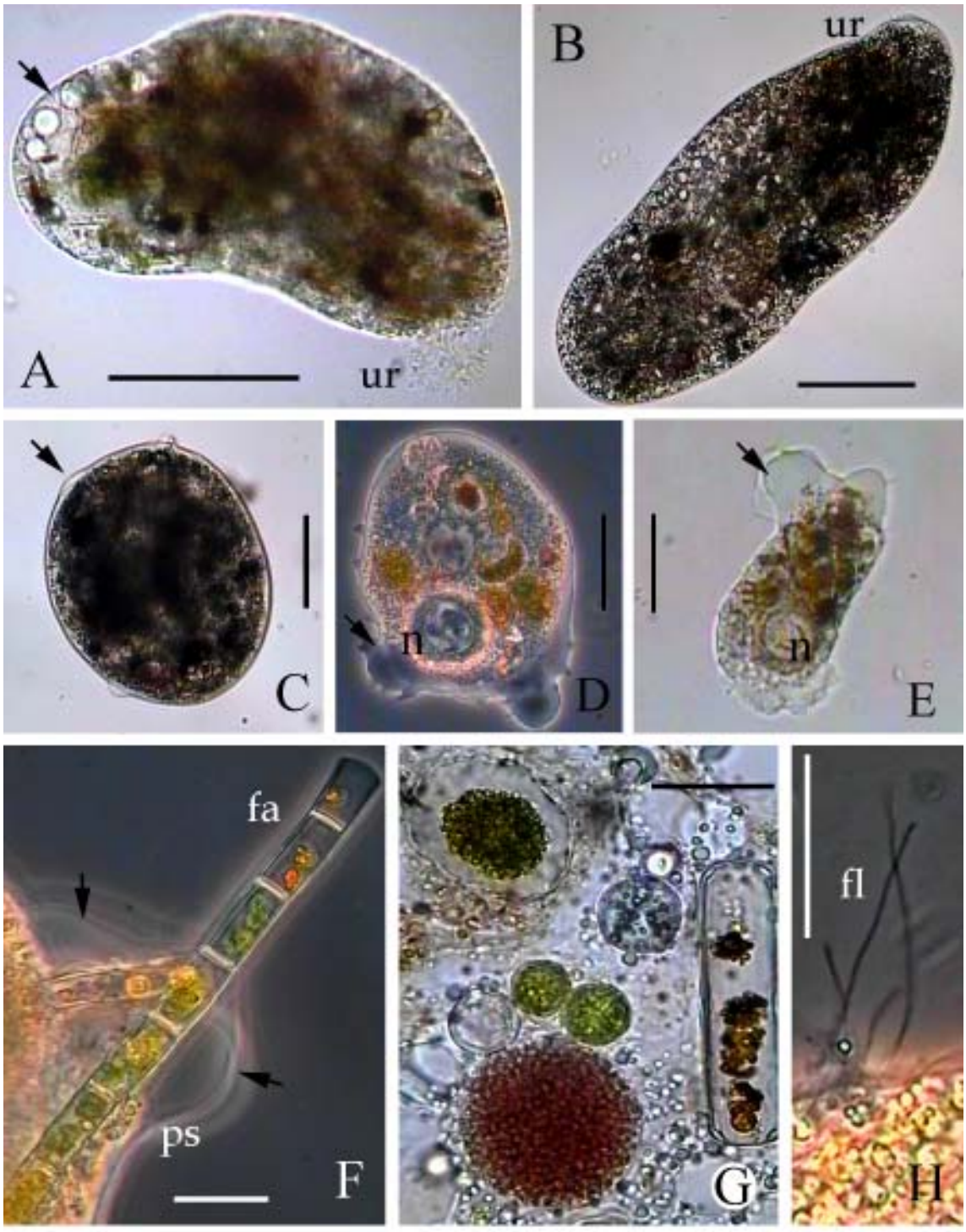

Fig. 1. The structure of *Pelomyxa binucleata* under light microscope. **A-C** - adult binucleate forms; **D-E** - young uninucleate individuals; **F** - pseudopodium engulfing a filamentous alga; **G** - food inclusions in cytoplasm; **H** - a group of flagella on cell surface. *Abbreviations*: fa - filamentous alga; fl - flagella; n - nucleus; ps - hyaline pseudopodium; ur - uroid. Arrows indicate hyaloplasm areas. F, H - phase contrast. Scale bars: A-C - 100 μm; D-E - 50 μm; F-G - 20 μm; H - 15 μm.

30 μm and more (Fig. 2 A). However, their diameter is constant, being about 1 μm in one of the species and not exceeding 0.5 μm in the other one.

As reflected in the species name, the key character of *P. binucleata* is the permanent presence of two nuclei in adult individuals (Fig. 2 C). Both nuclei of *P. binucleata* are spherical and have an almost identical structure and size (Fig. 2 C). Most part of the nucleus is occupied by the central nucleolus (Fig. 2 C, E), which has a complex spatial organisation. Possibly it is formed by several adjacent independent fragments or, on the contrary, it may be a single structure with lobes and deep depressions (Fig. 2 C, E). The diameter of the nucleolus is about 3/4 of that of the nucleus. A vast space between the nucleolus and the nuclear envelope is filled with a relatively homogeneous nucleoplasm (Fig. 2 C, E). In intact *Pelomyxa* amoebae the nuclei are hardly visible, being obscured by abundant food inclusions. The diameter of the nuclei varies from 38 to 40 μm. In amoebae that are slightly squeezed under the cover slip the diameter of the nuclei varies from 41 to 47 μm. Both nuclei move freely with cytoplasmic flows and are constantly changing their position against each other during the cell movement.

Young individuals of *P. binucleata* have only one nucleus (Fig. 2 D). In general its structure is similar to that of the nuclei of the adult individuals. The nucleolus is also central but its components appear to be more discrete. The diameter of nuclei in uninucleate *P. binucleata* amoebae squeezed under a cover slip varies from 49 to 54 μm, that of the nucleolus being 4/5 of this value. Correspondingly, the nucleoplasm layer surrounding the nucleolus is less pronounced that in the nuclei of the adult forms (Fig. 2 D, E).

Prokaryotic endocytobionts in adult binucleate individuals of *P. binucleata* do not form any visible agglomerations around the nuclei. On the contrary, endocytobionts of both types in young uninucleate pelomyxae concentrate around the nucleus.

Both young and adult individuals of *P. binucleata* bear numerous flagella on their surface (Fig. 1 H). *P. binucleat*a is the first species of pelomyxoids in which we have revealed motile flagella. The mode of motility is different in adult and young individuals. In binucleate amoebae the flagella perform either pendulum-like or rotating movements with a large amplitude. In both cases a flagellum is bent in its basal part near the body surface, whereas the undulipodium remains almost rigid. As noted above, the flagella of *P. binucleata* are often found in groups on the cell surface but no concurrence in the movement of the adjacent undulipodia has been observed. One may often see two neighbouring flagella perform pendulum-like movements in antiphase, whereas a third one in the vicinity is rotating. In uninucleate *P. binucleata* a true undulation of flagella has been noted. The analysis of the video-recorded movement of such flagella reveals that sinusoidal waves pass along an undulipodium about 15 μm in length with a frequency of up to 3 times per 10 s.

Electron microscopy

Ultrastructural organisation of *P. binucleata* was studied on adult binucleate individuals. The plasma membrane of the *P. binucleata* cells bears on its external surface a thin layer (5-7 nm) of non-structured amorphous glycocalyx (Fig. 3 A-C). Below the plasma membrane the ectoplasm layer is situated, devoid of most cell inclusions and organelles (Fig. 3 A-C). The thickness of this layer varies in a broad range, from 0.5 to 10 μm and more (Fig. 3 A-C). The ectoplasm is structured as a network, with thin fibrils (3-5 nm) being the main components. The ectoplasm of *P. binucleata* is separated from most of the cytoplasm with a well-developed layer of the endoplasmic reticulum channels, mostly oriented parallel to the plasma membrane (Fig. 3 A-C). The cytoplasm matrix, where cisterns and channels of the reticulum are concentrated, also has a net-like fine fibrillar structure, but the fibrils are much looser and much less organised than those in the ectoplasm. The ectoplasm zone may reach 10 μm and more in thickness; single endocytobionts may penetrate there but the border of ER channels turns out to be insurmountable for other cell inclusions and organelles (Fig. 3 A-C).

The covers and the underlying cytoplasm layer in *P. binucleata* are structurally and functionally associated with the flagellar apparatus (Fig. 4 A-I). The free parts of the flagella reach 20 μm in length. They are cytoplasmic protrusions of the cells covered with the plasma membrane and armed with the axoneme microtubules (Fig. 4 A-C). The number of microtubules in the axoneme is unstable. Unfortunately, we do not have any strictly transverse sections of axoneme in *P. binucleata* that would enable us to determine the exact arrangement of the microtubules. However, it can be seen in slightly oblique sections (not shown) that the central pair of microtubules in the axonemes of *P. binucleata* can be substituted with a single one or there may be no microtubules at all. At a longitudinal section of a flagellum (Fig. 4 B), additional microtubules successively join twice the peripheral microtubules of the axoneme towards the apical end of the flagellum. As mentioned above, both single flagella and their groups are found in *P. binucleata* (Fig. 4 A-B). The flagella are very often located on small papillae formed at the amoebae surface (Fig. 4 A-B).

The basal part of the flagellar apparatus in *P. binucleata* includes the transition zone and the kinetosome (Fig. 4 A-D, F, G). The transition zone

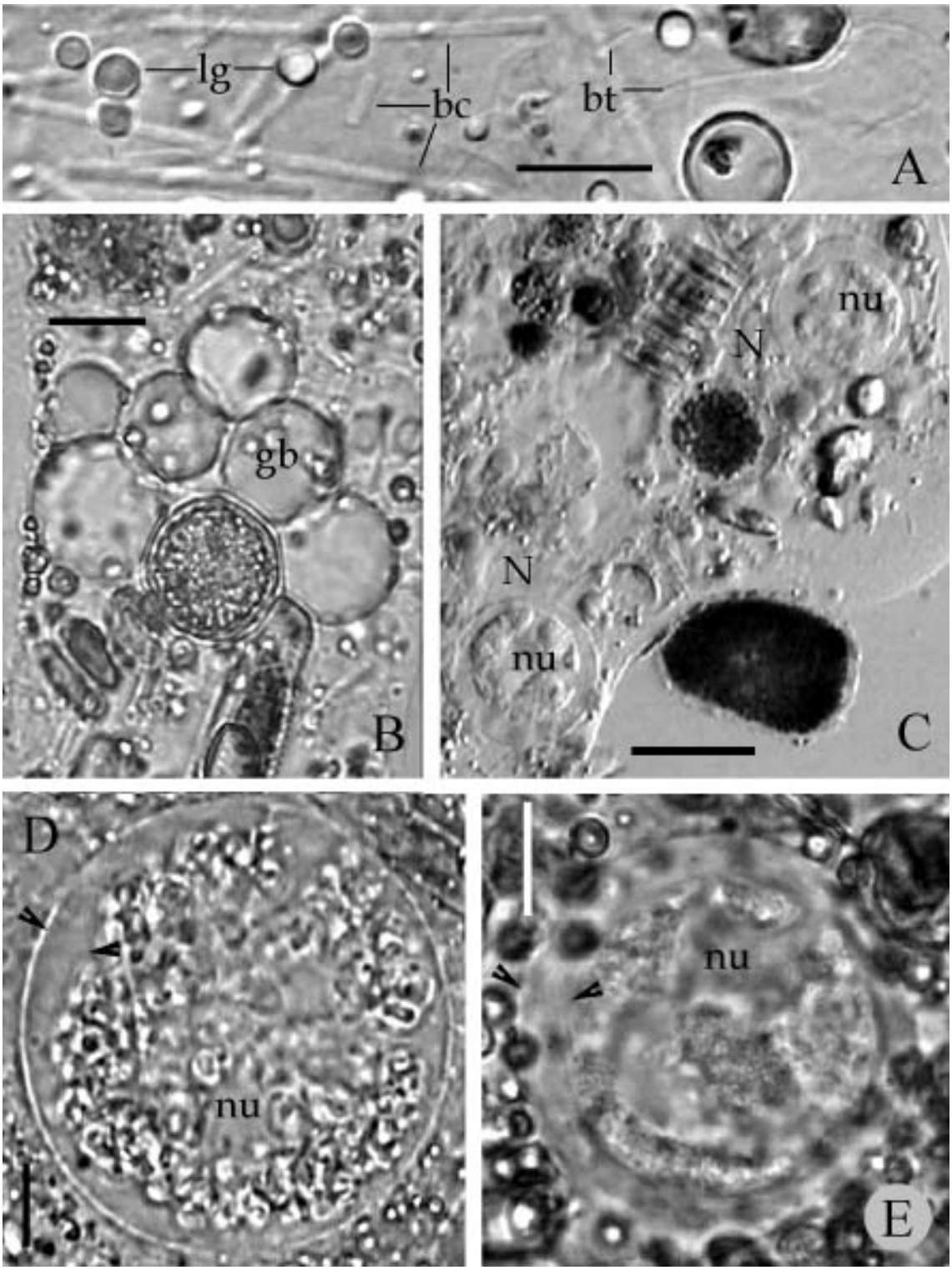

Fig. 2. The structure of *Pelomyxa binucleata* under light microscope. **A** - two types of prokaryotic symbionts in the cytoplasm; **B** - a group of 6 glycogen bodies in the cytoplasm; **C** - two nuclei of adult *P. binucleata* (the same individual as in Fig. 1 B); **D** - the nucleus of a young individual (the same as in Fig. 1 D); **E** - one of the nuclei of a binucleate adult amoeba. *Abbreviations*: bc - thick rod-like prokaryotic symbionts of various length with a cleft; bt - short and long thin rod-like Gram-positive prokaryotic symbionts; gb - spherical glycogen bodies; lg - light-refracting granules; N - nucleus; nu - nucleolus. Scale bars: A, B, D, E - 10 μm; C - 30 μm. Arrowheads indicate the zone of peripheral nucleoplasm around the nucleolus; A, C - differential contrast.

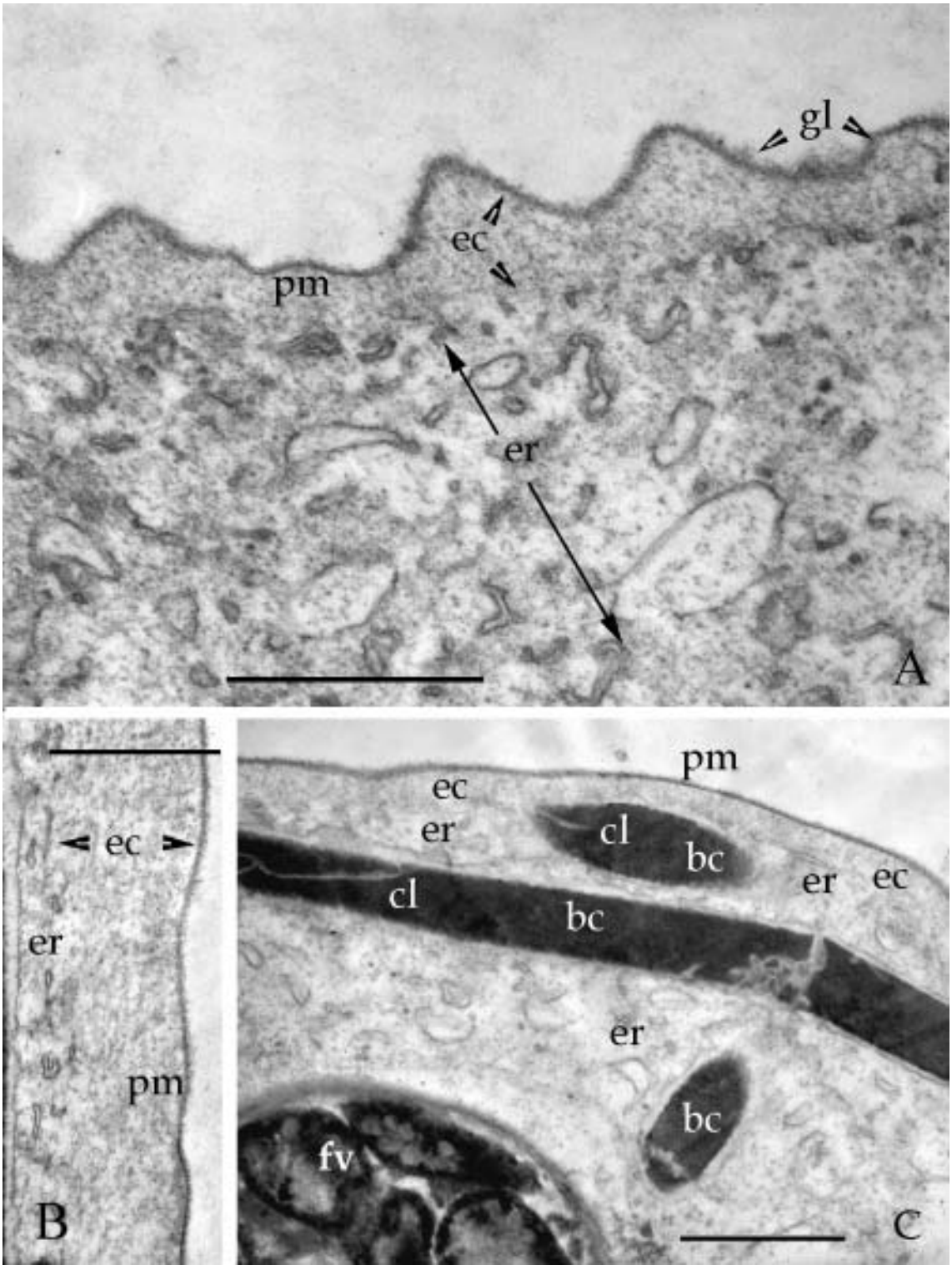

Fig. 3. The ultrastructure of *Pelomyxa binucleata*. **A-C** - organisation of the surface and cell periphery. *Abbreviations*: cl - cleft, longitudinal slit-like depression on the surface of thick rod-like symbiotic bacteria; ec - ectoplasm layer between the plasma membrane and the zone with ER channels; er - ER channels at the border of ecto- and endoplasm; fv - food vacuole; gl - amorphous glycocalyx on the surface of the plasma membrane; pm - plasma membrane. Scale bars: A, B - 1 μm; C - 2 μm.

starts at the level where the flagellum leaves the cell and protrudes above its surface by 0.15-0.2 μm. In the basal part of the transition zone the transition cylinder is located, about 100 nm high and about 80 nm in diameter. The central microtubules of the axoneme start from the upper boundary of this cylinder. Their bases are surrounded with electron-dense material (Fig. 4 B, D, F, G). A short kinetosome about 200 nm in length is wholly situated in the ectoplasm (Fig. 4 A-D). In *P. binucleata* the flagellar kinetosomes are not surrounded with a muff of electron-dense material. Along all the kinetosome 35-40 radial microtubules radiate directly from the kinetosomal microtubules at a small angle to their longitudinal axis (Fig. 4 A-I). The basal part of these microtubules is somewhat thickened due to the surrounding electron-dense fine granular material. The longitudinal kinetosome sections reveal that these microtubules are situated around the kinetosome in 3-4 rows interspersed with a distance of 20-30 nm (Fig. 4 A-D). It can be seen at a transverse section passing through a bundle of these microtubules directly under the kinetosome base (Fig. 4 E) that the bundle of radial microtubules there has the shape of a hollow cylinder. Thus, only lateral microtubules participate in its formation, there being no microtubules that radiate from the kinetosome bases in *P. binucleata*. Almost at once the microtubules initially oriented perpendicular to the cell surface bend abruptly and proceed as a more or less compact bundle below the plasma membrane (Fig. 4 B, F-I). The bundle of radial microtubules in *P. binucleata* is always located beneath the cell surface in the ectoplasm layer bounded with the plasma membrane on the one side and by the layer of the EPR channels and cisterns on the other side. These microtubules never penetrate into the cell beyond the ectoplasm border.

The endoplasm of *P. binucleata* contains the whole set of organelles and inclusions characteristic of pelomyxoids. The main component of the *P. binucleata* endoplasm are food vacuoles. Their size depends on that of the food particles and varies in a broad range (Fig. 3 C, 5 A). In the spaces between the food vacuoles prokaryotic symbionts are most numerous (Fig. 5 A, B). They lie in vacuoles bounded with two membranes, the external one continuing into the EPR channels (Fig. 5 A, B). Thick rod-like bacteria are 0.8-0.9 μm in diameter. A shallow longitudinal depression is formed on their surface (Fig. 3 A, B). Nucleoid-containing areas are well visible in the intracellular matrix (Fig. 5 A, B). Thinner bacteria are 0.3 μm in diameter. They also lie in vacuoles bounded with two closely adjacent membranes. The intracellular matrix of these cytobionts is electron-dense.

Structural vacuoles are few, not exceeding 2-2.5 μm in diameter, and are usually found in small groups (Fig. 5 A, C). Agglomerations of smaller channels and vacuoles can be seen sometimes near them (Fig. 5 C).

Large (up to 1.5-3 μm in diameter) spherical inclusions of high electron density are found throughout the cytoplasm (Fig. 5 D). They are not surrounded with a membrane and are likely to correspond to the refractive granules seen under the light microscope (Fig. 2 A). These inclusions are probably of lipid nature. Glycogen bodies are spherical, their internal structure is granular (Fig. 5 E, F). They are surrounded with flat cisterns of the reticulum (Fig. 5 E).

The two nuclei of the adult *P. binucleata* are identical ultrastructurally. They are bounded with a nuclear envelope bearing a multilaminar layer (~ 0.3 μm) at its external surface (Fig. 6 A-C). The heavily fragmented nucleolus is located in the centre of the nucleus (Fig. 6 A, D, E). Among the fragments of the nucleolus heterochromatin blocks are found (Fig. 6 A, E), with chromatin fibrils 9-10 nm in diameter being their main component. Analogous fibrils are the main structural component of the karyolymph (Fig. 6 A, D, E). The layer of karyolymph filling the space between the nucleolus and the nuclear envelope (~ 3.2 μm) is as well expressed at the ultrastructural level as it is at the light microscopic one (Figs 2 E; 6 A).

Discussion

In 1884 August Gruber published his "Studien über Amöben", where he gave detailed descriptions of 15 amoebae species illustrated with excellent colour plates. According to their characters, 8 of these 15 species: *Pelomyxa villosa* Leidy 1979, *Amoeba prima* Gruber 1884, *A. tertia* Gruber 1884, *A. lucida* Gruber 1884, *A. binucleata* Gruber 1884, *A. quarta* Gruber 1884, *A. quinta* Gruber 1884, *A. secunda* Gruber 1884 can be attributed to pelomyxoid amoebae. Four species described by Gruber: *A. lucida, A. binucleata, A. tertia, A. prima* were later transferred by Penard (1902) to the genus *Pelomyxa* Greeff 1874. Eventually all of them shared the fate of other pelomyxoid species, i.e. were declared synonyms of *P. palustris* (Whatley and Chapman-Andresen, 1990). Actually, *P. binucleata* was reduced to a synonym of *P. palustris* even earlier, when Chapman-Andresen (1982) published (as an abstract) the results of her observations upon the reproduction of *P. palustris*, proving, in her opinion, that binucleate stages were an obligatory stage in the developmental cycle of this species. We have already cited her conclusion in the Introduction.

However, comparison of the facts, that led Chapman-Andresen (1982) to synonymize *P. binucleata* with *P. palustris*, with the original description of *P. binucleata* by Gruber (Gruber, 1884) convinces us that these two works most probably deal with two different organisms. Chapman-Andresen (1982) described small pear-shaped amoebae, 300 μm in length and 200 μm in

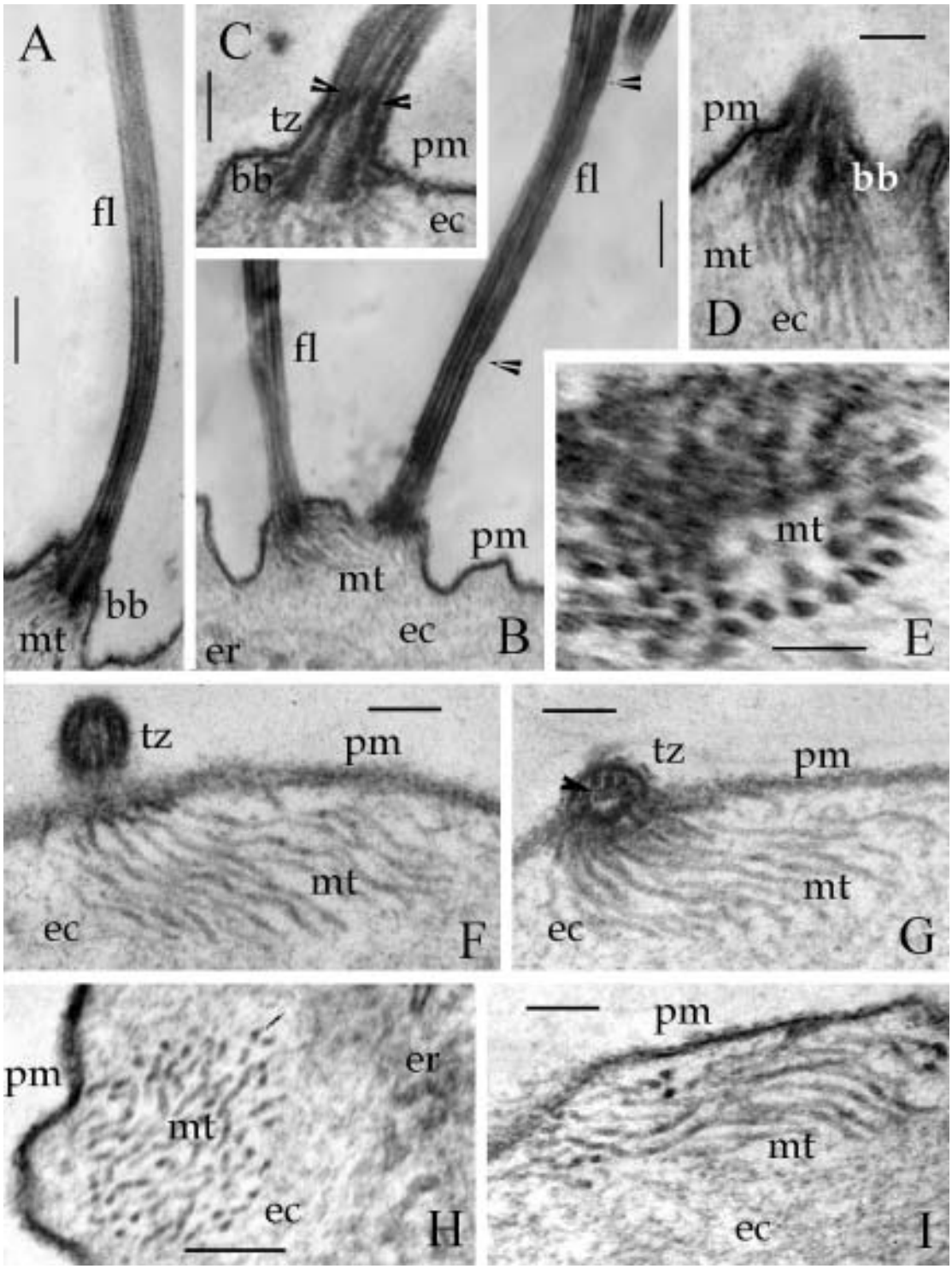

Fig. 4. The ultrastructure of *Pelomyxa binucleata*. **A-I** - organisation of the flagellar apparatus. **A** - a flagellum at the top of a small papilla on the cell body; **B** - two flagella on a small papilla (the site where additional microtubules appear in the axoneme is indicated by arrowheads); **C** - kinetosome and transition zone with an internal cylinder (arrowheads) in a flagellum of *P. binucleata*; **D** - kinetosome and kinetosome-associated microtubules; E - roughly perpendicular section across a microtubular bundle slightly below the kinetosome base; **F, G, I** - sections across the basis of the same flagellum, 2 sections skipped between G and I. The bundle of microtubules starting from kinetosome is located in the ectoplasm. **H** - a microtubular bundle in the ectoplasm. *Abbreviations*: bb - kinetosome of the flagellum; fl - flagella; mt - microtubules; tz - transition zone of the flagellum; other abbreviations as in figs 1-3. Scale bars: A, B - 0.5μm; C, D, F, G, H, I - 0.2 μm, E - 0.1 μm.

width, formed as a result of plasmotomy of large *P. palustris*. They contained 2 oval nuclei 50 μm long, did not have food vacuoles, glycogen bodies or mineral inclusions. These amoebae started feeding on filamentous algae and doubled their body volume in 14 days, the number of nuclei increasing to 8-16 in different individuals, whereas the size of the nuclei decreased down to 16-30 μm (Chapman-Andresen, 1982). The amoebae described by Gruber (1884) reached 0.2 mm in length, had 2 nuclei of regularly spherical shape and 0,003 mm in diameter (Gruber, 1884, Taf. XIV, figs. 28-32), contained glycogen bodies (ibid., Taf. XIV, fig. 29), numerous small refractive "drops", a large number of symbiotic bacteria of varying length (also very long ones) but of constant diameter (ibid., Taf. XIV, fig. 29). Though these amoebae did engulf filamentous algae, the basis of their diet were unicellular green algae (ibid., Taf. XIV, figs. 29, 31). Diatom frustules and rare mineral granules were also found in their cytoplasm. All these data were summarised by Gruber (1884) in the diagnosis of *A. binucleata*. Gruber observed these amoebae in the aquarium for 9 months, yet he never noted any increase in the number of their nuclei or a change in the mode of feeding. It is therefore evident that the observations of Chapman-Andresen (1982) were made not upon *P. binucleata* but upon another pelomyxoid species. On the contrary, Gruber's description (1884) corresponds perfectly to our organisms. The differences concern only the length of the amoebae and the diameter of their nuclei. According to Gruber (1884), the length of binucleate forms of *A. binucleata* was rather constant, 0.2 mm. In our observations, amoebae elongated during movement could reach 400 μm in length. Most probably, Gruber measured the forms of *P. binucleata* that we call broadly oval. At least, it is these forms that are shown on his original drawing (Gruber, 1884, Taf. XIV, fig. 29). The difference in the diameter of the nuclei of "our" and Gruber's amoebae (~ 10 μm) are likely to be coarsed by the error of measurement. Gruber expressed measurements in portions of a millimetre, using a relatively imprecise optics of his time.

The fact that we have found an organism, corresponding perfectly to Gruber's description, 120 years later and in another geographical area testifies to the following: first, that *P. binucleata* does exist in the nature and, second, that the description made by Gruber (1884) contains a set of characters necessary and sufficient for identification of this species.

P. binucleata is the second (after *P. prima*) species among those described by Gruber (1884) that we re-establish as a nominal species.

According to our data, ultrastructural organisation of *P. binucleata* is also very characteristic, allowing a reliable differentiation of this species among other pelomyxoids. The plasma membrane of the *P. binucleata* cell bears on its external surface only a thin layer of amorphous glycocalyx. Contrary to *P. binucleata*, both in *P. prima* and in *P. corona* the cell surface is well-developed; it is represented by a layer of structured glycocalyx reaching 80-100 nm in thickness (Frolov et al., 2004; Frolov et al., 2005). In *P. palustris* the covers, as well as other cell compartments, are described differently by different researchers. Griffin (1988) discovered in large "light" forms of *P. palustris* masses of amorphous glycocalyx up to 16 μm in thickness (Griffin, 1988). Goodkov observed amorphous glycocalyx (0.5 μm) in a "light" form but failed to find it in large grey and green *P. palustris* (A.V. Goodkov, unpublished obs.). In most studied pelobionts from the genera *Mastigamoeba* and *Mastigella*, the glycocalyx is not revealed at an ultrastructural level (Brugerolle, 1982; Chavez et al., 1986; Simpson et al., 1997; Walker et al., 2001). The two exceptions are *Mastigamoeba aspera* Schulze 1875 and *M. setosa* (Goldschmidt 1907). The cell surface in *M. aspera* is covered with a multilayered glycocalyx. Its thickness may reach 1 μm at some areas of the plasma membrane (A.O. Frolov, unpublished obs.). In the glycocalyx on the surface of *M. aspera* the prokaryotic ectobionts are located. Another, most peculiar organisation of covers is found in *M. setosa*. These amoebae have an amorphous glycocalyx 0.5 μm thick, into which organic scales and the bases of long organic spicules associated with the scales are embedded (A.O. Frolov, unpublished obs.). The diversity of the coverings in different pelobionts makes it possible to use this character for diagnostic purposes. Among the *Pelomyxa* species studied ultrastructurally, *P. binucleata* has the least developed cell surface complex. This character allows one to distinguish it reliably from *P. palustris, P. prima* and *P. corona* (Griffin, 1988; Frolov et al., 2004; Frolov et al., 2005).

A pronounced differentiation of the cytoplasm into the ecto- and the endoplasm appears to be characteristic of all pelomyxoids, but the degree of the differentiation varies between the species. In *P. binucleata* the ectoplasm contains a dense network of fine (3-5 nm) fibrils and is separated from the rest of the cytoplasm with a zone where the ER channels are concentrated. In *P. corona* the ectoplasm is also clearly expressed but, contrary to *P. binucleata*, its internal boundary is defined by the cytoplasmic zone filled with thick (25-30 nm) microfibrils, not the reticulum channels (Frolov et al., 2004). In *P. palustris* the ectoplasm is less distinct than in the above two species. There is no structured border zone between the ecto- and the endoplasm in *P. palustris* (Goodkov and Seravin, 1991). Griffin (1988) determined the hyaline ectoplasm of *P. palustris* as the zone of cytoplasm between the plasma membrane and the structural vacuoles. Though formation of separate, sometimes considerable areas of hyaloplasm can be

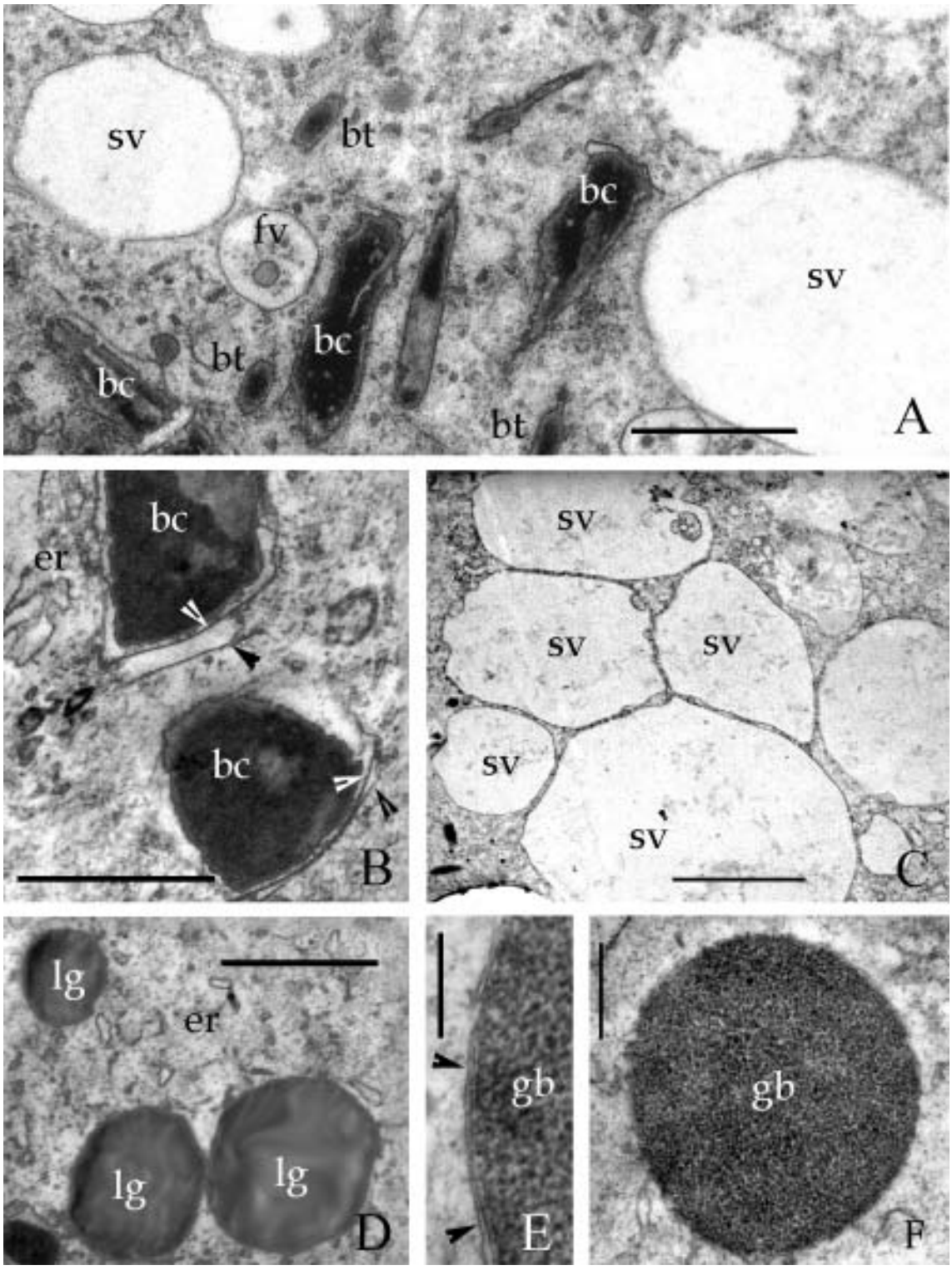

Fig. 5. The ultrastructure of *Pelomyxa binucleata*. **A-F** - organisation of cytoplasm. **A** - area of cytoplasm containing endosymbiotic bacteria and structural vacuoles. **B** - thick rod-like symbiotic bacteria with a cleft (not seen) surrounded with two membranes (arrowheads). **C** - a group of structural vacuoles in the cytoplasm. **D** - large "refractive granules" (the same as in Fig. 2 A). **E, F** - spherical glycogen bodies surrounded by two membranes (arrowheads). *Abbreviations*: sv - structural vacuoles; other abbreviations as in figs 1-4. Scale bar: A, C, D, F - 2 μm, B - 1 μm, E - 0.8 μm.

observed in *P. prima* under the light microscope, we failed to reveal any differentiation of the cytoplasm into the ecto- and the endoplasm at the ultrastructural level (Frolov et al., 2005). Thus, a characteristic organisation of the ectoplasm in *P. binucleata* is yet another valuable diagnostic character of this species.

The endoplasm of *P. binucleata* is filled with food vacuoles; in the space between them the main cell organelles and various inclusions are situated. *P. binucleata* has relatively few structural vacuoles in its cytoplasm, contrary to *P. palustris, P. prima* and *P. corona*. According to different authors, structural vacuoles in *P. palustris* may occupy from 40 to 75% of the cell volume (Fortner, 1934; Chapman-Andresen and Hamburger, 1981; Goodkov and Seravin, 1991). In *P. prima* and *P. corona* structural vacuoles also are the dominant elements in the cytoplasm (Frolov et al., 2004; Frolov et al., 2005). In the endoplasm of *P. binucleata* groups of 5-7 adjacent structural vacuoles are sometimes observed. Among mastigamoebid pelobionts the presence of structural vacuoles (vacuolarisation of the cytoplasm) was observed (Goldschmidt, 1907; Penard, 1909) at the light microscopic level only in two *Mastgamoeba* species (earlier attributed to the genus *Mastigina*): *Mastigamoeba lacustris* (Penard 1909) Lemmermann 1914 and *Mastigamoeba setosa* (Goldschmidt 1907) Lemmermann 1914. Our ultrastructural investigations of *M. setosa* have supported the presence in the cytoplasm of these mastigamoebae of a well-developed network of structural vacuoles, morphologically identical to those in *Pelomyxa* (A.O. Frolov, unpublished obs.). Therefore, the presence of structural vacuoles in the cytoplasm of pelobionts is not a specific character of pelomyxoid amoebae. At the same time, the degree of development of the system of structural vacuoles may characterise certain pelomyxoid species, in particular, *P. binucleata*.

Glycogen bodies of *P. binucleata* have a regular spherical shape and smooth surface and are enveloped by the flat cisterns of the endoplasmic reticulum. Until recently it has been thought that the glycogen bodies of pelomyxoids are not membrane-bounded. For instance, Daniels et al. (1966) did not reveal any membrane structures around the glycogen bodies in *P. palustris*, and neither did Goodkov and Seravin (1995). We have earlier discovered membrane-enveloped glycogen bodies in *P. corona* (Frolov et al., 2004). However, they differ greatly from the glycogen bodies of *P. binucleata* in having an oval shape and a wrinkled surface. The morphology of glycogen bodies and their location in the cytoplasm are possibly different in various *Pelomyxa* species and may be a distinctive character.

The presence in the cytoplasm of *P. palustris* of three species of prokaryotic symbionts is considered as a characteristic feature of this pelomyxoid species (Van Bruggen et al., 1988; Whatley and Chapman-Andresen, 1990; Goodkov and Seravin, 2000). Two species of metanogenic bacteria among them, Gram-positive *Methanobacterium formicium* and unidentified Gram-negative bacteria, are represented by thin rod-like forms of similar size. The third species is represented by thick rod-like bacteria with a characteristic deep longitudinal furrow (the so-called cleft) reaching the middle of the cell. Identification and comparison of pelobionts-derived symbiotic bacteria is to a large extent provisory, since their isolation and cultivation are still not feasible. According to the data available, only *Mastigella vitrea* Goldschmidt 1907 and *Mastigella nitens* Penard 1909 have a set of symbiotic bacteria similar to that of *P. palustris* (Penard, 1909; Van Bruggen et al., 1988; A.O. Frolov, unpublished obs.). Thin rod-like Gram-positive bacteria were found in *Mastigella commutans* (Meyer 1897; Walker et al., 2001). We have earlier discovered three species of prokaryotic symbionts in *P. corona*. Only two of them, thin Gram-negative rod-like bacteria and thick bacteria with a pronounced longitudinal cleft, corresponded morphologically to a reduced set of *P. palustris* symbionts. The third species of *P. corona* symbionts was represented by large ellipsoidal Gram-positive bacteria. Contrary to the rod-like symbionts, the ellipsoidal bacteria were several in a vacuole (Frolov et al., 2004). In *P. prima* only Gram-positive rod-like bacteria and thick bacteria with a longitudinal cleft were found. Thin rod-like Gram-positive bacteria and thick bacteria have also been found in *P. binucleata*. However, both symbiotic forms are very long in this species (10 and more times longer than the symbionts of other species of *Pelomyxa*). Thick bacteria have a longitudinal slit-like depression but it is shallow and appears to be less long than the body. Morphological data do not allow us to make a conclusion about the species of the prokaryotic symbionts of *P. binucleata*. However, the fact that such long bacteria have not been revealed in any other *Pelomyxa* species, makes it possible to use their presence as yet another specific criterion of *P. binucleata*.

The nuclei of *P. palustris* were shown to have a characteristic organisation (Daniels et al., 1966; Whatley and Chapman-Andresen, 1990; Goodkov and Seravin, 1995). Fragmented nucleolar material occupies a peripheral position, being often adjacent to the internal membrane of the nuclear envelope; separate heterochromatin blocks are found both amongst the nucleolar material and lying freely in the karyolymph. The only exception are the nuclei of one of the cells studied by Daniels and colleagues (Daniels et al., 1966, figs 11, 12). In these large (~18 μm) spherical nuclei strongly condensed chromatin occupies a central position as a compact fibrillar ball, whereas the fragmented nucleolar material containing nucleolar

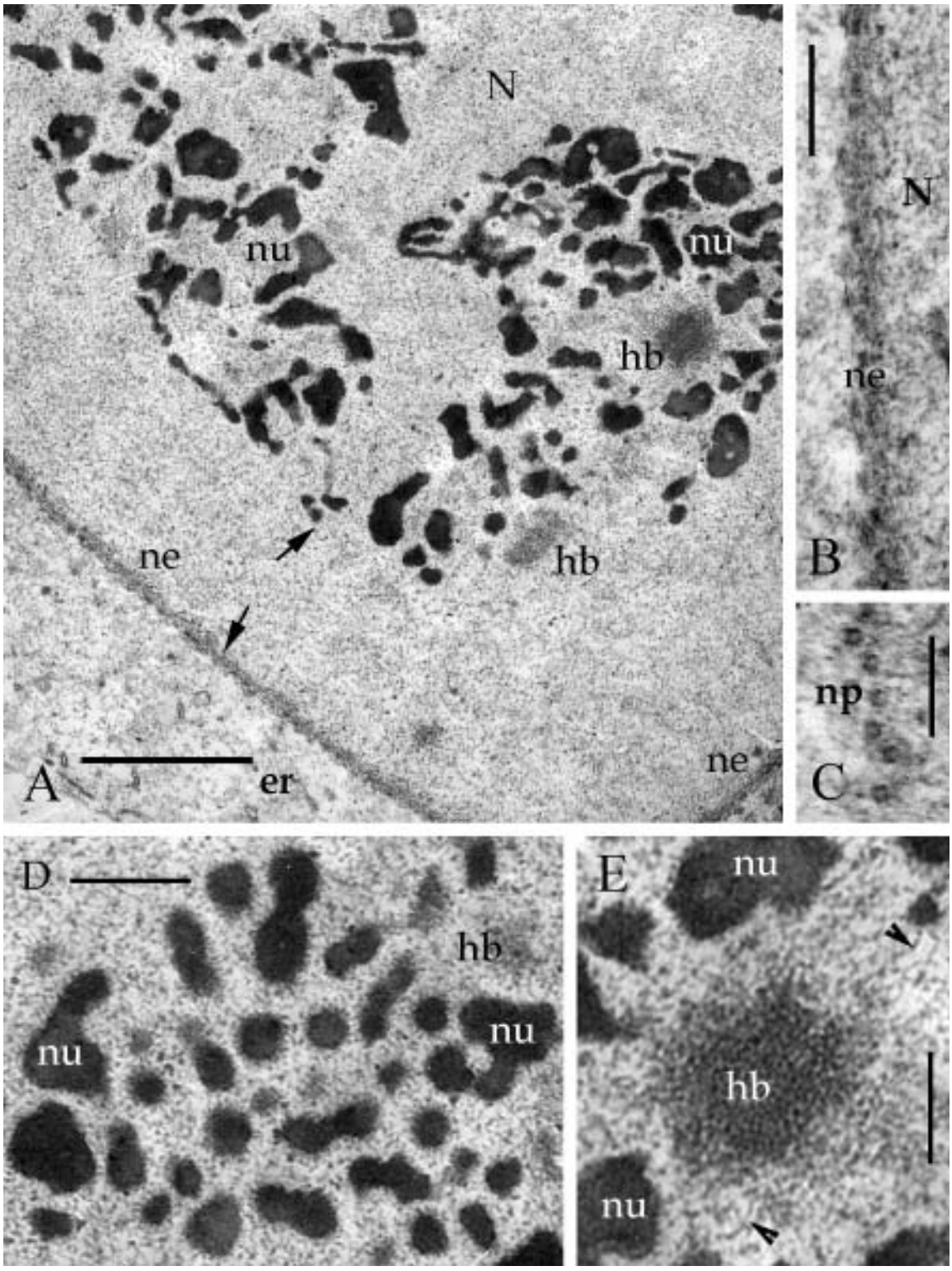

Fig. 6. The ultrastructure of *Pelomyxa binucleata*. **A-E** - organisation of nucleus. **A** - a fragment of *P. binucleata* nucleus at low magnification, arrows indicate the layer of peripheral karyoplasm. **B, C** - multilaminar nuclear envelope with pore complexes. **D, E** - central part of the nucleus containing nucleolar material and heterochromatin blocks. Arrowheads indicate separate chromatin fibrils. *Abbreviations*: hb - heterochromatin blocks, ne - nuclear envelope, np - pore complexes; other abbreviations as in figs 1-4. Scale bar: A - 3 μm, B - 0.6 μm, C - 0.5 μm, D - 2 μm, E - 0.8 μm.

clusters surrounds this ball from all sides. A vast zone between this central structure and the nuclear envelope is filled with a relatively homogeneous karyolymph. The nuclear envelope of the nuclei of this type is multilayered and bears on its external surface numerous electron-dense stellate structures (Daniels et al., 1966). Externally these nuclei are somewhat similar to those of *P. binucleata*, but there are several essential differences. The nuclei of *P. binucleata* are twice larger in diameter. They lack the central compact ball of dense chromatin, their nucleolar material does not contain nucleolar clusters, the multilayered envelope on the surface of the nuclei of *P. binucleata* is almost two times thinner than that in the above form of *P. palustris*, and, finally, there are no electron-dense stellate structures on the surface of the multilayered envelope. To sum up, the nuclei of *P. binucleata* are clearly different from all the types of nuclei described earlier in *P. palustris*. They are equally distinct from the nuclei of *P. corona* and *P. prima* (Frolov et al., 2004; Frolov et al., 2005). In the latter two species the size of the nuclei never exceeds 15 μm, and there is no multilayered envelope. Besides, in *P. corona* fragmented nucleolar material is situated at the periphery of the nuclei, similarly to *P. palustris*. In *P. prima* a distinct large nucleolus occupies a central position in the nuclei, morphologically resembling, most of all, the nucleolar organisation of uniflagellate pelobionts (Brugerolle, 1982; Chavez et al., 1986; Simpson et al., 1997; Walker et al., 2001).

Until recently only fragmentary data were available on the flagellar apparatus structure in pelobionts (Brugerolle, 1982; Seravin and Goodkov, 1987; Griffin, 1988; Goodkov, 1989; Goodkov and Seravin, 1995). It was shown, in particular, that immobile flagella of *P. palustris* and *Mastigina hylae* have an unstable set of microtubules in the axoneme, different from the classical 9+2 scheme. Information about the structure of the basal part of the flagellum in pelobionts was very contradictory. For instance, *M. hylae* was shown to have a lateral microtubular rootlet and a microtubular cone connecting the kinetosome and the nucleus (Brugerolle, 1982). Griffin (1988) found a kinetosome with a microtubular cone similar to that in *M. hylae* in only one of the four isolates of *P. palustris*. Seravin and Goodkov (1987) and Goodkov (1989) did not observe a microtubular cone in *P. palustris*; according to their data, the kinetosomes of *P. palustris* formed a bundle consisting of a few microtubules that was located in the ectoplasm parallel to the cell surface.

Further investigations, carried out on uniflagellate pelobionts from the genera *Mastigamoeba* and *Mastigella*, elucidated this issue considerably (Simpson et al., 1997; Walker et al., 2001). It has been shown that the mobile flagellum of these pelobionts used for movement has an axoneme with a typical 9+2 set of microtubules. A cylinder has been found in the transition zone of such flagella. At the same time, it has been demonstrated that the kinetosome of mastigamoebae may have a structure different from the classic scheme (doublets instead of triplets) (Simpson et al., 1997). These investigations also revealed a relatively conservative organisation of the basal part of the flagellar apparatus. In general, this part of the flagellum may be represented in the following way. The kinetosome is associated with three groups of microtubules: 1, a microtubular cone usually formed by the microtubules starting from the kinetosome base; 2, radial cytoplasmic microtubules starting from the kinetosome above the microtubules of the cone and radiating into the cytoplasm in a fan-like fashion; 3, a lateral microtubular rootlet starting from an electron-dense MTOC adjacent to the lateral surface of the kinetosome (Walker et al., 2001). The microtubular cone and the lateral rootlet have been found in all the *Mastigamoeba*-like pelobionts studied, whereas the degree of development of the radial cytoplasmic microtubules varies greatly, up to their absence in *M. simplex*.

New data have also been obtained on the organisation of the flagellar apparatus of the pelomyxae. In *P. prima* we have found all the three kinetosomal microtubular derivatives characteristic of uniflagellate pelobionts (Frolov et al., 2004). Interestingly, mobile flagella of *P. binucleata* are structurally much more similar to the immobile flagella of *P. palustris* than to the mobile flagella of mastigamoebae. The information on the structure of the flagellar apparatus in *P. binucleata* given in the present work shows that discrepancies between the data obtained by Griffin (1988) and by Seravin and Goodkov (1987, 1989, 1995) were due to neither chance nor method. Contrary to *P. prima, P. binucleata* lacks the lateral rootlet and the microtubular cone, whereas its radial cytoplasmic microtubules form a bundle passing in the ectoplasm below the cell surface. Thus, the structure of the basal part of the flagellar apparatus turned out to be less conservative in pelomyxoids than in uniflagellate pelobionts. At least two significantly different forms of the cytoskeleton organisation have been revealed. It may demonstrate a general tendency to degeneration of the flagellar apparatus in pelomyxoids accompanying the loss of its locomotory function (Walker et al., 2001). This tendency may ultimately result not only in the loss of some elements of the flagellar apparatus but also in its total disappearance (Frolov et al., 2004; Goodkov et al., 2004). *P. binucleata* differs clearly in the structure of the flagellar apparatus from *P. prima* and *P. palustris* sensu Griffin (1987), the latter two species having kinetosomes associated with radial cytoplasmic microtubules. *P. binucleata* is different from *P. palustris* sensu Goodkov (1989) in having a twice as large number

of the kinetosome-associated microtubules (40 and 20 at the kinetosome base, respectively) that form a bundle passing below the cell surface.

In conclusion, we would like to emphasize that both light and electron microscopic data prove conclusively systematic validity of the species *Pelomyxa binucleata* (Gruber, 1884). The three species of not very large (< 1 mm) pelomyxoid amoebae recently investigated by us, *P. binucleata, P. prima* and *P. corona*, can be reliably distinguished by a complex of characters both from each other and from various forms and strains united by the name of *P. palustris*. This fact resolves the dispute about monotypy of the genus *Pelomyxa* Greeff 1874. In addition, all the above supports our previous supposition (Goodkov et al., 2004) that the diversity of the state of different characters in *P. palustris*, including ultrastructural ones, cannot be explained by an exceptional polymorphism of its developmental stages. Therefore, the next step in the faunistic studies of pelomyxoids should be the investigation of biodiversity of large (> 1mm) species of the genus *Pelomyxa* Greeff 1874.

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