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ORIGINAL ARTICLE

New data on the fine structure of *Chaos nobile* CCAP 1511/2 (Amoebozoa, Tubulinea)

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Summary

The genus *Chaos* is one of six amoebae genera belonging to the family Amoebidae (Amoebozoa, Tubulinea). Together with members of the genus *Amoeba*, representatives of this genus are known as "classical" amoebae and are used as laboratory objects for a variety of cytological, physiological and biochemical studies. Non-surprisingly, these amoebae early became objects of ultrastructural investigations, and most of those studies were performed in the 1960-1980s. However, the majority of them were devoted to the "model" objects: *Chaos carolinense* and *Chaos illinoisense*. Other species received little attention. Currently, many previously described strains of the genus *Chaos* are lost. DNA samples and molecular data on them are absent. Morphological comparison remains the only way to identify these species, if they are re-isolated. Such a comparison requires as much data as possible. In this context, re-investigation of available materials on early studied strains using modern technical facilities is of great value.

In this paper, we report a study of the ultrastructure of *Chaos nobile* CCAP 1511/2 strain. This strain was the only available live representative of the species in culture collections and is now lost. For our study, we used embeddings of these amoebae made by A. Smirnov in 1999, during his stay at CCAP. Our study revealed mitochondrial heteromorphism in this species and some new details of the cell membrane and nuclear structure, including the presence of clusters of RNP helices in the karyoplasm (characteristic of some other Amoebidae species). Our images complement data on this species obtained by Gromov and Page in the 1980s and provide a comprehensive picture of the ultrastructure of this species.

Key words: Amoebidae, *Chaos*, Amoebozoa, ultrastructure, mitochondrial heteromorphism

Introduction

The genus *Chaos* was established by Linnaeus (1758) for the amoeboid organism *Chaos chaos* unidentifiable from his description. Later, Schaeffer (1926) transferred into this genus the amoeba, identified earlier as *Pelomyxa carolinensis* by Wilson (1900). This proposal was not universally accepted by researchers. For a long time, simultaneously with the name "*Chaos carolinensis*", the names "*Chaos*

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chaos" and "P*elomyxa carolinensis*" were used (this nomenclature confusion was described in detail by Goodkov et al., 2004). Further, with the studies by Bovee (1985), Page (1986, 1987, 1988), Whatley and Chapman-Andresen (1990), the genus *Chaos* was finally established and diagnosed.

Nowadays, the genus *Chaos* belongs to the family Amoebidae and includes four species: *Ch. carolinense* (Willson, 1900), *Ch. illinoisense* (Kudo, 1950), *Ch. nobile* (Penard, 1902), and *Ch. glabrum* (Smirnov et Goodkov, 1997). The most extensively studied species is *Ch. carolinense*, which was for a long time the popular laboratory model organism for physiological (Griffin, 1964; Daniels and Breyer, 1968; Deng et al., 2017; Chong et al., 2018, 2021; Almsherqi, 2021), biochemical (Bruce and Marshall, 1965; Friz, 1968; Sopina, 1993, 1999) and cytological studies (Andresen, 1956, 1973; Brandt and Pappas, 1960; Christiansen and Marshall, 1965; Chapman-Andresen, 1976; Gromov, 1986a). The species *Ch. Illinoisense* has also been widely investigated (Daniels and Roth, 1955, 1964; Daniels, 1958, 1962, 1964; McClellan, 1958, 1959; Daniels and Breyer, 1965, 1966). It was re-isolated and accurately described by Goodkov et al. (1999). The species *Ch. glabrum* was described by Smirnov and Goodkov (1997) and has not been seen since that time.

There are little data on the morphology of the last species of the genus *Chaos* – *Ch. nobile*. Page (1981, Figs 16–21) provided microphotographs of fixed amoebae from the stained preparations deposited by E. Penard in the collection of the British Museum of Natural History (London). Page (1986) studied a strain isolated in the USA by I.J. Lorch in 1972 and identified as belonging to this genus and species by C. Chapman-Andresen (Page, 1986, p. 302). He provided data on the organization of the cell coat (Fig. 11), the fine structure of the nucleus, and the internal nuclear lamina (Figs 26–28). Descriptions of live amoebae, supplied with microphotographs of locomotive forms and nuclei, were provided by F. C. Page in 1988 (Fig. 13, D–F) and 1991 (Fig. 15, C, E). The main focus of Gromov's study (1986b) was the ultrastructure of mitosis, so he provided only images of the nucleus. The overall ultrastructure of *Ch. nobile* was never studied.

The strain of *Chaos nobile* studied by Page (1986) was deposited in the Culture Collection of Algae and Protozoa (CCAP, UK) under the number 1511/2, but is now lost. Alexey Smirnov, during his stay at CCAP in 1999 collected some light microscopic data and prepared embeddings of this strain for electron-microscopic study. Here we report the results of the examination of these embeddings. Our images complement the data on this species obtained earlier by Gromov (1986b) and Page (1986) and provide a comprehensive picture of the ultrastructure of this species.

Material and methods

The strain CCAP 1511/2 was maintained on the Chapman-Andresen's modified Pringsheim's solution with wheat grains (Chapman-Andresen, 1962; Page, 1986). Amoebae in the culture fed on *Colpidium striatum*, other eukaryotes and accompanying bacteria (Page, 1986). Light microscopic observations, imaging and video records were performed on the glass object slides under room conditions using Olympus BH2 microscope, equipped with the phase contrast and DIC optics. Video records were done using JVC sVHS camera. Images were made in the year 1999.

For transmission electron microscopy, amoebae were collected and transferred using tapered-tips glass Pasteur pipettes. The cells were fixed individually in glass embryo dishes with 2.5% glutaraldehyde made in 0.05M phosphate buffer (pH 7.4) for 40 min. After the fixation, the cells were washed in the same buffer 3 times for 5 min. Then, postfixed for 1 hour with osmium tetroxide prepared in the same buffer at the final concentration of ca. 2%. Further, amoebae were washed again, prior to dehydration, 3x5 min in the same buffer. All fixation procedures were carried out under room temperature. Dehydration was in a grade ethanol series followed by propylene oxide and embedding in Spurr's resin according to the manufacturer's instructions.

Embeddings were done in the year 1999. Sections were cut in 2022 and 2023 using a Leica Ultracut 7 ultramicrotome and thereafter double-stained with 2% aqueous solution of uranyl acetate and Reynolds' lead citrate. The samples were examined with a JEOL JEM-1400 (JEOL, Ltd., Tokyo, Japan) electron microscope at 80 kV.

Results

Light microscopic observations

The results of our light microscopic observations of the strain *Chaos nobile* CCAP 1511/2, as well as the cell size and the size of nuclei generally corresponded to the data obtained for this strain by Page (1986).

During rapid locomotion, the cells of the strain CCAP 1511/2 were orthotactic (Fig. 1, A–E, L, M), with broad tubular pseudopodia, consisting of granulo- and hyaloplasm (Smirnov and Brown, 2004). The anterior hyaline zone looked like a thin crescent-like area on the tip of the pseudopodium (Fig. 1, C, F). Cells often had small but distinct lateral wrinkles (Fig. 1, A–C, F, G) and/or remnants of several tiny lateral pseudopodia (Fig. 1, B, C, F, G). The latter branched from a single leading pseudopodium (Fig. 1, B, C, G). Usually, they were located at the anterior part of the cell (Fig. 1, F). Lateral pseudopodia never participated in locomotion. When the cell changed the direction of movement, one of the lateral pseudopodia could become a leading one (Fig. 1, C–E). Slower moving cells had several short pseudopodia mainly in the anterior part of the cell (Fig. 1, I–K). If such polypodial amoebae started to move faster, they adopted the orthotactic form with additional lateral hyaline lobes (Fig. 1, L, M). Most cells during locomotion demonstrated a distinct bulbous (Fig. 1, F, G) or morulate uroid (Fig. 1, A–E, M).

Numerous nuclei had elongated, ellipsoid forms (Fig. 1, H). They were granulated, with abundant compact particles of nucleolar material diffused along the periphery of the nucleus. The cytoplasm of cells contained numerous truncate bipyramidal crystals (Fig. 1, H). Cysts were never seen.

Transmission electron microscopy

The cell surface of *Chaos nobile* strain CCAP 1511/2 was covered with glycocalyx differentiated into two layers. The total thickness of the cell coat in our sections ranged from 90 to 100 nm (Fig. 2, A). The basal amorphous layer, 10–15 nm in thickness, was electron-dense and tightly fitted to the plasma membrane. The crinkled filaments originating from the basal layer formed the outer layer. Some filaments had dendritic shapes (with a trunk extending from the basal layer and branches, directed apically). The thickness of this layer was 80–90 nm. During the fixation procedures, glycocalyx was fixed not homogeneously. The basal layer was always successfully preserved, but the filamentous layer could occasionally coagulate and the overall layer looked slightly compressed from above in the distal part.

The cytoplasm was subdivided into the hyaloplasm, located mostly in the periphery of the cell, and the granuloplasm, occupying its inner volume. At the border between the granuloplasm and the hyaloplasm, no distinct bundles of microfilaments were noticed, as is typical for many amoebae species, for example: *Polychaos centronucleolus* (Kamyshatskaya et al., 2021, Fig. 5, B), *Deuteramoeba mycophaga* (Kamyshatskaya et al., 2022, Fig. 2, B). At the same time, individual microfilamentous bundles were abundant in the granuloplasm. We also observed a huge curved strand of microfilaments in the area of highly vacuolated cytoplasm (Fig. 2, C).

The granuloplasm contained mitochondria and food vacuoles (Fig. 2, C). The contractile vacuole was surrounded by numerous vesicles, forming the spongiome (Fig. 2, B). Endocytobiotic rod-shaped bacteria found in the cytoplasm were not surrounded by a membrane. They were not numerous, the length of a bacterium varied from 1.5 μm to 2.5 μm, the diameter – 250–300 nm (Fig. 2, D). The dictyosomes of the Golgi complexes were composed of 2–3, occasionally 4 cisternae surrounded by vesicles (Fig. 2, E). Dictyosomes were not numerous, and we never saw them grouped together.

All observed mitochondria had tubular branched cristae. By the size of cristae, their pattern of organization and matrix density, they can be divided into two basic types. Mitochondria of the first type had more electron-dense matrix, comparatively wide cristae and irregular or elongated profiles in sections (Fig. 3, A, E). Mitochondria of the second type demonstrated a matrix of lower electron density, narrow and crimped cristae (Fig. 3, B). They consistently displayed regular rounded or only slightly elongated shapes in the sections. However, most of the mitochondria in the cells were not easily assigned to one of the types described above (Figs 2, C; 3, C, E). Apparently, they represented the transitional forms. Such mitochondria had less dense matrix than mitochondria of the first type, but had elongated profiles and wider cristae, characteristic to the mitochondria of the second type. They often were arranged in groups (approximately 5–20) accompanying mitochondria of the first type (Fig. 3, E). In a single case, we found a rounded mitochondrium with highly anastomosing cristae (Fig. 3, D).

Nuclei in our sections were elongated (Fig. 4, A, B), with uneven edges (Fig. 4, C). Tight granules of nucleolar material occupied the periphery of the nucleus (Fig. 4, B, C). Small loose patches of this material were also localized in its central part (Fig. 4, C). The inner honeycomb-like nuclear lamina, about 100 nm in thickness, was better visible in tangential sections (Fig. 4, D). It was organized in loose, rounded hexagonal cellules, 125–160 nm in

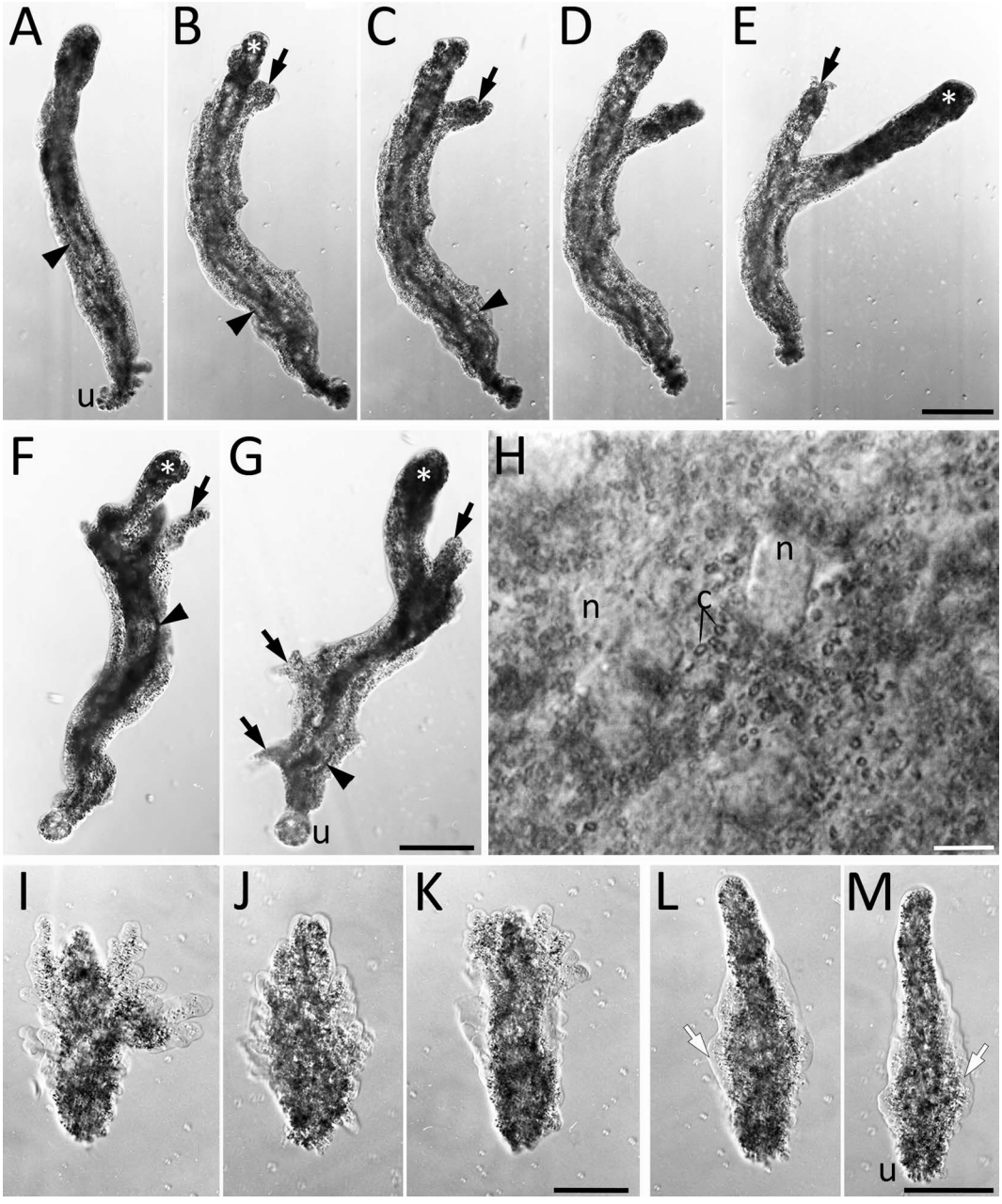

Fig. 1. Light microscopic images of *Chaos nobile* strain CCAP 1511/2. DIC. A-E, L-M – Orthotactic locomotive forms; C-E – cell, changing the direction of locomotion; H – nuclei and bipyramidal crystals in cytoplasm; I-K – polypodial cell with short pseudopodia in its anterior part; L-M – the orthotactic cell with lateral hyaline lobes. Distinct lateral pseudopodia are marked with black *arrows*, lateral wrinkles – with black *arrowheads*, leading pseudopodium – with white *asterisk*, lateral hyaline lobe – with white *arrow*. *Abbreviations*: u – uroid, n – nucleus, c – crystals. Scale bars: A-E, F-G, I-K, L-M – 100 μm; H – 10 μm.

size. Each cellule encircled a single typical nuclear-pore complex, showing the peripheral spoke ring assembly and the central plug (Fig. 4, D). In several nuclei, we observed the protrusions of the nuclear envelope outwards into the cytoplasm (Fig. 4, E). The karyoplasm of observed nuclei contained clusters of intranuclear helices (Fig. 4, F–H). These helices were predominantly localized near the nuclear envelope and close to the nuclear pore complexes (Fig. 4, H).

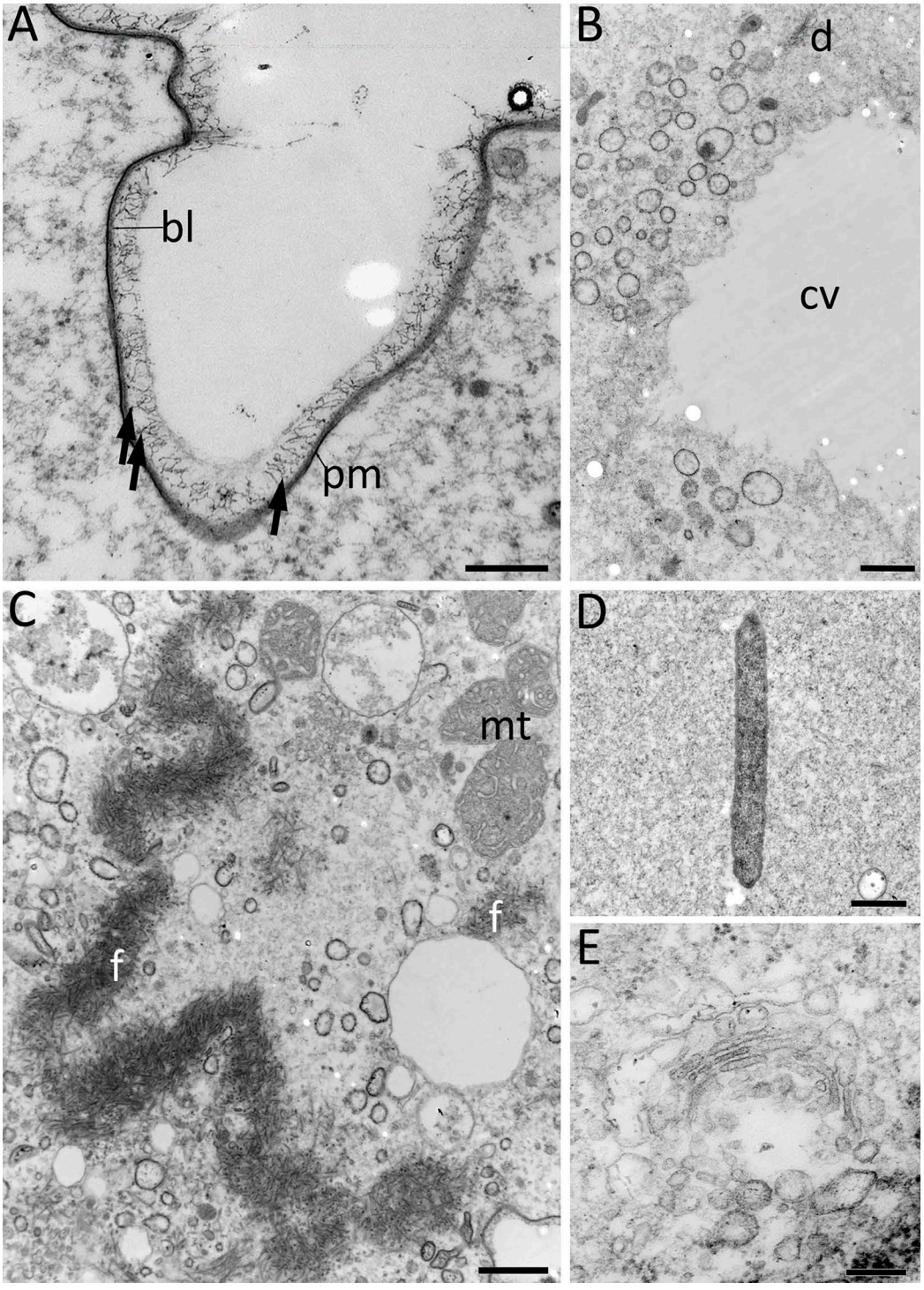

Fig. 2. General ultrastructure of *Chaos nobile* strain CCAP 1511/2. TEM. A – Plasma membrane of the cell, covered with two-layered glycocalyx: electron-dense basal layer and filamentous layer (individual filament is marked with black *arrow*); B – section through the contractile vacuole, and numerous vesicles in its surrounding; C – an area of cytoplasm containing huge curved cord consisted of the bundles of microfilaments, numerous mitochondria and vacuoles of different sizes; D – endocytobiont, localized freely in the cytoplasm; E – dictyosome of the Golgi complex. *Abbreviations*: bl – basal layer of glycocalyx, pm – plasma membrane, cv – contractile vacuole, d – dictyosome of the Golgi complex, f – a bundle of microfilaments, mt – mitochondria. Scale bars: A – 250 nm; B, C – 1 μm; D – 500 nm; E – 200 nm.

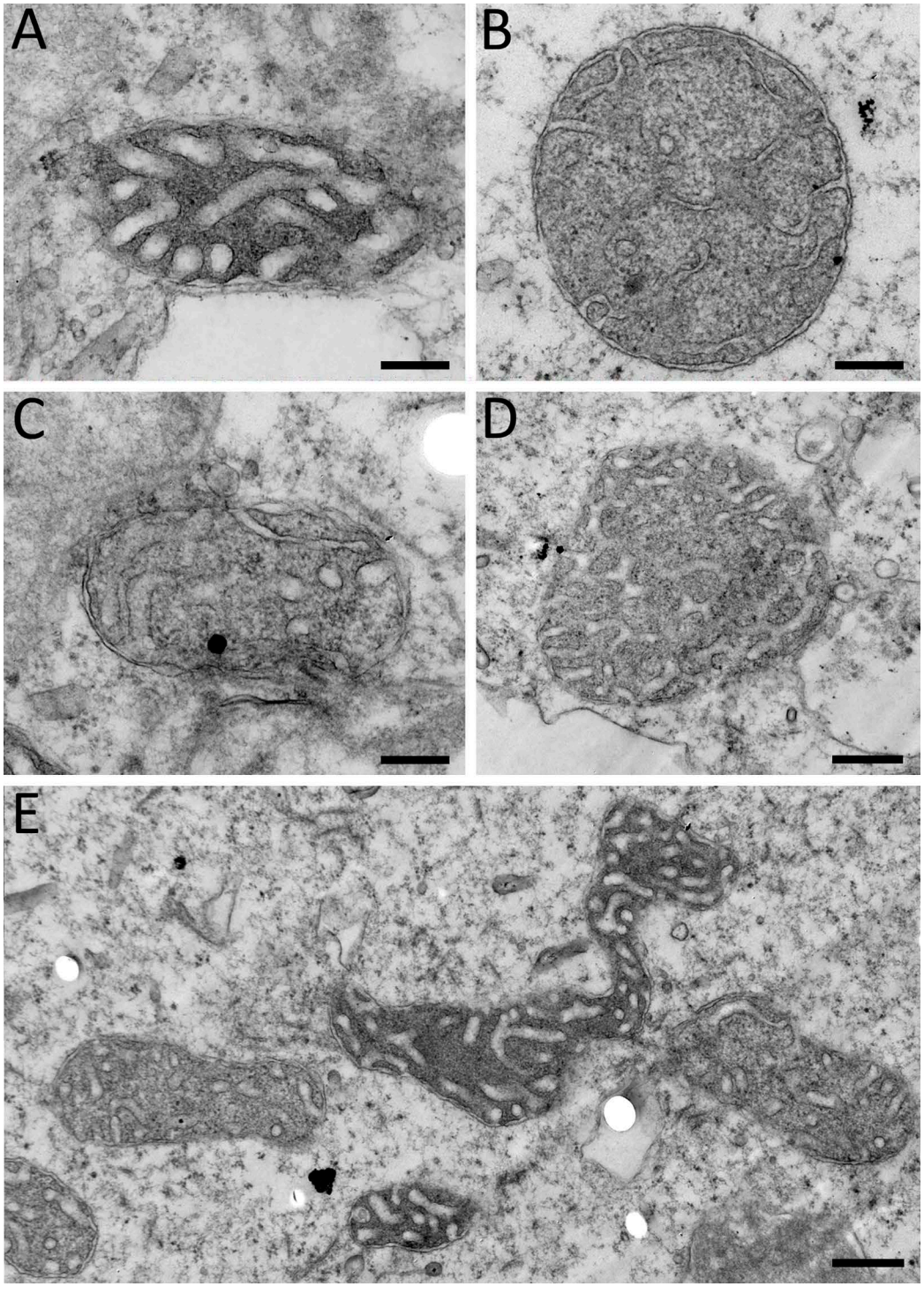

Fig. 3. Ultrastructure of the mitochondria of *Chaos nobile* strain CCAP 1511/2. TEM. A – Mitochondrium of type I with electron-dense matrix and wide cristae; B – mitochondrium of type II with more electron-transparent matrix and narrow, crimped cristae; C – mitochondrium of intermediate type; D – mitochondrium with unique pattern of organization of highly anastomosing cristae; E – an area of cytoplasm containing both the mitochondria of the type I and of the type II. Scale bars: A-C – 250 nm; D, E – 500 nm.

Discussion

Generally, the ultrastructure of *Ch. nobile* presented in this study is congruent with previous data (Page, 1986, 1988, 1991; Gromov, 1986b). However, some of our findings differ from previously reported results or complement them.

The basal and filamentous layers of glycocalyx look the same as in the electronograms of Page (1986, Fig. 11 and 1991, Fig. 15, G). The note by Page (1986) that the filaments of *Ch. nobile* are "more crinkled and more like those of *A. proteus*, than in *Ch. carolinense*", is confirmed by our observations. We noted the dendritic shapes of some filaments (Fig. 2, A), but we have to reserve that an image like this could be a result of overlaying of two or more filaments. According to our data, the total thickness of the glycocalyx of *Ch. nobile* strain CCAP 1511/2 is 90–100 nm, against 176–240 nm (200 nm) in the study of F. Page (1986). This mismatch probably is the result of differences in the filament preservation and fixation quality. In our images, the filamentous layer looks slightly pressed from the top. Reviewing data on the fine structure of the cell coat among species of the genus *Chaos*, we denote that glycocalyx of all currently known species is organized in two layers. The thin basal layer is always amorphous. The distal layer can also be amorphous – in *Ch. glabrum* (Smirnov and Goodkov, 1997), "appeared to be filamentous" in *Ch. illinoisense* (Goodkov et al., 1999), or consisting of filaments: crinkled in *Ch. nobile* (our investigation; Page, 1986) or wavy in *Ch. carolinense* (Page, 1986).

All known species of the genus *Chaos* have the inner nuclear honeycomb-like lamina (Gromov, 1986a, 1986b; Page, 1986; Smirnov and Goodkov, 1997; Goodkov et al., 1999). It is a fibrous layer underlying the nuclear envelope and organized in cellules, each of them encircling a single nuclear pore complex. The thickness of the lamina in *Chaos nobile*, according to our data, is smaller than in Page (1986). That also can be caused by the differences in preservation on this structure during the fixation procedure.

The distinguishable finding is the detection of protrusions of the nuclear envelope to the cytoplasm (Fig. 4, E). The similar phenomenon was recently noted for three strains of the genus *Amoeba* (Goodkov et al., 2020, Fig, 4, B). This study reveals that such protrusions are stages of the formation of "extrusion buds" containing the chromatin and are related with the elimination of excess DNA accumulated in the nuclei of amoebae while hyper-replication proceeds. In our sections, the space inside the protrusion looks "empty" (electron-transparent) (Fig. 4, E), while the extrusion bud in the *Amoeba* nucleus carries electron-dense content. Thus, we cannot stay that the observed protrusions are related to the process of chromatin extrusion. However, it is necessary to note this fact, to get an idea about how widespread this phenomenon could be.

Intranuclear helices, observed as individual units or arranged in clusters, were earlier described in *Ch. nobile* (Gromov, 1986b) and in other amoebae as RNP particles: *Amoeba proteus, A. discoides, Ch. carolinense, Ch. illinoisense* (Stevens and Prescott, 1965; Daniels and Breyer, 1966; Wolstenholme, 1966; Minassian and Bell, 1976). We detected the presence of clusters of RNP helices in the nuclei of *Ch. nobile*, and revealed the presence of individual helices in the close proximity to the nuclear envelope and nuclear pores (Fig. 4, H).

One of the important observations is the finding of mitochondrial heteromorphism in *Ch. nobile*. This feature was never detected previously in this species, but was described in *Amoeba proteus* (Flickinger, 1974; Ord, 1976; Smith and Ord, 1979), *Chaos glabrum* (Smirnov and Goodkov, 1997) and *Chaos illinoisense* (Goodkov et al., 1999). The interesting fact is that the examined cells contained predominantly the mitochondria of the intermediate type (Fig. 3, C, E).

The analysis of the literature revealed that the proper taxonomic diagnosis of the species *Chaos nobile* was never formulated. The first mentioning of multinucleate proteus-like amoebae, resembling a species nowadays dedicated as *Ch. nobile*, is dated back to Bütschli (1876) and Ehrenberg (1838). Schaeffer (1941) cited more mentions that are ancient; however, none can be accepted nowadays as a reliable species description. E. Penard (1902, pp. 65–70) described a multinucleate species, "*Amoeba nobilis*". He provided line drawings and an extensive set of light microscopic characteristics, but did not publish a distinct taxonomic diagnosis. Noteworthy, E. Penard isolated several specimens infected with parasitic fungus, and the most of his discussion of "*Amoeba nobilis*" was dedicated to this finding, while the comparison of his species with ancient descriptions was brief and mostly dedicated to Wilson's "*Pelomyxa carolinensis*" (Wilson, 1900) and Leidy's "*Amoeba proteus*" (Leidy, 1879). For the latter species, he argued that some of the specimens illustrated by Leidy as *A. proteus* in fact

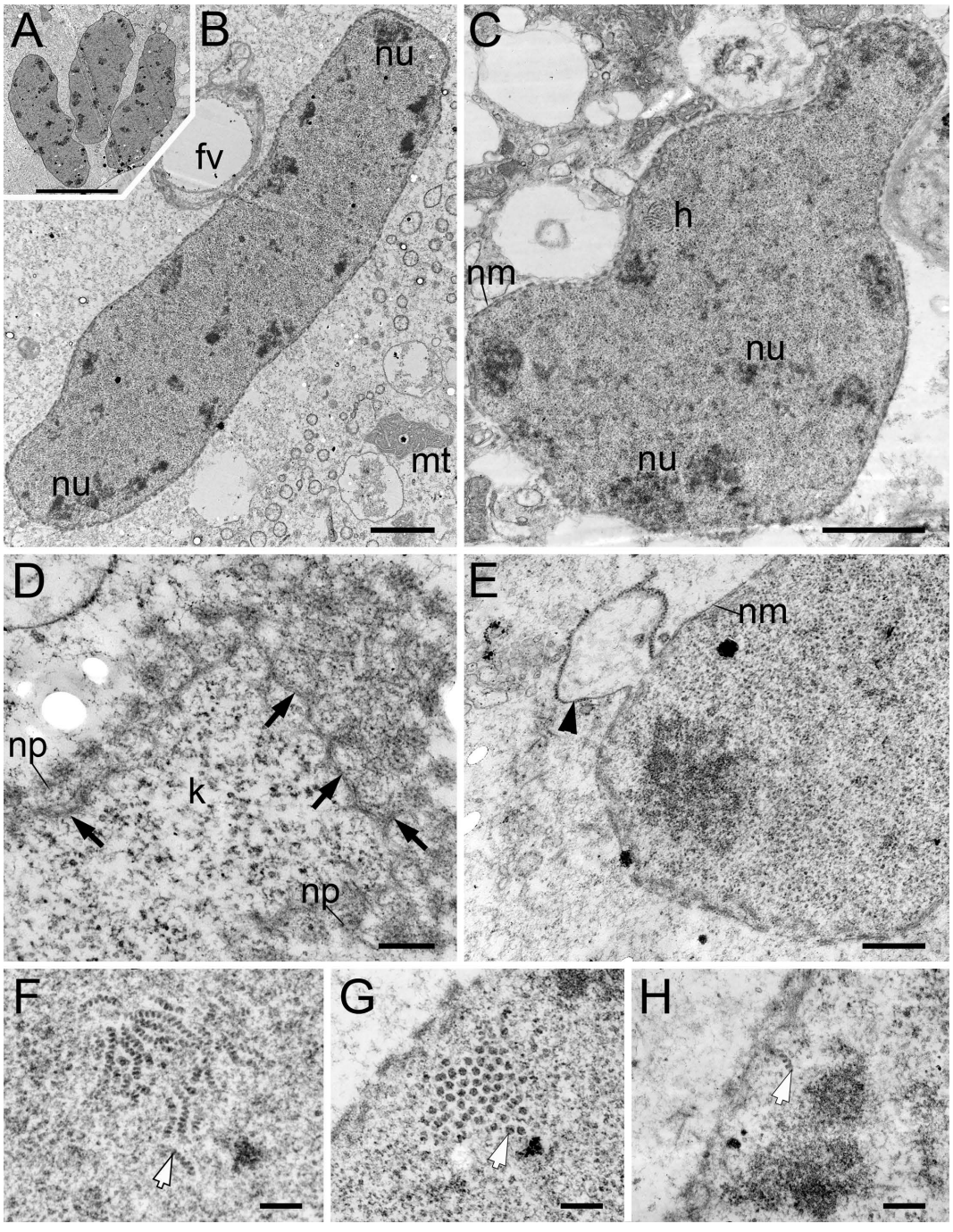

Fig. 4. Ultrastructure of the nucleus of *Chaos nobile* strain CCAP 1511/2. TEM. A – Section across the three nuclei; B – longitudinal section through the nucleus, showing a localization of nuclear material in the karyoplasm; C – tangential section through the nucleus, showing the transverse sectioned nuclear envelope and a cluster of RNP helices in the karyoplasm; D – tangential section through the nuclear envelope and nuclear lamina, showing the nuclear pores, cellules of the fibrous nuclear honeycomb-like lamina (black *arrows*); E – the protrusion of nuclear envelope in the cytoplasm; black *arrowhead* marks the different "damaged" part of the envelope; F – cluster of longitudinally sectioned RNP helices; G – cluster of cross-sectioned RNP helices; H– individual RNP helix at the short distance with the nuclear envelope and nuclear pore. *Abbreviations*: fv – food vacuole, nu – nucleolar material, mt – mitochondria, nm – nuclear envelope, h – cluster of RNP helices, np – nuclear pores, k – karyoplasm. Scale bars: A – 10 μm; B-C – 2 μm; D, F-H – 250 nm; E – 500 nm.

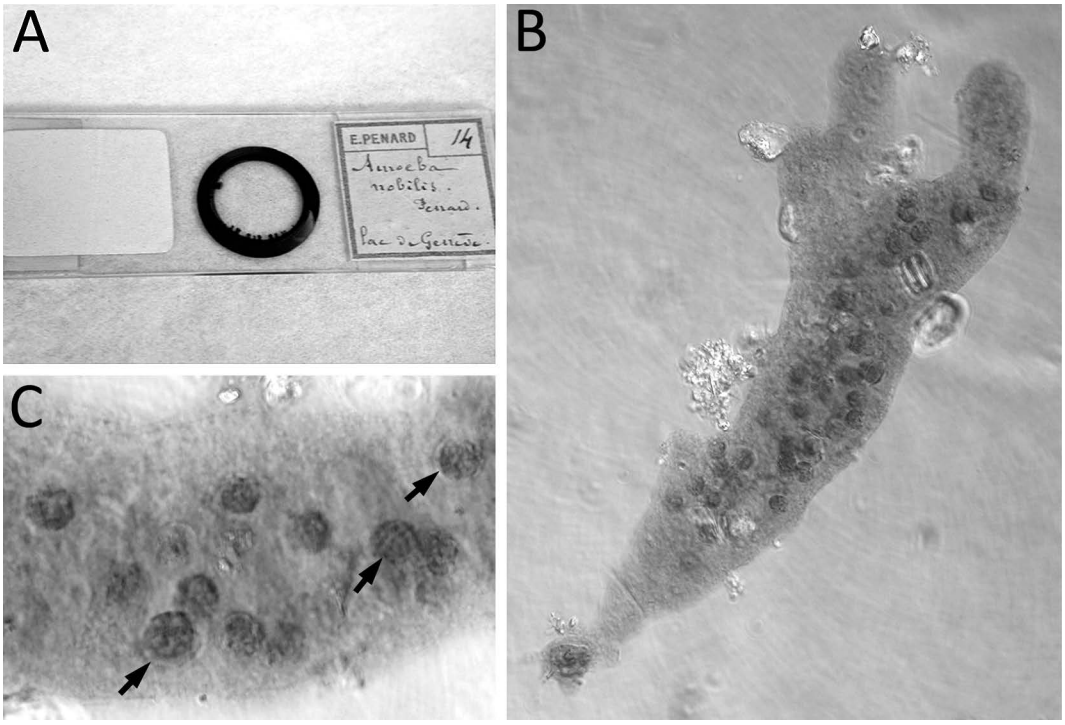

Fig. 5. Permanent stained preparation of "*Amoeba nobilis*" (slide 20.12.8.14) deposited with the collection of E. Penard's slides (British Museum of Natural History, London). A – Actual view of this slide, showing the high quality of mount and good conditions of the preparation; B – locomotive form; C – nuclei with peripherally arranged bodies of nucleolar material (several of them are *arrowed*).

were multinucleate (Penard, 1902, p. 70). However, in contrast to predictors, Penard left stained preparations of his amoebae (Fig. 5). Based on the examination of these preparations, deposited with the collection of the British Museum of Natural History (London), F. Page (1981) formally transferred this species into the genus *Chaos*, as *Chaos nobile* comb. nov. (op. cit. p. 10).

However, it should be noted that the combination "*Chaos nobilis*" appeared much earlier in literature: it was used by Schaeffer (1941) with general (without dates) references to some previous authors and, closer to modern times, by Bovee and Jahn (1973). For example, in the catalogue of the Culture Collection of Algae and Protozoa (CCAP), the studied strain is listed as "*Chaos nobile* (Penard) Bovee et Jahn, 1973". However, to the best of our knowledge, nobody of the earlier authors (including Bovee and Jahn in their book chapter from 1973) formed this combination as a taxonomic act. Therefore, for convenience, we suggest retaining the naming by Page and using this species under the taxonomic name *Chaos nobile* (Penard, 1902) Page, 1981.

Chaos nobile perhaps is not a rare species; it was isolated several times. Multinucleate amoebae resembling this species were seen by Carter (1919) and Vonwillier (1913). The latter author wrote that he demonstrated his isolate to E. Penard and it was recognized by him as co-specific with "*Amoeba nobilis*" (Vonwillier, 1913, p. 407). Schaeffer (1941) isolated this species the same year from Willow Grove, Pennsylvania, USA, and Siemensma (1980) – in the Netherlands. According to CCAP records, the strain 1511/2 (now dead), studied in the present paper, was isolated by Lorch in 1972 from the site designated as Dresser Creek, Erie County, New York, USA and identified as belonging to this species by C. Chapman-Andresen (Page, 1986). It was further lost in CCAP and replaced in 1993 with the same strain maintained by C.F. Friz. The archived CCAP page on this strain contains a note "does not travel well". The low vitality of *Chaos nobile* was mentioned by other authors (Penard, 1902; Schaeffer, 1941). It also contains a note "type strain", but we were not able to find a formal reason for this designation. Morphological characters of this strain are congruent with Penard's description

(Page, 1981; present data). So, though the CCAP strain is not the same isolate that was studied by E. Penard and preserved in his stained preparations, we have reliable reasons to recognize them as co-specific with Penard's "*Amoeba nobilis*". Since the "type" culture is now lost, Penard's slides deposited with the British Museum of Natural History (London) should be considered as the type ones for this species. Below we provide a formal taxonomic diagnosis of *Chaos nobile* based on all available data, both ancient and modern.

Diagnosis: *Chaos nobile* (Penard, 1902) Page, 1981

Orthotactic, rarely monothactic in rapid locomotion, usually with lateral/dorsal wrinkles and several short lateral pseudopodia branching from the leading one and not involved in locomotion. Polypodial in slower movement. Usually with morulate or bulbose uroid. Length in locomotion 240–900 μm. Numerous bipyramidal crystals. Multinucleate, up to hundred nuclei per cell. Ellipsoid nuclei of granulate type, 15–23 μm in diameter, with honeycombe-like nuclear lamina. Bilayered cell coat: an amorphous basal layer of glycocalyx and a distal layer of crinkled filaments. Possesses mitochondrial heteromorphism. No cysts observed.

Observed habitats: Freshwater; reported from Switzerland, USA, possibly, the Netherlands.

Type material: permanent stained preparations deposited with the British Museum of Natural History (London), numbers: 04.5.9.21, 04.5.9.22; 20.12.8.14 (hapanthotype). One specimen from the slide 20.12.8.14 is represented in the Figire 5 A–C. Images were made by A. Smirnov in 2005, during his stay in the Natural History Museum.

Sequence of the 18S rRNA gene: GenBank AJ314606, this sequence is made from CCAP 1511/2 strain studied by Page (1986) and in the present paper.

Comparison with closely related species. Light microscopic level: differs from *Ch. carolinense* and *Ch. illinoisense* by smaller size of locomotive form and a fewer number of nuclei. In contrast to *Ch. glabrum*, frequently has morulate rather than bulbous uroid. Ultrastructure level: unlike *Ch. glabrum* and *Ch. illinoisense*, has distinct filamentous distal layer of cell coat. The filaments in this layer are shorter and look crinkled (more like those in *Amoeba proteus* than wavy filaments of *Ch. carolinense*). At the molecular level could be differentiated using the 18S rRNA gene sequence.

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