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Structure in the *Pelomyxa* nuclei: key morphological patterns

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ABSTRACT: The pelomyxae are archamoebian multinuclear protists with a specific cellular organization, including a peculiar nuclear structure. It is generally accepted that all species belonging to the genus *Pelomyxa* are characterized by exceptional morphological diversity of their nuclei. However, a detailed comparative morphological analysis of 16 species of *Pelomyxa*, including one species that has not yet been formally described, allowed us to divide them into four clearly defined groups within one fundamentally common type of nuclear organization. No obvious correlation has been established between nuclear structure and other important species-specific features of pelomyxae, such as the organization of the flagellar apparatus, nuclear envelope, etc. It is possible that the observed differences in nuclear organization may reflect the peculiarities of cellular metabolism of certain species, which arose as a result of fine adaptation to the different microniches of the anoxic environment in which they all live.
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KEY WORDS: nuclear structure, ultrastructure, Archamoebae, *Pelomyxa* spp.

Структура ядер *Pelomyxa*: ключевые морфологические закономерности

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РЕЗЮМЕ: Пеломиксы — это архамебоидные многоядерные протисты с загадочной клеточной организацией, включая структуру ядра. Общепризнано, что все виды, относящиеся к роду *Pelomyxa*, характеризуются исключительным морфологическим разнообразием ядер. Однако детальный сравнительно-морфологический анализ 16 видов *Pelomyxa*, включая один вид, который пока формально не описан, позволил разделить их на четыре четко очерченные группы в пределах одного принципиально

общего типа ядерной организации. Не установлено очевидной корреляции между строением ядра и другими важными видоспецифическими признаками пеломикс, такими как организация жгутикового аппарата, состав эндоцитобионтов и т.д. Не исключено, что наблюдаемые различия в организации ядра могут отражать особенности клеточного метаболизма отдельных видов, возникшие в результате тонкой адаптации к различным микронишам бескислородной среды, в которой все они обитают.
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КЛЮЧЕВЫЕ СЛОВА: структура ядра, ультраструктура, Archamoebae, *Pelomyxa* spp.

Introduction

Representatives of the genus *Pelomyxa* (Amoebozoa, Archamoebae, Pelobiontida) are free-living anaerobic protists with an amoeboid type of cellular organization. In most cases, they have several or numerous non-motile or slightly mobile flagellae that do not participate in locomotion of the cell. The cytoplasm of pelomyxae is usually highly vacuolated and is characterized by the presence of numerous obligate prokaryotic endocytobionts. The life cycle of pelomyxae typically includes a multinucleate stage (Frolov, 2011; Ptáčková *et al*., 2013; Chistyakova *et al*., 2013; Walker *et al*., 2017).

The *Pelomyxa* species differ from each other in a complex of morphological features, including the peculiarities of the organization of the flagellar apparatus, cytoplasm, as well as the structure of the glycocalyx, the composition of prokaryotic endocytobionts, and the shape of cells during locomotion. It is believed that one of the most important features that allows differentiating distinct *Pelomyxa* species is the structure of their nuclei, which differ significantly in the organization of the nuclear envelope, the size and structure of the intranuclear inclusions/bodies, as well as the distribution pattern of these bodies in the nucleoplasm (Chistyakova *et al*., 2013, 2024a). The differences in the nuclear architecture *Pelomyxa* between the species appear so significant that the species can be identified quite accurately even with a light microscope (Table 1).

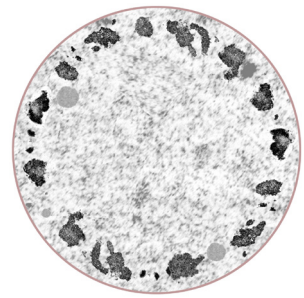
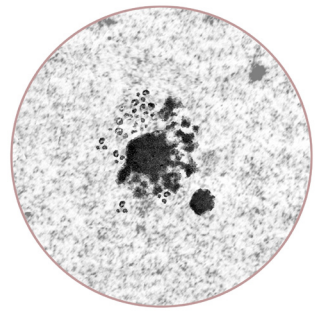
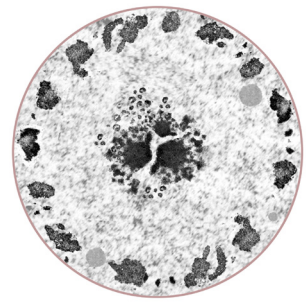
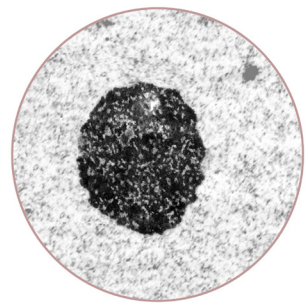
It cannot be ruled out that all currently known species of the genus *Pelomyxa* can in fact be divided into several independent taxa of at least genus rank. It is obvious that performing such a taxonomic revision should be based on an integrated approach using data from both comparative morphological and molecular phylogenetic analyses. However, the implementation of the latter, unfortunately, does not yet seem possible, since SSU sequences are available only for three species, *P. palustris*, *P. belevskii* and *P. stagnalis* (Ptáčková *et al*., 2013), and the genome has been assembled for only one species, *P. shiedti* (Záhonová *et al.*, 2022). It is all the more relevant to conduct a comparative analysis of pelomyxae according to a number of morphological characteristics.

Previously, the results of a similar analysis were published regarding the structural features of the flagellar apparatus, the composition of obligate prokaryotic endocytobionts, and the nature of the accumulation of reserve polysaccharides (Chistyakova *et al*., 2016, 2020a, b). The present study is devoted to a comparative analysis of the structural features of the nuclei in 16 representatives of the genus *Pelomyxa*. This number of species includes one representative of the genus (*Pelomyxa* sp.), which undoubtedly represent an independent species, but do not yet have a formal description. The structure of *P. shiedti* nuclei was assessed based on publications by others (Zadrobílková *et al*., 2015; Treitli *et al*., 2023).

Material and Methods

Samples of bottom sediments containing pelomixae were collected in the spring-autumn period of 2004–2023 from the following water reservoirs: 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 (*P. corona*, *P. prima*, and *P. flava*), 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 (*P. binucleata* and *Pelomyxa* sp.), Ceratophyllum Pond, Sergievka Park, St. Petersburg, Russia, 59°53′

Table 1. The representatives of the genus *Pelomyxa*, grouped in four categories depending on the structure of nuclear biomolecular condensates (BMCs).

Group	Species	Signature morphology of nuclear BMCs	Schematic representation of typical nuclear structure
I	***P. palustris**** *P. paradoxa* *P. belevskii* *P. corona* *P. flava* *P. schiedti*** *P. secunda*	Marginal BMCs of various morphologies; the central part of the nucleus is devoid of noticeable BMCs to a large extent	
II	***P. stagnalis*** *P. binucleata* *P. pilosa* *Pelomyxa* sp.	Central, usually amorphic BMC mass(es)	
III	***P. doughnuta*** *P. tarda*	Central mass(es) + marginal BMCs	
IV	***P. prima*** *P. gruberi* *P. quarta*	A single, large, compact, spherical BMC with complex internal ultrastructure	

*Typical/header species in each group are shown in bold.
**According to Zadrobílková *et al*. (2015) and Treitli *et al*. (2023).

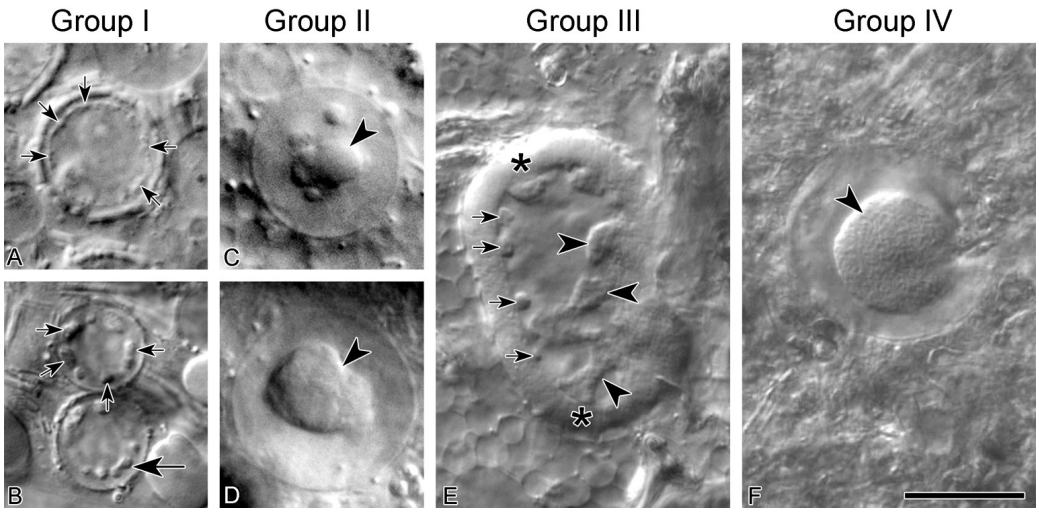


Fig. 1. Nuclei of representative members of four *Pelomyxa* groups, differing in morphology, as observed under a light microscope. A — *P. palustris*; B — *P. corona*; C — *P. stagnalis*; D — *P. binucleata*; E — *P. doughnuta*; F — *P. gruberi*. Arrows indicate marginal nuclear BMCs, presumably nucleoli; arrowheads point to BMCs in the central part of the nucleus; asterisks indicate a perinuclear layer of glycogen, specific for *P. doughnuta* (Chistyakova *et al*., 2022). Scale bar 20 μm is the same for all images.

N, 29°50′ E (*P. stagnalis*, *P. tarda*, *P. paradoxa*, *P. secunda*, *P. pilosa,* and *P. doughnuta*), Lake Osinovskoe, North-West of Russia, Leningrad Region (*P. palustris*, *P. belevskii*, *P. gruberi*, and *P. quarta*). The methods of collecting, storing and processing samples have been described in detail previously (Chistyakova *et al*., 2024a).

Fresh unfixed *Pelomyxa* cells were rapidly observed using a Leica DM2500 microscope equipped with DIC optics and a Leica DFM 495 digital camera. For fluorescent microscopy, non-fixed *P. stagnalis*, *P. belevskii* or *P. tarda* cells were simultaneously stained with 4',6-diamidino-2-phenylindole (DAPI) and 3,6-bis(dimethylamino)xanthylium (pyronin Y) as proposed (Bogolyubov *et al*., 2025a), and then immediately examined under an Axio S1 fluorescent microscope (Carl Zeiss) equipped with a set of appropriate filters.

For transmission electron microscopy (TEM), *Pelomyxa* specimens were collected individually using a glass pipette under a stereomicroscope. The specimens were immediately fixed in a mixture of 4% glutaraldehyde and 1% OsO_4 (1:1) in 0.1 M cacodylate buffer. Fixation, embedding, and sectioning were performed according to the protocol described in detail earlier (Frolov *et al*., 2005). Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined under a Morgagni 268 (FEI, the Netherlands), Tesla BS-500 (Czech Republic) or Libra 120 (Carl Zeiss, Germany) transmission electron microscopes.

Results and Discussion

Numerous microscopically visible nuclear substructures are now collectively referred to as membraneless organelles, which facilitate biochemical reactions by co-concentrating various factors and/or creating interchromosomal hubs that contribute to the regulation of gene expression (Hirose *et al*., 2023). They are also often referred to as biomolecular condensates (BMCs), as they arise from liquid-liquid phase separation of polymeric macromolecules, primarily intrinsically disordered, multivalent proteins (Banani *et al*., 2017). The nuclei of archamoeboid protists belonging to the genus *Pelomyxa* contain a great variety of BMCs, still poorly characterized even at the morphological level. Despite their impressive morphological diversity, among the 16 studied species of pelomyxae, four groups can be distinguished already at the light level, based on the BMCs' morphology and their location in the nucleus (Table 1). Ultrastructural morphology allowed us to clarify the fine structure of these BMCs in representative specimens of each group (see below). The morphology of *P. schiedti* nuclei was analyzed based on TEM images presented in the papers of other authors (Zadrobílková *et al*., 2015; Treitli *et al*., 2023).

The pelomyxae of Group I exhibit numerous nuclear BMCs located peripherally (Fig. 1A–B). The rest of the nucleoplasm looks rather "empty", for it is devoid of prominent inclusions. These peripheral BMCs vary in size in different species, being from minute-rushed and barely noticeable in *P. palustris* (Fig. 1A) and to a certain degree in *P. paradoxa* till more perceptible lumpy structures in *P. belevskii*, *P. secunda*, *P. flava* and *P. corona* (Fig. 1B). If, for example, in *P. corona* these BMCs are always clearly separated by a space from the nuclear envelope, then in *P. secunda*, on the contrary, they are closely associated with it. In some species, such as *P. belevskii*, the association of BMCs with the nuclear envelope is not always obvious at the light level.

Group II includes four species, *P. stagnalis*, *P. binucleata*, *P. pilosa* and *Pelomyxa* sp., which lack peripheral nuclear BMCs (Fig. 1C–D). Instead, their nuclei contain conspicuous inclusions, usually one or sometimes several. If there are several such inclusions, one is always noticeably larger than the others.

Group III includes two species, *P. doughnuta* and *P. tarda*, exhibiting both small peripheral nuclear BMCs located in a close vicinity of the nuclear envelope and an amorphous mesh of prominent lumpy structures in the inner part of the nucleus (Fig. 1E). A relatively low density of the centrally located BMC material compared to the marginal BMCs does not always allow it to be clearly seen at the light level. However, the central BMC material is apparent when examined using TEM (see below). In addition to the specific nuclear structures, *P. doughnuta*, a representative of Group III, is distinguished by the presence of a perinuclear layer of glycogen (Chistyakova *et al*., 2022), which masks the nuclear boundary in this species.

Group IV includes three species, *P. prima*, *P. quarta* and *P. gruberi*, which are distinguished from all other species of pelomyxae by the presence in the nuclei of a single compact BMC (Fig. 1F). The voluminosity, solitariness and ideally spherical shape of this BMC allowed us to distinguish representatives of Group IV, especially from Group II, separating it into a separate covey. Notably, the nuclei of Group IV pelomyxae with their characteristic single BMCs, clearly resemble those of some other amoeboid protists, such as the naked amoebae *Dermamoeba fibula* (Mesentsev *et al*., 2023) and *Paradermamoeba levis* (Kamyshatskaya, Smirnov, 2016).

Simultaneous fluorescent staining of non-fixed pelomyxae with pyronin and DAPI (Bogolyubov *et al*., 2025a) allowed us to make four key observations (Fig. 2A–C): (1) the nuclear BMCs of *Pelomyxa* are highly enriched in RNA regardless of their morphology; (2) these BMCs are not clumps of condensed chromatin, since they poor in DNA; (3) regardless of the diversity of nuclear architecture, chromatin in different *Pelomyxa* species is in a highly decondensed state, the network of which fills almost the entire nuclear volume, with the exception of areas where BMCs are located. Thus, despite the apparent morphological diversity, the nuclei of most of the studied pelomyxae can generally be classified as "ovular" type according to Raikov (1982), with the exception of representatives of Group IV with their large vesicular nuclei. According to Raikov (1982), vesicular nuclei, unlike ovular ones, never contain multiple nucleoli.

It is highly likely that most of the BMCs we observe are nucleoli. However, at this stage of nuclear research on pelomyxae, we cannot yet say this with certainty. Furthermore, it should be understood that (1) not all RNA-rich nuclear BMCs are nucleoli and (2) a portion of pyronin signal is obviously associated with chromosomal transcription. For example, a clear pyronin signal is observed on *P. belevskii* and *P. tarda* chromatin (Fig. 2A, C), suggesting chromosomal transcription. However, it was not as obvious in *P. stagnalis* (Fig. 2B), which may be due to differences in the life cycle.

To confirm the nucleolar nature of *Pelomyxa* nuclear BMCs, first, the nucleolus organizer regions (rDNA sequences) should be established and characterized, which is currently impossible given the paucity of data on the *Pelomyxa* genomes. Second, it is necessary to confirm the ongoing rDNA transcription, which leads to the formation of nucleoli in the cell, and the involvement of specific BMCs in the production of ribosomes (Lafontaine *et al*., 2021). The difficulty of identifying *Pelomyxa* nucleoli is also due to the fact that some evolutionarily conserved nucleolar proteins, such as nucleolin, even though they can be detected immunocytochemically using commercial antibodies against mammalian proteins, are present in the nucleus in much lower

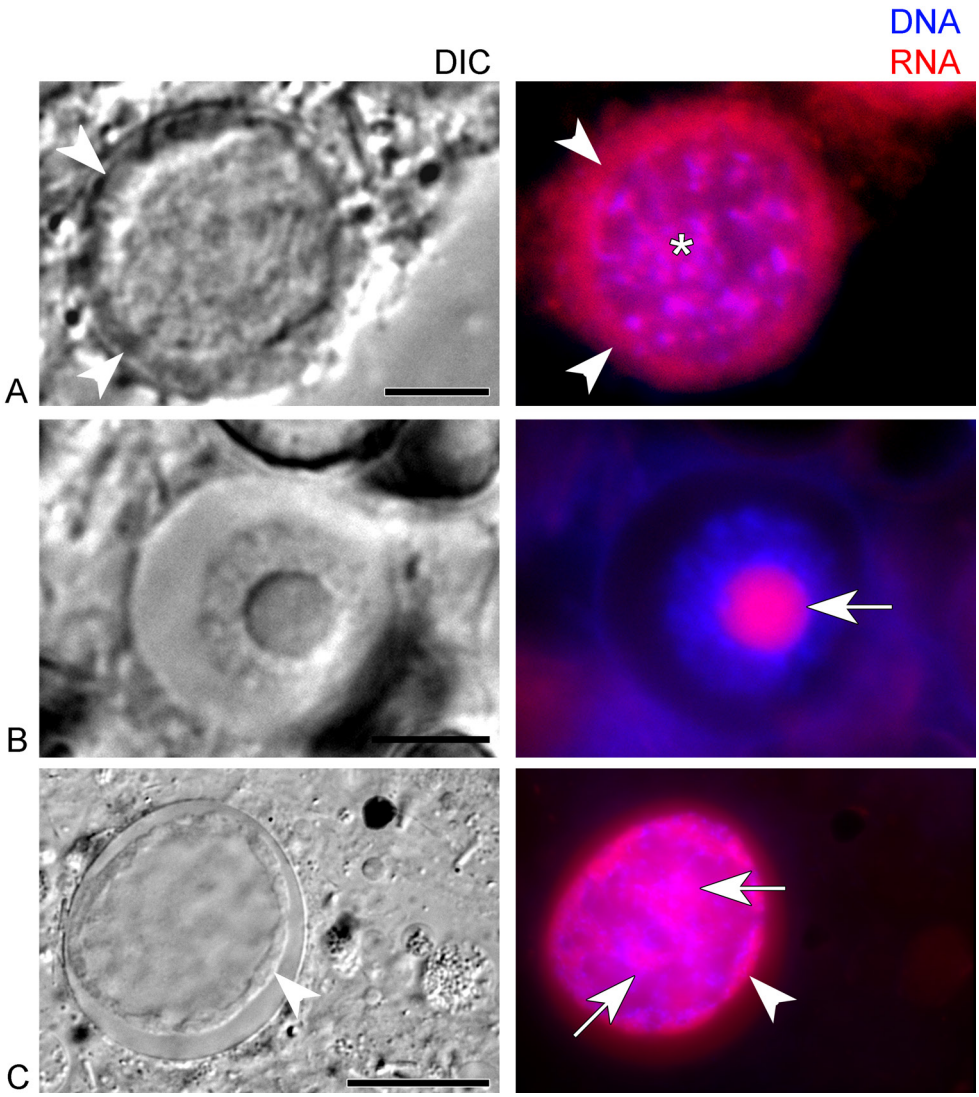


Fig. 2. Nuclei of three *Pelomyxa* species simultaneously stained with pyronin (red) for RNA and DAPI (blue) for DNA. A — *P. belevskii*, asterisk indicate pyronin signal on chromatin; B — *P. stagnalis*; C — *P. tarda*. Note to different morphology of nuclear RNA-containing BMCs, presumable nucleoli, but similar chromatin meshwork in these species. Marginal (arrowheads) and centrally located nuclear BMCs (arrows) are indicated. Scale bars: A, B — 5 μm; C — 20 μm.

quantities than would be expected, for example in *P. belevskii*, which exhibits a structurally complex and highly developed putative nucleolar apparatus (Bogolyubov *et al*., 2025b).

A comparative TEM study has elucidated the fine structure of *Pelomyxa* BMCs, presumably nucleoli, and verified the assignment of a particular species to a particular group. In Group I, the marginal nuclear BMCs can be either irregularly-shaped finely-fibrillar and loosened clumps as in *P. palustris* and *P. flava* (Fig. 3A, C) or reticular nucleolonema-like formations as in *P. belevskii* (Fig. 3B). In *P. secunda*, these BMCs exhibited the highest electron and packing densities of the fibrillar material. Electron microscopy also confirmed

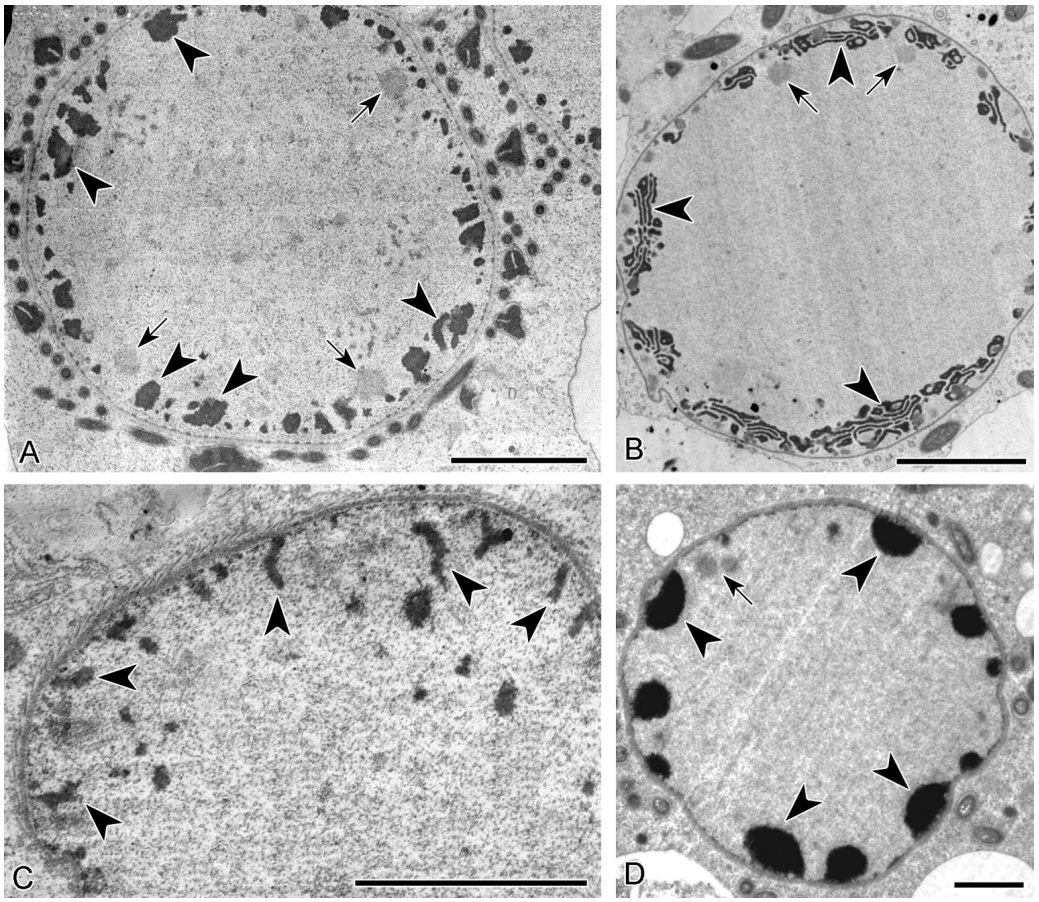


Fig. 3. Nuclear morphology of some *Pelomyxa* species of Group I. A — *P. palustris*; B — *P. belevskii*; C — *P. flava*; D — *P. secunda*. Arrows indicate fine-fibrillar spherical BMCs, distinct from the putative nucleoli (arrowheads). Scale bars: A–C — 5 μm; D — 2 μm.

their close association with the nuclear envelope in this species (Fig. 3D). It should be noted that *P. schiedti*, not explored in our study, appears to belong to the same group, judging by the TEM images presented in the papers by Zadrobílková with coauthors (2015) and Treitli with coauthors (2023). Since there are currently no approaches to reliably identify key components of putative *Pelomyxa* nucleoli, such as fibrillar centers and the dense fibrillar component (Hernandez-Verdun, 2011), it is not yet possible to conclude that the reticular nucleoli of *P. belevskii* correspond to canonical nucleolonemic nucleoli (Sato *et al*., 2005), as already suggested by the study with nucleolin immunogold labeling (Bogolyubov *et al*., 2025b).

TEM confirmed the absence of marginal BMCs and the presence of one or, less commonly, several massive formations localized in the central part of the nuclei of Group II pelomyxae (Fig. 4A–D). In a particular species belonging to this group, the latter masses may have a reticulate structure, predominantly at their periphery (Fig. 4A, D), or represent more or less compact conglomerates of small "bodies" (Fig. 4B), or a combination of both (Fig. 4C).

Group III pelomyxae somewhat combine the features of previous two ones. They simultaneously display multiple compact BMCs at the nuclear periphery and reticular mass(es) in the rest of the nucleoplasm (Fig. 5A–B).

Finally, all three *Pelomyxa* species that we assigned to Group IV contain a single, large and compact BMC that exhibits a highly complex internal ultrastructure (Fig. 6A–C). It is obvious that it appears by the coexistence of distinct,

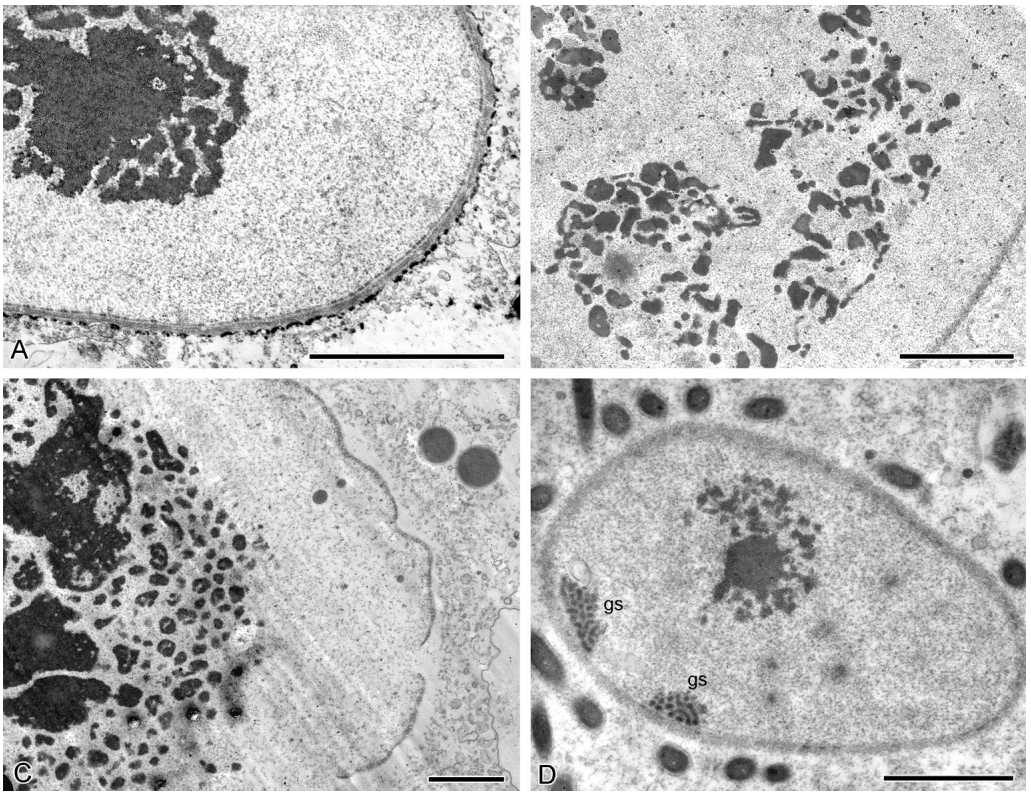


Fig. 4. Nuclear morphology of Group II *Pelomyxa* species. A — *P. stagnalis*; B — *P. binucleata*; C — *P. pilosa*; D — *Pelomyxa* sp. Abbreviation: gs — glomerulosomes. Scale bars 5 μm.

liquid phases of constituting polymer macromolecules, as is the case of segregated components of the nucleolus (Feric *et al*., 2016) and other RNA-containing nuclear bodies. It is important that not only proteins but also RNA may play a key role in the formation of BMCs and the creation of their complex internal organization due to phase transitions inside the liquid droplet (Sawyer *et al*., 2019).

However, in the BMC of Group IV pelomyxes it is not possible to identify typical nucleolar subcompartments, namely fibrillar centers, dense fibrillar and granular components, which are well documented morphologically in canonical eukaryotic nucleoli (Hernandez-Verdun, 2011). This BMC represents a dense, compact skein of heteromorphic fibrils and granules of varying electron density (Fig. 6A–C), the nature of which is unclear, as is the nucleolar nature of this nuclear body itself. Despite certain doubts, it is well-known that rRNAs are the most abundant in the cell, accounting for ~80% of the total RNA content (Feng, Manley, 2022). This makes it likely that most of the specific BMCs found in all *Pelomyxa* groups, regardless of their morphology, may represent nucleoli or be genetically related to them, given that these BMCs are highly enriched in RNA at least in *P. belevskii*, *P. stagnalis* and *P. tarda* (Bogolyubov *et al*., 2025a; and this paper).

At the same time, regardless of the group to which a species belongs, its nuclei may contain various types of non-nucleolar BMCs, such as glomerulosomes (Bogolyubov *et al*., 2022) (Fig. 4D) or fine fibrillar "spheres" (Raikov, 1982) (e.g., Figs 3A–B, D; 5B), some of which are associated with the putative nucleolar material, while others are observed separately from it in the nucleoplasm. Although their nature is still elusive, such "spheres" may represent counterparts of Cajal bodies (Gall *et al*., 1995). To date, each of the *Pelomyxa* BMCs, including nucleoli, requires special study to prove and/or clarify their nature.

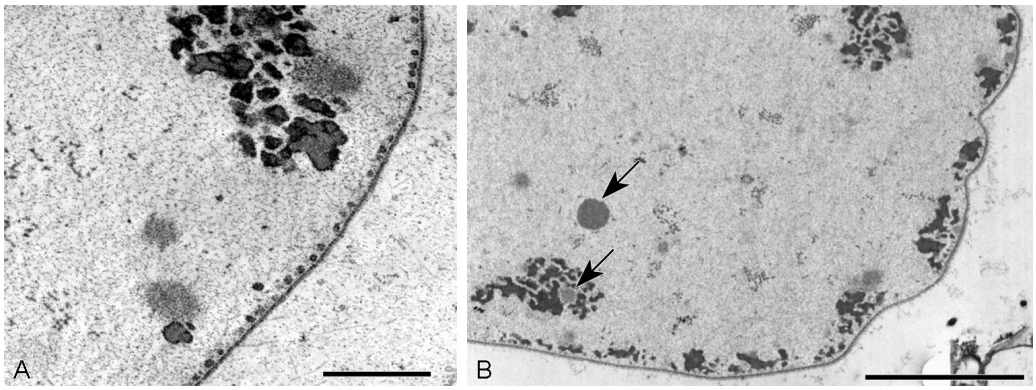


Fig. 5. Nuclear morphology of Group III *Pelomyxa* species. A — *P. doughnuta*; B — *P. tarda*. Arrows indicate spherical non-nucleolar BMCs. Scale bars 5 μm.

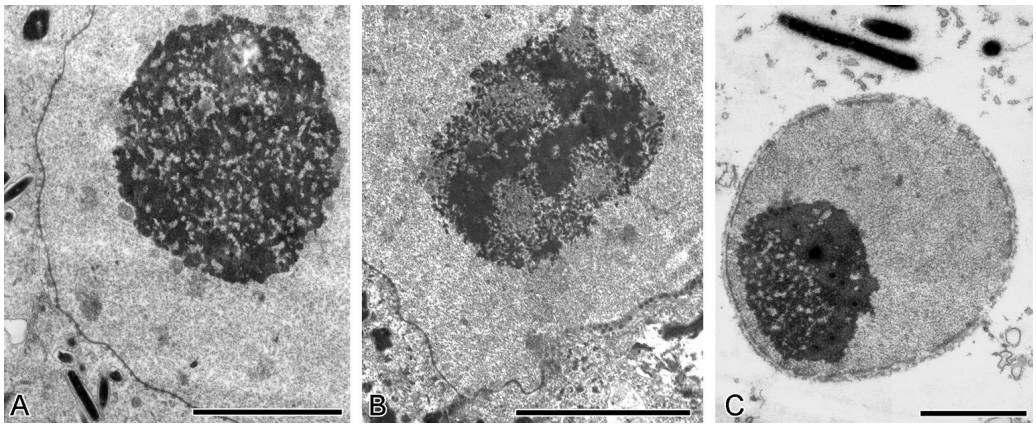


Fig. 6. Nuclear morphology of Group IV *Pelomyxa* species. A — *P. prima*; B — *P. gruberi*; C — *P. quarta*. Note the single, large BMC with a spherical shape and complex internal structure, presumably the nucleolus. Scale bars: A, B — 5 μm; C — 2 μm.

Our proposed *Pelomyxa* grouping is primarily based on the morphological characteristics of nuclei, especially nuclear BMCs, which are considered typical for specific species of the genus *Pelomyxa* and are used in their identification (Chistiakova *et al*., 2021, 2024a). Below we will consider how these nucleus-based nomenclature fit together with other important morphological features of *Pelomyxa*.

One of the most important taxonomic features is the structure of the basal flagellar apparatus (Chistyakova *et al*., 2020a). A comparison of the results of analyses of the features of nuclear organization in representatives of the genus *Pelomyxa* with the available data on the structure of the flagellar apparatus showed that within each group, different variants of the organization of the basal zone of the flagellum are observed. For example, in *P. flava*, a representative of Group I, three groups of microtubules are associated with the kinetosome: radial, basal, and the lateral rootlet (Frolov *et al*., 2011). In *P. palustris*, *P. belevskii* and presumably *P. schiedti*, there are radial microtubules (Ptáčková *et al*., 2013; Zadrobílková *et al*., 2015). However, it is necessary to note here that the flagellar apparatus of pelomyxae is an unstable structure. It shows signs of reduction in these protists, which is probably associated with the loss of locomotory function of the flagellae (Goodkov, 1989; Chistyakova *et al*., 2020a). For example, flagella are not found at all in *P. tarda*, a representative of Group III (Chistyakova *et al*., 2021). Although the organization of the basal apparatus of the flagellum

is a significant systematic feature, in a number of *Pelomyxa* species, flagellae with a normal set of rootlet microtubules, as well as flagellae practically devoid of them, can be observed within a single cell (Chistyakova *et al*., 2020a).

A similar situation occurs with respect to another important feature used in the differentiation of species of the genus *Pelomyxa*, namely the organization of the nuclear envelope. Thus, within Group I, all the main types of nuclear envelope complication found in *Pelomyxa* are observed: a layer of small vesicles surrounding the nucleus in *P. corona*; short microtubules extending at equal distances from the nuclear envelope in *P. secunda*; and a multilamellar layer associated with the layer of small vesicles in *P. paradoxa* (Frolov *et al*., 2004; Chistyakova *et al*., 2014; Berdieva *et al*., 2015).

It cannot be excluded that differences in the organization and distribution of individual components in the nucleoplasm, including RNA-containing BMCs, in different species of *Pelomyxa* reflect the peculiarities of their metabolism associated with species-specific adaptation to various microenvironments formed in the anaerobic zone of bottom sediments of fresh water bodies where these protists live. Metabolic interactions between the host cell and prokaryotic endocytobionts, the set of which in the cytoplasm is often specific for different species of pelomyxae, may also play a certain role (Chistyakova *et al*., 2016; Walker *et al*., 2017). In this context, it is interesting to note that the ability to accumulate glycogen in the form of well-formed cytoplasmic clusters is not observed in pelomyxae belonging to group IV, which have a compact spherical nucleolus-like BMC (Frolov *et al*., 2005, 2006; Chistyakova *et al*., 2024b). The specificity of the biology and metabolism of *Pelomyxa* may also be associated with the appearance in the cytoplasm of some types of unusual inclusions that accumulate the nucleolar protein nucleolin (Bogolyubov *et al*., 2025b).

In conclusion, the proposed classification, which made it possible to divide all studied species of *Pelomyxa* into groups based on the nuclear/BMC morphology, is rather formal and takes into account only superficial morphological characteristics. When the nature, molecular composition and, most importantly, the functions of *Pelomyxa* nuclear BMCs are established, this classification will undoubtedly require revision. In addition, there is no doubt that this classification will also be improved with the emergence of new knowledge about the systematics and phylogeny of representatives of the genus *Pelomyxa* – archamoeboid protists, amazing in their biology, ecology and diversity.

Compliance with ethical standards

CONFLICT OF INTEREST: The authors declare that they have no conflict of interest.

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