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Protistology

A new pelobiont protist *Pelomyxa corona* sp. n. (Peloflagellatea, Pelobiontida)

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Summary

A new species of multinucleate pelobionts, *Pelomyxa corona*, is described. Morphology of the amoebae has been studied with the use of the light and the transmission electron microscope. The cells of *P. corona* are rounded. The cytoplasm is clearly differentiated into the marginal hyaloplasm and the endoplasm. Numerous hyaline pseudopodia of approximately equal length make the amoeba look like a "crown", which is reflected in the specific name. Ultrastructurally *P. corona* is rather similar to *P. palustris*, but a number of unique characters allows a reliable differentiation of these two species. The study of *P. corona* revealed a well-developed structured glycocalyx, the dictyosomes of the Golgi apparatus and fine pseudopodia armoured with bundles of microtubules. One species out of the set of the symbiotic bacteria characteristic of *P. palustris* is lacking in *P. corona*, but another bacterial species is present which has not been reported from pelobionts so far. Flagella have not been found.

Key words: pelobionts, Peloflagellatea, *Pelomyxa corona* sp. n., *Pelomyxa palustris,* ultrastructure

Introduction

Most pelobionts inhabit bottom sediments of freshwater, mainly stagnant basins; some few species have passed to parasitism. In both cases oxygen content in their environment is low. At present pelobionts comprise the genera *Mastigamoeba, Mastigella, Mastigina* and *Pelomyxa* (Walker et al., 2001; Brugerolle and Patterson, 2002; Goodkov et al., 2004). The representatives of the former three possess one, rarely two nuclei and an apical flagellum, whose kinetosome, together with other cytoskeletal elements, forms a characteristic microtubular cone, which may be associated with the nucleus (Simpson et al., 1997; Bernard et al., 2000; Walker et al., 2001). On the contrary, *Pelomyxa* representatives are multinuclear and possess numerous immobile flagella, whose kinetosomes have been reported both to form typical cones

and to form no cones at all (Griffin, 1988, Seravin, Goodkov, 1987a; Goodkov, 1989; Goodkov and Seravin, 1991, 1995; Goodkov et al., 2004). All pelobionts lack mitochondria and most, with the exception of *Mastigamoeba punctachora* and *P. palustris*, the Golgi apparatus. The former is shown to have separate non-stacked cisterns with characteristic terminal puffs and vesicles associated with these cisterns (Walker et al., 2001), and the latter is suggested to have dictyosomes (Seravin and Goodkov, 1987b). However, the data on *P. palustris* are not convincing enough and are contested by many authors. Contradictory information about the organisation of *P. palustris* is aggravated by taxonomic confusion, traditionally accompanying all *Pelomixa*-related studies. Since it is still unclear whether *P. palustris* is a nominal species or a group of species erroneously united into a single taxon, the scarce data on its organisation may well apply to different organisms (Goodkov et al., 2004). Therefore, one of the principal questions behind this problem is: Are there true multinuclear pelobionts other than *P. palustris* (sensu Griffin, 1988)? A positive answer to this question opens the way for the faunistic study of pelomyxoid pelobionts. It was this objective that we put forward when starting the research.

Material and methods

We first found the individuals of *Pelomyxa corona* in the autumn of 1999 in the samples of silt sediments from a small bogged basin formed by a broadening of a stream flowing into the Plyussa River in the vicinity of the Lyady Village (the Pskov Region) at approximately 58°35′ N and 28°55′ E. Spherical, not very motile amoebae with a crown of hyaline pseudopodia were found in the organic remains alongside with other pelobionts such as *Pelomyxa palustris* and *Mastigina setosa*. In subsequent years the amoebae in question were discovered in the samples of bottom sediments of the basins at the site of the former Plyussa riverbed. All attempts to establish *P. corona* in culture failed. The amoebae survived in the samples from 1 to 1.5 months in 100-500 ml vessels at a temperature of app. 10°C. They continued to feed but did not reproduce. 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. In this way isolated amoebae could be observed for up to three weeks.

Investigations were conducted with the use of Ergoval and Leika microscopes equipped with visualisation systems on the basis of Panasonic 650 CCTV и PC PIII. All measurements were made on live intact individuals with the image analysis system IT v.2.2.

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% OsO_4 on 0.1 M cacodylate buffer in the dark on melting ice. After a transition through a graded ascending alcohol series the material was embedded in Epon-Araldite. 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 (Fig. 1).

The amoebae in samples usually move very little. They lie among detritus particles and are often surrounded by them. Such individuals are spherical, with their cytoplasm clearly divided into transparent homogenous ectoplasm and very dense central endoplasm, containing food remnants, nuclei and inclusions. No food specialisation has been revealed. The cytoplasm contains a set of inclusions characteristic, in particular, of *Pelomyxa palustris*: diatom frustules and testate amoebae shells, spores of coniferous plants, mineral particles, etc. The whole surface of the amoebae is covered with hyaline pseudopodia (Fig. 1 A, B, D) of different shape. They may branch slightly and often fork at the ends (Fig. 1 A, D). Several times we have observed the amoebae engulfing with their pseudopodia large food particles, mostly diatom frustules.

Disturbed amoebae change the body shape from spherical to cylindrical and start locomotion (Fig. 1 B). During this process the anterior end forms a strong hyaline pseudopodium, whereas the hyaline pseudopodia covering the body gradually shift to the posterior end, where they retract. At the posterior end the uroid is formed (Fig. 1 C) or, to be precise, the uroid zone, consisting of numerous small cylindrical papillae of almost equal length. Very rarely small immobile bristle-like structures were observed among the papillae (Fig. 1 C), but it was impossible to ascertain at the light microscopic level whether or not they were true flagella. The main role in locomotion is apparently played by the strong lobopodium constantly crawling over the substrate and short forked pseudopodia formed at the ventral body surface. Reaching a large agglomeration of detritus particles the amoeba penetrates into it, stops

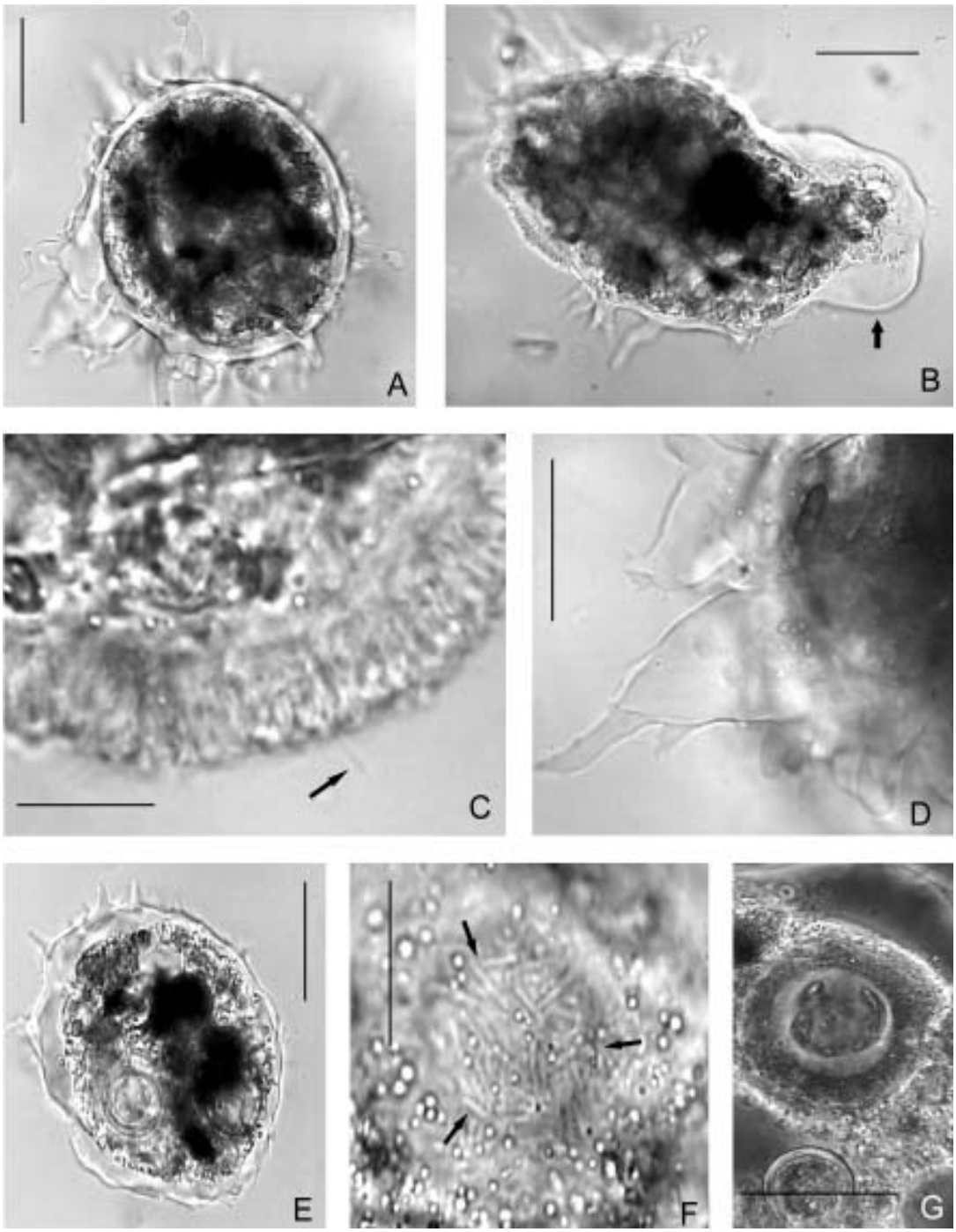

Fig. 1. *Pelomyxa corona*, light micrographs. A - large rounded cell with a "crown" of hyaline pseudopodia; B - elongate locomotive cell with a distinct frontal hyaline lobopodium (*arrow*) and residual (posterior) pseudopodia; C - uroidal zone with a fine bristle-like projection (*arrow*); D - irregular lobed hyaline pseudopodia; E - small, uninucleate cell with distinct hyaline border and a few conical pseudopodia; F, G - nucleus surrounded with symbiotic bacteria (*arrows*) closely apposed to it. Scale bars: A, B - 100 μm; C - 25 μm; D, E - 50 μm; F, G - 10 μm.

moving, becomes rounded and forms radial pseudopodia.

The size of amoebae varies greatly. Rounded forms are 50–350 μm in diameter, rarely reaching 500 μm. Cylindrical forms reach 400 μm in length, the breadth being 50-100 μm. The length of pseudopodia usually does not exceed half of the body diameter.

We observed the amoebae from March to October. In March-May small (about 50 μm in diameter) uninucleate amoebae are found in the samples (Fig. 1 E). In summer and autumn large individuals are found (Fig. 1 A), with 100 and more nuclei. The nuclei (Fig. 1 F, G) are granular, 10-15 μm in diameter. They are slightly larger in uninucleate individuals than in multinucleate ones. Agglomerations of endosymbiotic bacteria have been noted at the cytoplasmic surface of the nuclear envelope.

Electron microscopy

The plasma membrane of the protists bears on its external surface a well-developed layer of structured glycocalyx, up to 100 nm thick (Fig. 2 A-D). Below the plasma membrane there lies the ectoplasmic zone. The ectoplasm has a relatively homogenous fine granular structure. The main cell organelles (except ribosomes) and inclusions are absent in this zone, the only constant element being thick bundles of ordered microtubules, sometimes found in the submembrane zone (Fig. 2 B-D). Each of these bundle contains about 30-40 microtubules, their profiles forming a circle or an ellipse on cross-sections. Microtubular bundles pass under the plasma membrane and may armour thin cylindrical pseudopodia (Fig. 2 B). The ectoplasm is separated from the endoplasm with a layer of rather compactly arranged microfibrills 25-30 nm in diameter, the thickness of the layer varies from 0.3 to 0.5 μm.

The endoplasm is complexly organised. Alongside with the cell organelles, it contains many diverse food inclusions of varying size and density which hinder considerably its TEM investigation. A very well-developed network of the endoplasmic reticulum penetrates the endoplasm and numerous vacuoles of varying diameter are present there (Fig. 3 A, B). Cell organelles mostly concentrate between large structural vacuoles. Numerous microtubular bundles are also present in the cytoplasm (Fig. 3 A). They are oriented at different angles to each other and appear more loose than those in the ectoplasm. We have not observed penetration of microtubular associates or single microtubules from the endoplasm to the ectoplasm across the microfibrillar border.

A dictyosome of the Golgi apparatus has been found in the endoplasm. It is a stack of 11 very short (about 0.2 μm) cisterns, with vesicles 50-100 nm in diameter budding from their ends.

Glycogen bodies, reaching 1 μm in diameter, have a plicate external surface and are surrounded with numerous channels of the endoplasmic reticulum (Figs 3 D and 4 B). Three types of endosymbionts, abundant in the endoplasm, are present in almost equal proportion (Fig. 4 A-D): large bacteria with a pronounced cleft and smaller rod-like bacteria lie in symbiontophoric vacuoles whose membranes are closely apposed to the surface of the bacteria, whereas large ellipsoid bacteria lie freely in large vacuoles, 2-3 individuals in a vacuole (Fig. 4 A-C). In one and the same vacuole bacteria of different size and dividing bacteria were often present (Fig. 4 A-C). While in the endoplasm bacteria of all three types form mixed accumulations, in the perinuclear space and in direct contact with the nuclear envelope only large endosymbionts with a pronounced cleft and smaller rod-like bacteria were found (Fig. 4 C, D).

Nuclei of the amoebae are spherical, surrounded with the nuclear envelope perforated with nuclear pores (Fig. 4 C, D). Numerous channels of the endoplasmic reticulum and agglomerations of vesicles 70-100 nm in diameter with electron-dense contents are accumulated close to the nuclear surface (Fig. 4 C, D). Nucleoplasm is relatively homogenous, nucleolar material is fragmented and scattered mostly at the periphery of the nucleus (Figs 1 G, 4 C, D).

Discussion

Pelobionts are an original group of amoeboid protists inhabiting bottom sediments of freshwater basins under microaerobic or anaerobic conditions (Page, 1976; Griffin, 1988, Whatley and Chapman-Andersen, 1990, Brugerolle and Patterson, 2000; Goodkov and Seravin, 2000). The representatives of three pelobiontid genera, *Masigina, Mastigella* and *Mastigamoeba*, share a high structural similarity and their relatedness is undoubted (Brugerolle, 1982; Simpson et al., 1997; Walker et al., 2001). The attribution of *Pelomyxa palustris*, still a mysterious organism, to this group is based, in essence, on three characters: anaerobic habitats similar to those of mastigamoebae, outwardly similar organisation of the microtubular apparatus associated with flagellar kinetosomes (after Griffin, 1988) and the presence of flagella at amoeboid stages (Griffin, 1988, Brugerolle and Patterson, 2000; Goodkov et al., 2004). However, these statements are based on rather contradictory factual material. In fact, we do not even know what the name "*Pelomyxa palustris*" stands for: a single polymorphic species, a complex of close but indepen-dent species, or even several genera (Goodkov et al., 2004). This question is not discussed in the modern literature, especially after most *Pelomyxa* species described earlier have been united into a single species as stages of its complex life cycle (Whatley and Chapman-Andersen, 1990) and after a microtubular cone associated with the flagellar kinetosome was described in *Pelomyxa* (Griffin, 1988). However, some facts require a deeper analysis.

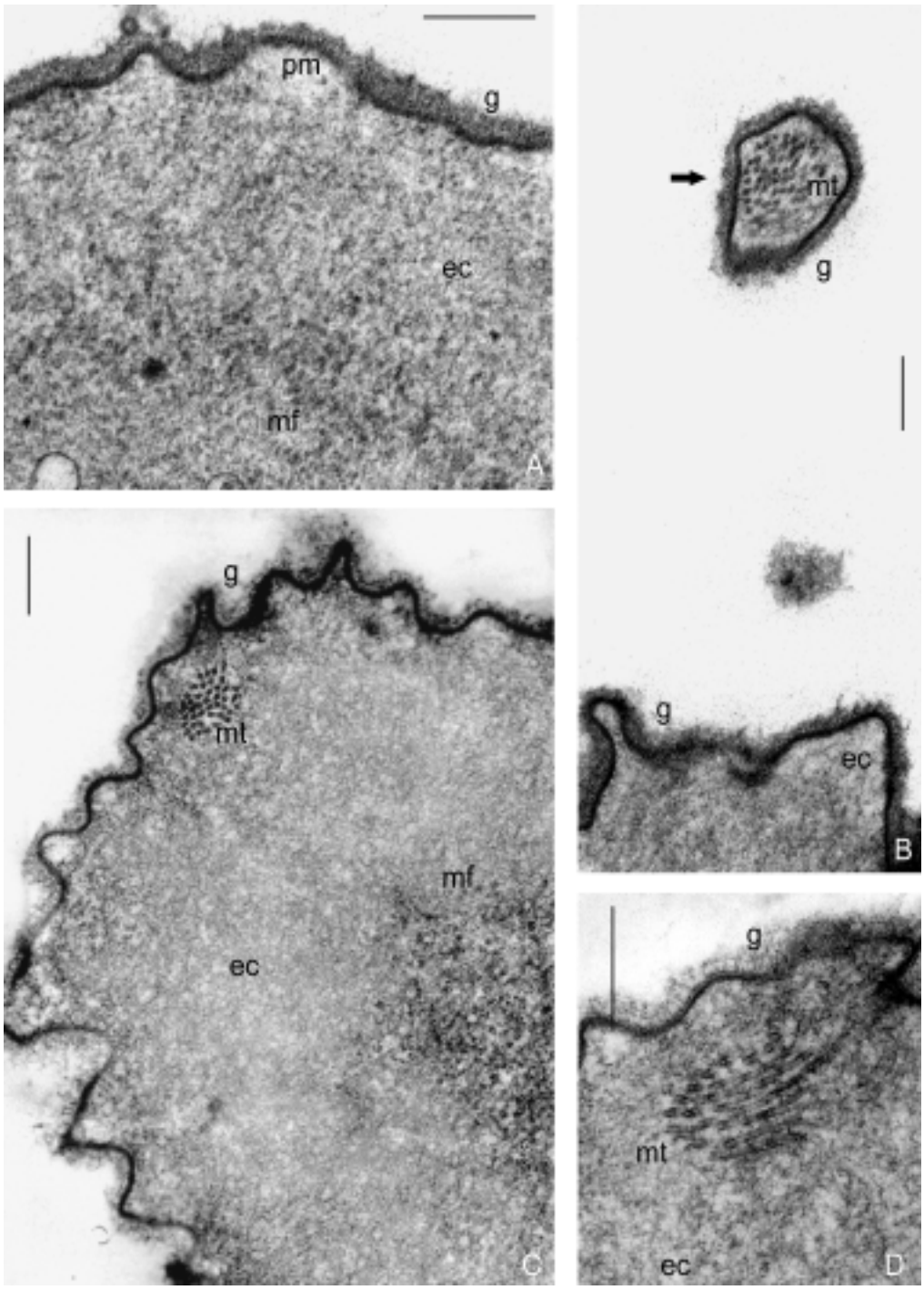

Fig. 2. Fine structure of *Pelomyxa corona*. A-D - cell surface and ectoplasmic organisation (*arrow* indicates a portion of thin hyaline projection). *Abbreviations*: ec - ectoplasm, g - glycocalyx, mf - microfibrills on the border of ecto- and endoplasm, mt - microtubules, pm - plasma membrane. Scale bars: 0.4 μm.

For examples, the flagellar apparatus of *Pelomyxa palustris* was described at the electron microscopic level independently almost at the same time in the USA (Griffin, 1988) and in Russia (Seravin, Goodkov, 1987a; Goodkov, 1989; Goodkov and Seravin, 1991), and in some "American" amoebae kinetosomal cones were described, whereas in the "Russian" ones, only lateral kinetosomal rootlets. It is very unlikely that lack of

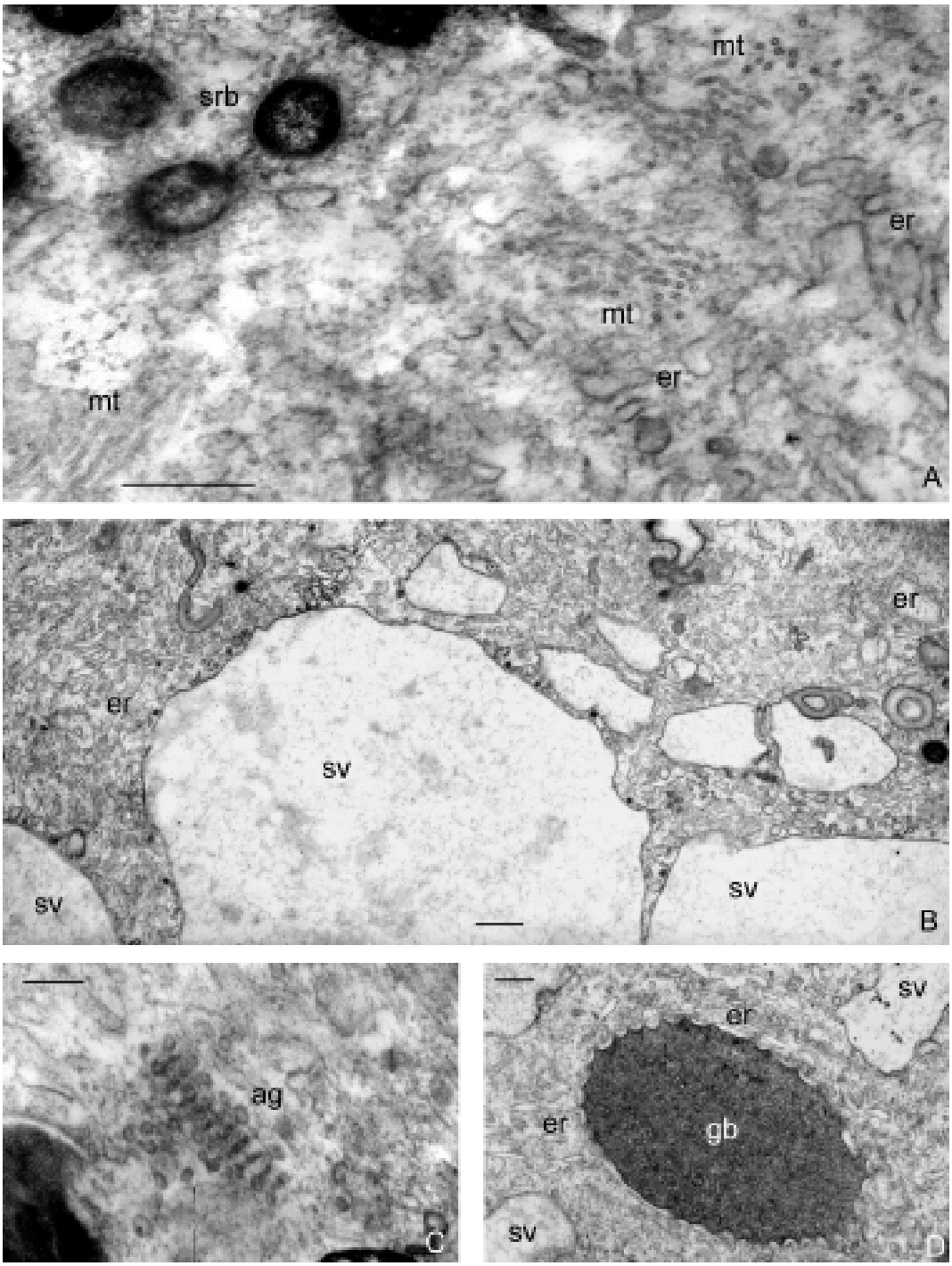

Fig. 3. Fine structure of *Pelomyxa corona*. A - a portion of endoplasm with prominent bands of microtubules; B - structural vacuoles; C - dictyosome of the Golgi apparatus; D - glycogen body surrounded by channels of the endoplasmic reticulum. *Abbreviations*: ag - Golgi apparatus, er - endoplasmic reticulum, gb - glycogen body, srb - small rod-like bacteria, sv –structural vacuole. Scale bars: 0.5μm.

cones in *Pelomyxa palustris* (sensu Seravin and Goodkov) is associated with morphological changes in the course of its life cycle, since these authors investigated the amoebae of different age in different seasons for many years with the same reproducible results. Thus, two organisms, corresponding well to the description of *P. palustris* (Greeff, 1874) at the light microscopical level, have considerable differences in

the flagellar rootlet system and appear to be two different species.

The amoebae under investigation are often found together with *P. palustris* but these organisms cannot be confused one with the other even under the light microscope, mostly due to the "crown" of hyaline pseudopodia formed by former. Light microscopic characters (relatively large size, vacuolated cytoplasm, multinuclearity, numerous bacterial endocytobionts, etc.) allowed us to suppose that the organism in question was a peculiar *Pelomyxa*, though flagella were not revealed.

Ultrastructural investigation yielded unexpected results. On the one hand, some of the features found were characteristic of *Pelomyxa* (Whatley, 1976; Griffin, 1988; Goodkov and Seravin, 2000; Brugerolle and Patterson, 2000). They were: well-developed microtubular cytoskeleton, two out of three species of bacterial endocytobionts found in *Pelomyxa palustris*, a characteristic organisation of endoplasm containing structural vacuoles, nuclear organisation, the position of symbiotic bacteria around the nuclei, the presence of glycogen bodies, lack of mitochondria. On the other hand, the dictyosomes of the Golgi apparatus and no traces of the flagellar apparatus were characters precluding, considering current ideas (Griffin, 1988; Brugerolle and Patterson, 2000), the attribution of this organism to the genus *Pelomyxa*. Moreover, its attribution to pelobiontids is also to be doubted.

The character "lack of the Golgi apparatus", included into the description of the taxon uniting pelobiontids, is really based on scarce factual material. In particular, the endoplasm of *P. palustris* is poorly studied, since previous ultrastructural investigations (Griffin, 1988; Seravin and Goodkov, 1987a; Goodkov, 1989) laid emphasis on the flagellar apparatus and the nuclei. The results of pioneer ultrastructural investigations of *P. palustris*, e.g., Andresen et al. (1968), Andresen (1973), Daniels et al. (1966), Daniels and Breyer (1967), Daniels (1973), are mostly inadequate for technical reasons; for the same reasons the researchers doubt the report of Seravin and Goodkov (1987b) on the presence of dictyosomes in the cytoplasm of *P. palustris*. Among mastigamoebids investigated ultrustructurally, in one species, *Mastigamoeba punctachora*, flattened sacs with terminal puffs were found in the cytoplasm, very similar to separate cisterns of the Golgi apparatus (Walker et al., 2001). The authors are very careful about interpretation of these structures, since they do not form a classic dictyosome with "cis" and "trans" poles. In our opinion, these structures look like a slightly disassembles dictyosome. The Golgi apparatus found in *P. corona* differs from the typical dictyosomes in proportions but otherwise has a classic organisation. Thus, it may considered as established that pelobionts may have true dictyosomes of the Golgi apparatus.

Flagella have also been thought of as a key character of pelobiontids. We do not rule out the possibility that the scarce bristle-like structures observed in the uroidal zone of *P. corona* under the light microscope may turn out to be true, though immobile flagella. However, though the microtubular cytoskeleton is well-developed in *P. corona*, MTOCs have not been found. In *P. palustris* the role of MTOC is played by kinetosomes or electron-dense structures associated with them (Goodkov and Seravin, 1987a; Griffin, 1988; Goodkov, 1989; Walker et al., 2001). Though the search for flagella in *P. corona* should be continued, we consider their absence in this pelobiont species as probable. The flagellar apparatus in *P. palustris* shows clear signs of degeneration (Seravin and Goodkov, 1987a; Goodkov, 1989; Goodkov and Seravin, 1991, Goodkov et al., 2004). The loss of locomotor function by flagella is accompanied by a considerable morphological reconstruction. It mostly results in a non-stable set of microtubules in the axoneme, but the flagellar kinetosome and its derivatives also change. If the organisation of the basal part of flagellum in uniflagellate pelobiontids, with developed microtubular cone and a lateral rootlet (Simpson, et al., 1997; Walker et al., 2001), is considered as an initial evolutionary state, the following changes may have happened in *P. palustris*. It may have retained the microtubular cone probably losing the lateral rootlet (Griffin, 1988), or, on the contrary, it may have lost the microtubular cone, retaining the lateral rootlet (Seravin and Goodkov, 1987a, Goodkov, 1989; Goodkov and Seravin, 1991). In other words, degeneration of the flagellar apparatus, associated with the evolutionary transition from the flagellate cell organisation to the amoeboid one, may be accompanied by loss of different elements (Goodkov and Seravin, 1991; Goodkov et al., 2004). In the extreme the degeneration may lead to the loss of the flagellar apparatus as a whole. Noteworthy, even among *Mastigamoeba*-like pelobionts there are species whose life cycle includes amoeboid forms lacking any signs of flagella (Simpson et al., 1997).

To conclude, morphological differences between *P. palustris* and *P. corona* are considerable and may exceed specific differences. These organisms may belong to different genera or even different taxa of higher rank. This problem calls for a detailed study of other pelobiontids, especially of multinuclear forms.

Diagnosis

Pelomyxa corona sp. n. (type Fig. 1 A).

Spherical *Pelomyxa*-like amoeba with pronounced hyaloplasm. Radiating hyaline pseudopodia of various

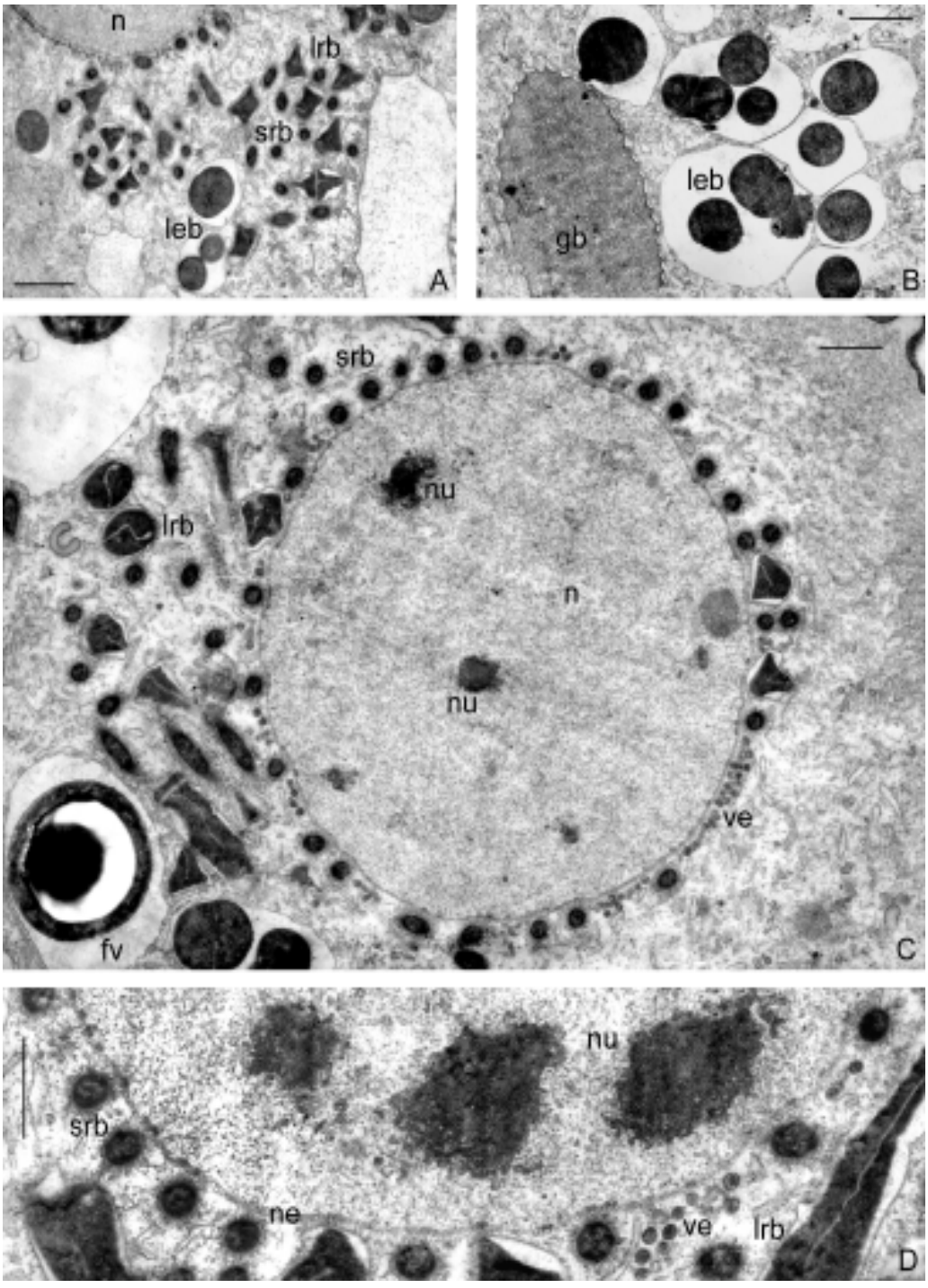

Fig. 4. Fine structure of *Pelomyxa corona* (continuation). A - three types of endocytobiotic bacteria; B - large ellipsoidal endocytobionts; C, D - nuclei surrounded by small and large rod-like bacteria. *Abbreviations*: fv - food vacuole, leb - large ellipsoidal bacteria (without cleft), lrb - large rod-like bacteria (with cleft), n - nucleus, ne - nuclear envelope, nu - nucleolus, p - nuclear pores, ve - dense vesicles associated with the nuclear envelope. Scale bars: A - 2,0 μm; B, C - 1,5 μm; D - 1,0 μm.

shape slightly branched, often forked at ends. Strong hyaline lobopodium formed during locomotion. Uroid consisting of numerous hyaline cylindrical papillae. Nuclei granular, numbering from 1 in young individuals to 100 and more in mature ones. Flagella not found. Diameter of uninucleate amoebae 50-70 μm, that of

multinucleate ones, 300-500 μm.

Habitat: silt, small freshwater basins overgrown with water plants.

Found in North-West Russia at approximately 58°35′ N and 28°55′ E.

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