Structure and development of *Pelomyxa gruberi* sp. n. (Peloflagellatea, Pelobiontida)

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Summary

The general morphology, ultrastructure, and development of a new pelobiont protist, *Pelomyxa gruberi*, have been described. The entire life cycle of this eukaryotic microbe involves an alteration of uni- and multinucleate stages and is commonly completed within a year. Reproduction occurs by plasmotomy of multinucleate amoebae: they form division rosettes or divide unequally. Various surface parts of this slowly-moving organism characteristically form finger-shaped hyaline protrusions. Besides, during the directed monopodial movement, a broad zone of hyaline cytoplasm with slender finger-shaped hyaline protrusions is formed at the anterior part of the cell. In multinucleate stages up to 16 or even 32 nuclei of a vesicular type may be counted. Individuals with the highest numbers of nuclei were reported from the southernmost part of the investigated area: the North-West Russia. Each nucleus of all life cycle stages is surrounded with microtubules. The structure of the flagellar apparatus differs in individuals of different age. Small uninucleate forms have considerably fewer flagella per cell than do larger or multinucleate amoebae but these may have aflagellated basal bodies submerged into the cytoplasm. In young individuals, undulipodia, where available, emerge from a characteristic flagellar pocket or tunnel. The basal bodies and associated rootlet microtubular derivatives (one radial and one basal) are organized similarly at all life cycle stages. There is a thin-walled cylinder in the flagellar transition zone, and an electron-dense column above that zone. In the separate non-motile undulipodia the arrangement of axoneme microtubules deviates from the typical 9+2 eukaryotic pattern. In the cytoplasm of *P. gruberi* two types of rod-shaped endocytobionts are present: (1) large bacteria with a pronounced longitudinal cleft, and (2) smaller methanogen-like bacteria.

Key words: systematics, life cycle, Pelobiontida, *Pelomyxa gruberi* sp. n., ultrastructure, cytoskeleton

Introduction

The systematics of the genus *Pelomyxa* Greeff 1874 was influenced for a long time by two opposite views with regard to both qualitative and quantitative composition of this taxon. On the one hand, nearly 20 species had been described within a century resulting from numerous observations of *Pelomyxa*-like amoebae (Gruber, 1884; Penard, 1902; Goodkov et al., 2004). On the other hand, the validity of most of these species was often doubted (Page, 1981, 1988; Whatley and Chapman-Andresen, 1990). Results of studies into the life cycle of *P. palustris* (the type species) seemed to resolve the question in favour of monotypy of the genus *Pelomyxa* (Chapman-Andresen, 1978, 1982). It was postulated that all *Pelomyxa* species ever described were merely particular stages of the complex life cycle of the sole polymorphic species *P. palustris* (Whatley and Chapman-Andresen, 1990). Since then no other detailed studies of multinucleate pelobionts followed, and until recently the above concept remained dominating in special protistological literature (Whatley and Chapman-Andresen, 1990; Page and Siemensma, 1991; Brugerolle, 1991; Brugerolle and Patterson, 2002; Goodkov et al., 2004).

The situation has dramatically changed when Frolov et al. (2005a, 2005b) reported results of their ultrastructural studies of such "life cycle stages" of *P. palustris* as *P. binucleata* (Gruber 1884) ("division product of large, grey type of *P. palustris*" *sensu* Whatley and Chapman-Andresen, 1990) and *P. prima* (Gruber 1884) ("young growth phase of *P. palustris*" *sensu* Whatley and Chapman-Andresen, 1990). The obtained morphological characters allowed reliable differentiation between *P. binucleata*, *P. prima*, and the type species *P. palustris*. The most considerable differences involved basal parts of flagella, nuclear organization and cell surface of examined species of *Pelomyxa*. The provided evidence (Frolov et al., 2004, 2005a, 2005b) resumed an interest to the previous question about the true biodiversity of multinucleate pelobionts.

In the present paper, a new species of pelobionts, *Pelomyxa gruberi*, is described. The species name is given in honour of Professor August Gruber (University of Freiburg, Germany), whose detailed descriptions of *Pelomyxa*-like amoebae (Gruber, 1884) anticipated modern studies of pelomyxoid diversity.

Material and Methods

Pelomyxa amoebae were collected in freshwater basins in the North-West Russia (Sosnovo Village, the Leningrad region, 60° 30' N, 30° 30' E and Lyady Village, the Pskov Region, about 58° 35' N, 28° 55' E). The amoebae were found in silt samples from fully or almost stagnant permanent water bodies. The samples were taken near the bank, at a depth of 5-70 cm. Attempts to establish *Pelomyxa* cultures failed, but the collected amoebae survived for up to 14 months in samples put in hermetically sealed 300 ml vessels and kept at about 10°C. Alive individuals were investigated in closed microaquaria, with a volume of 2.5 ml³, connected via a running system with a 0.5 l vessel filled with water and silt from their natural habitat. The system was maintained at 10°C and transferred to room temperature only for observation.

Leika microscope equipped with visualisation systems on the basis of Panasonic 650 CCTV + Canon EOS350D and PC P4 was used. All measurements were made on live individuals, with the help of the image analysis system IT v.2.2 (UTHSCSA).

For electron microscopy the amoebae were fixed with a cocktail of 5% glutaraldehyde and 0.5% OsO_4 (1:1) on 0.1 M cacodylate buffer. Fixation was performed on melting ice in the dark for 4 h, followed by the fixative replacement in 15 min. Then fixed amoebae were washed in 0.1 M cacodylate buffer (15 min) and postfixed with 2% OsO_4 on 0.1 M cacodylate buffer in total darkness on melting ice (1 h).

After a transition through a graded ascending alcohol series, the material was embedded in Epon-Araldite mixture. 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

Pelomyxa gruberi **sp. n.** (Figs 1-10).

Diagnosis: Rounded (feeding stages) or elongated cylindrical (locomotive stages) amoeboid organisms. A well-developed broad zone of hyaline cytoplasm with slender finger-like hyaline protrusions formed at the anterior body end during locomotion. Cell diameter varies from 80 µm (young uninucleate individuals) to 350 µm (multinucleate locomotive forms). In the life cycle, uninucleate developmental stages alternate with multinucleate ones. Reproduction occurs by plasmatomy of multinucleate amoebae: they form division rosettes or divide unequally. The cytoplasm clearly differentiated into ectoplasm and endoplasm. Vesicular nuclei (1-32 per cell) with a single central nucleolus. Nuclear envelope surrounded with microtubules in all life cycle stages. Flagella are numerous, non-motile; axoneme with a non-standard microtubular pattern. Long basal bodies deeply submerged into the cytoplasm and associated with microtubular derivatives of two types. First type derivatives (over 150 radial micro-

tubules) start from the lateral basal body surface. Second type derivatives (about 60 microtubules) are represented by one or two compact bundles originating from the bottom of the basal body. A thin-walled cylinder is present in the transition zone, an electron-dense column is located above it. In the cytoplasm two types of rod-shaped endocytobionts are present: (1) large bacteria with a pronounced longitudinal cleft, and (2) smaller methanogen-like bacteria.

Amoebae feed on small detritus particles, mostly of vegetative origin. No glycogen bodies are formed in any life cycle stages. Cytoplasm colour varies from light beige to dark brown.

Type locality: Sosnovo Village, the Leningrad Region, 60° 30' N, 30° 30' E, North-West Russia.

Type material: Holotype, slide N 934; Paratypes, slides N 935 and N 936; deposited in the Type Slide Collection, Laboratory of Unicellular Organisms, Institute of Cytology RAS, St. Petersburg, Russia.

Etymology: The species is named after Professor August Gruber (University of Freiburg, Germany).

Differential diagnosis. At the light microscopic level, *Pelomyxa gruberi* differs clearly from *P. palustris, P. corona, P. binucleata, P. belewski* and *P. prima* in the organization of the locomotive form (namely, in the presence of a well-developed frontal zone of hyaline cytoplasm with additional finger-like protrusions), and in the number and structure of nuclei. At the ultra-structural level, *P. gruberi* displays the greatest similarity with *P. prima*, but differs from it in having a weakly developed system of vacuoles, a greater number of radial microtubules associated with the basal body, absence of the lateral microtubular rootlet, presence of a dense column above the transition zone of the flagellum, and in a considerably smaller number of nuclei.

LIGHT MICROSCOPY

Pelomyxa gruberi can be found in water bodies of the North-West Russia throughout the year. These amoebae are silt dwellers at a depth of 5-70 cm. They are always surrounded with detritus particles, adhering to short hyaline pseudopodia. *P. gruberi* feeds on small detritus particle of vegetative origin. Mineral inclusions in the cytoplasm are infrequent. The cytoplasm is light beige to dark brown, depending on the prevailing food inclusions, which makes the amoebae difficult to distinguish inside silt lumps or during cursory exami-nation of samples. However, 30-40 min after putting samples into open Petri dishes at room temperature, amoebae freed from detritus particles and are easily spotted at the vessel bottom.

After numerous observations within the latest three years, we established in the life cycle of *P. gruberi* the alternation of uninucleate and multinucleate stages (Fig. 1). Prevalence of uninucleate individuals and mass appearance of multinucleate stages in *P. gruberi* micropopulations occurred approximately once a year. Interestingly, this cyclic recurrence was not season-related. Indeed, five micropopulations of *P. gruberi* were observed weekly in the Osinovskoye Lake from October to December, 2004. The five examined habitats were small silted anthropogenic bays on the west shore of the lake. The bays, located 30-50 m apart, had similar depth and bottom relief, in addition to identical shore landscapes. Table 1 summarises data on the proportion of uninucleate and multinucleate forms in these micropopulations. Multinucleate amoebae were registered only in one of the five above habitats (bay 4). Subsequent observations showed that in bays 1-3 and 5 the multinucleate stages became abundant as early as in April-May 2005, with the peak in July. Unlike, at that particular time only uninucleate forms were present in bay 4. Thus, *P. gruberi* micropopulations differing in the number of nuclei per cell were found in habitats within the same lake being only some tens of meters apart. In four of the 5 populations only uninucleate amoebae were present, whereas in one of them multinucleate stages were intensively formed. *P. gruberi* micropopulations made only by uninucleate individuals were observed for 7-8 months. Four to five months usually passed between the first appearance of multi-nucleate cells in such populations and their complete disappearance.

The size of uninucleate *P. gruberi* individuals varies from 79.2 to 215.1 μm. These are spherical or slightly oval in shape (Fig. 2, A-C, E). Their opaque cytoplasm is filled with many fine food inclusions. Numerous finger-shaped hyaline protrusions, reaching half the cell diameter in length, are formed on the cell surface (Fig. 2, C, D). On being gently pressed with a cover slip to the bottom of the microaquarium, amoebae retract

Table 1. Relative number* of multinuclear amoebae in five *P. gruberi* micropopulations from Lake Osinovskoye (Leningrad region) in October **– December 2004.

Week № / Bay №	1	2	3	4	5	6	7	8	9	10	11	12
1	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	1	0	0	0	0
4	5	3	17	30	41	35	56	51	56	51	63	57
5	0	0	0	1	0	0	0	0	0	0	0	0

Notes: * - in each case, the number of multinuclear amoebae in a random sampling of 100 *P. gruberi* individuals is given; ** - observations began on 03.10.2004, samples were collected weekly and examined in the day of sampling.
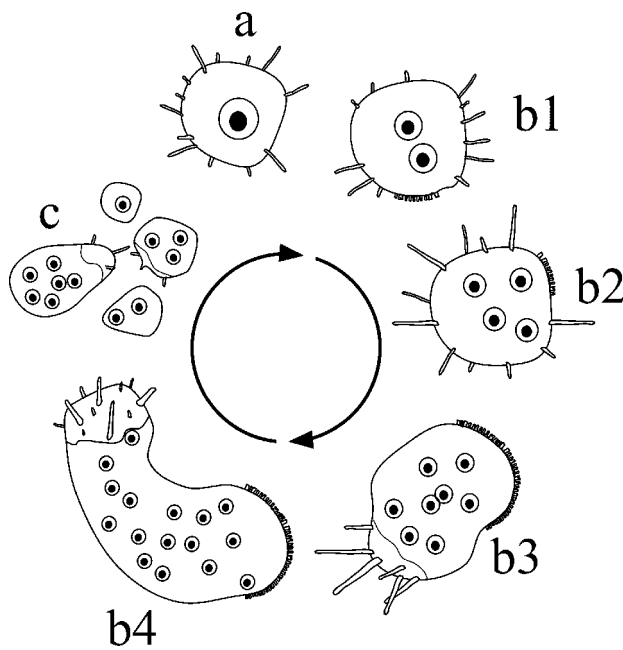
Fig. 1. Diagrammic representation of the life cycle of *Pelomyxa gruberi*. a - uninucleate stages; b1-b4 - multinucleate stages; c - stages of plasmotomy.

pseudopodia and develop a pronounced peripheral layer of hyaline cytoplasm (Fig. 2, E).

Cell polarity in uninucleate forms is poorly expressed. Opposite cell poles may be distinguished only in largest individuals, whose hyaloplasm zone is equipped with long finger-shaped protrusions at one pole and short papillae of the uroid zone at the other (Fig. 2, C).

No flagella can be discerned at the light microscopic level in small amoebae (less than 110 µm in diameter). Larger individuals (150-215 µm) display numerous short non-motile flagella easily distinguishable under a light microscope.

The spherical nucleus of *P. gruberi* is usually located centrally in the cell (Fig. 2, F). The nucleus diameter varies from 19.7 to 52.1 µm. In uninucleate forms there is a direct correlation between cell size and nucleus size: the larger amoebae, the larger their nuclei (Table 2). A compact central nucleolus is spherical or, rarely, irregular in shape. It usually has a pronounced granular structure (Fig. 2, F).

The nucleus of *P. gruberi* is surrounded with numerous prokaryotic endocytobionts. Under the light microscope, considerable agglomerations of rod-like bacteria radiating from the nuclear surface into the cytoplasm can be seen (Fig. 2 F). Such bacteria are also abundant in the cytoplasm but do not form any agglomerations there.

At the light microscopic level, multinucleate individuals look identical to uninucleate ones with regard to both their cytoplasm morphology and the range of food objects.

When moving not actively, multinucleate individuals are spherical (Fig. 3, A), 123.0-227.3 µm in diameter. The cell surface bears numerous small hyaline finger-shaped protrusions (Fig. 3, A).

Transition to locomotion is accompanied by considerable changes in the general cell morphology. Spherical amoebae become first egg-shaped (Fig. 3, B) and then cylindrical (Fig. 3, D). The latter forms may reach 300-350 µm in length. During directed movement, the anterior part of a multinucleate amoeba develops a pronounced zone of hyaline cytoplasm, with distinct hyaline finger-shaped subpseudopodia projecting from it (Fig. 3, C, D). Sometimes similar protrusions occur as well on the lateral cell surfaces but without any hyaloplasm layer. The posterior end of locomotive forms is wrapped with numerous small papillae, which form together the uroid zone (Fig. 3, B, D). Numerous optically empty vacuoles of varying diameter occur near the cell surface in this region (Fig. 3, E).

Numerous short non-motile flagella are scattered throughout the body surface of a moving amoeba except the frontal zone of the hyaline cytoplasm.

Nuclei of multinucleate individuals (Fig. 3, F) are situated both in the centre and on the periphery of the cell. The number of nuclei exceeded 16 only in 7 of more than 3000 multinucleate *P. gruberi* individuals in samples from the water bodies near the Sosnovo Village. In these, 4 individuals had 17 nuclei each, and the rest three displayed respectively, 21, 23 and 27 nuclei. However, 20-32 nuclei per cell in multinucleate individuals are commonly found in samples from the Pskov Region, the southern part of the *P. gruberi* distribution area (the Lyady Village, 400 km to the South of the Sosnovo Village).

The size of nuclei in multinucleate *P. gruberi* cells correlates with their number. The size of nuclei

Table 2. Correlation between nuclear and cell diameters * in uninucleate *P. gruberi* stages**.

Cell diameter (µm)	70-90	91-111	112-132	133-153	154-174	175-195	196-216
Nuclear diameter (µm)	19.23 ± 0.76	26.90 ± 1.04	27,73 ± 1.11	34,05 ±0.89	38.82 ± 1.23	43.62 ± 1.10	45,12 ± 0.98

Notes: * - in each size class a random sampling of 25 individuals was examined; ** - uninucleate stages examined came from the *P. gruberi* micropopulation that contains no multinucleate amoebae (the Lake Osinovskoye, bay No. 1, 11.10.2004, see Table 1).
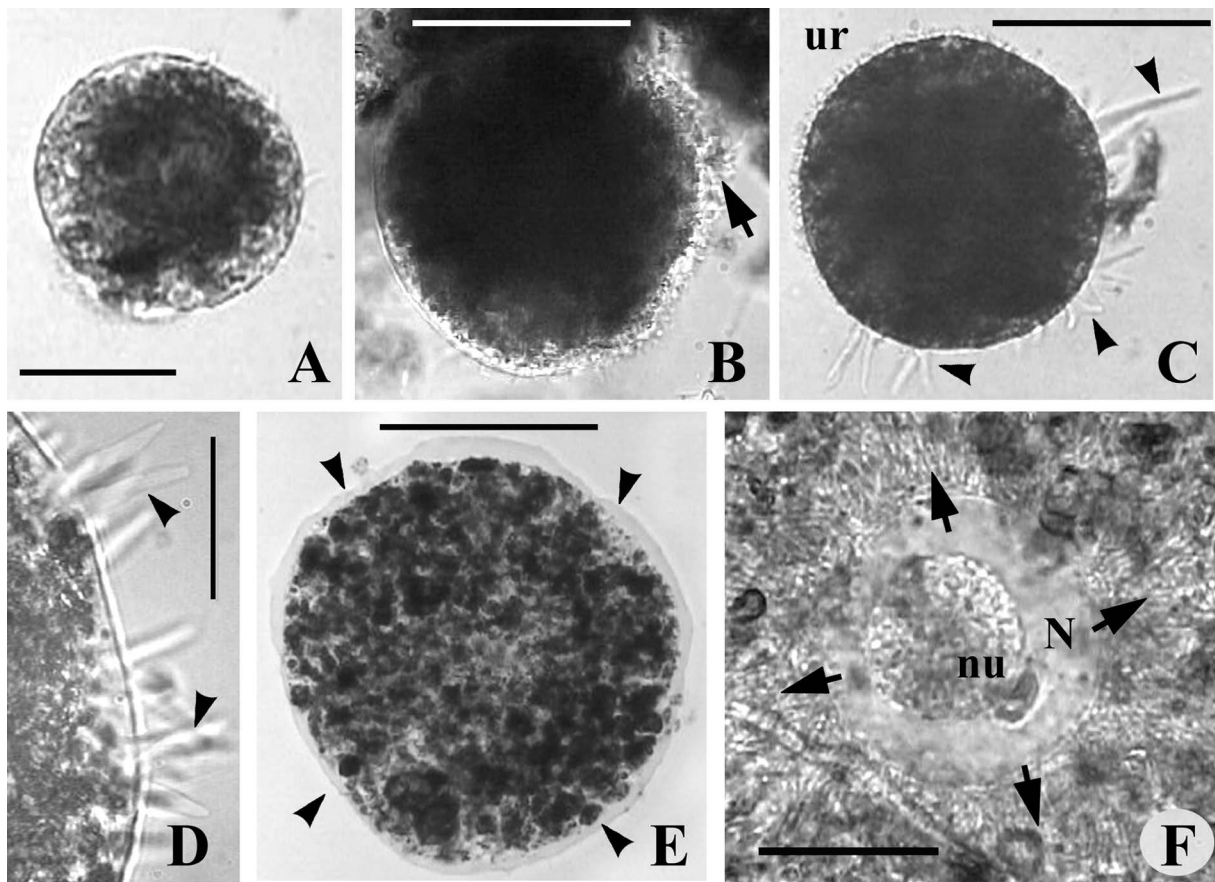
Fig. 2. Photomicrographs of uninucleate stages of *P. gruberi*. A - young uninucleate amoebae; B - a uninucleate amoeba partly cleared from the surrounding detritus, arrow points to a zone of short pseudopodia at the site of contact between the cell and the substrate; C - a mature uninucleate amoeba with a pronounced polarity: arrowheads point to pseudopodia thrown out at the pole opposite to the uroid; D - finger-shaped pseudopodia on the surface of a uninucleate amoeba; E - a uninucleate amoeba pressed with a cover slip, arrowheads point to the peripheral hyaloplasm; F - nucleus of a uninucleate cell slightly pressed with a cover slip. Arrows point to aggregations of prokaryotic cytobionts radiating from the nuclear surface into the cytoplasm. *Abbreviations*: N - nucleus; nu - nucleolus; ur - uroid zone. Scale bars: A, D - 50 μm; B - 100 μm; C, E - 150 μm; F- 25 μm.

decreases progressively as their number in the cell increases from 1 to 16 (Table 3). The exceptions are associated with reproduction of *P. gruberi*, which occurs in the form of plasmotomy. So, in the micropopulation of *P. gruberi*, where in the autumn of 2004 intensive formation of multinucleate cells took place (see Table 1, the Lake Osinovskoe, bay 4), in February-March 2005, a large number of individuals with various number of nuclei was found (e.g., 1, 3, 6, 7, 9, 12 and more per cell). The diameter of the latter was similar to that of 16-nucleate individuals. Such amoebae presumably resulted from 16-nucleate individuals.

Plasmotomy in *P. gruberi* occurs by either formation of symmetric rosettes or unequal divisions. Fig. 4

Table 3. Correlation between diameter of nuclei* and their number in multinucleate *P. gruberi* stages**.

Number of nuclei	1	2	4	8	16
Diameter of nuclei	42,21 ± 0.56	36,01 ± 0.74	30,01 ± 0.74	24,35 ± 0.43	18,40 ± 0.36

Notes: * - nuclear diameter was taken into account in the cells with completed nuclear divisions, i.e. those with 1, 2, 4, 8, 16 nuclei. ** - *P. gruberi* amoebae were examined in the period of intensive formation of multinucleate stages (the Lake Osinovskoye, bay no. 4, 30.11.2004, see Table 1)
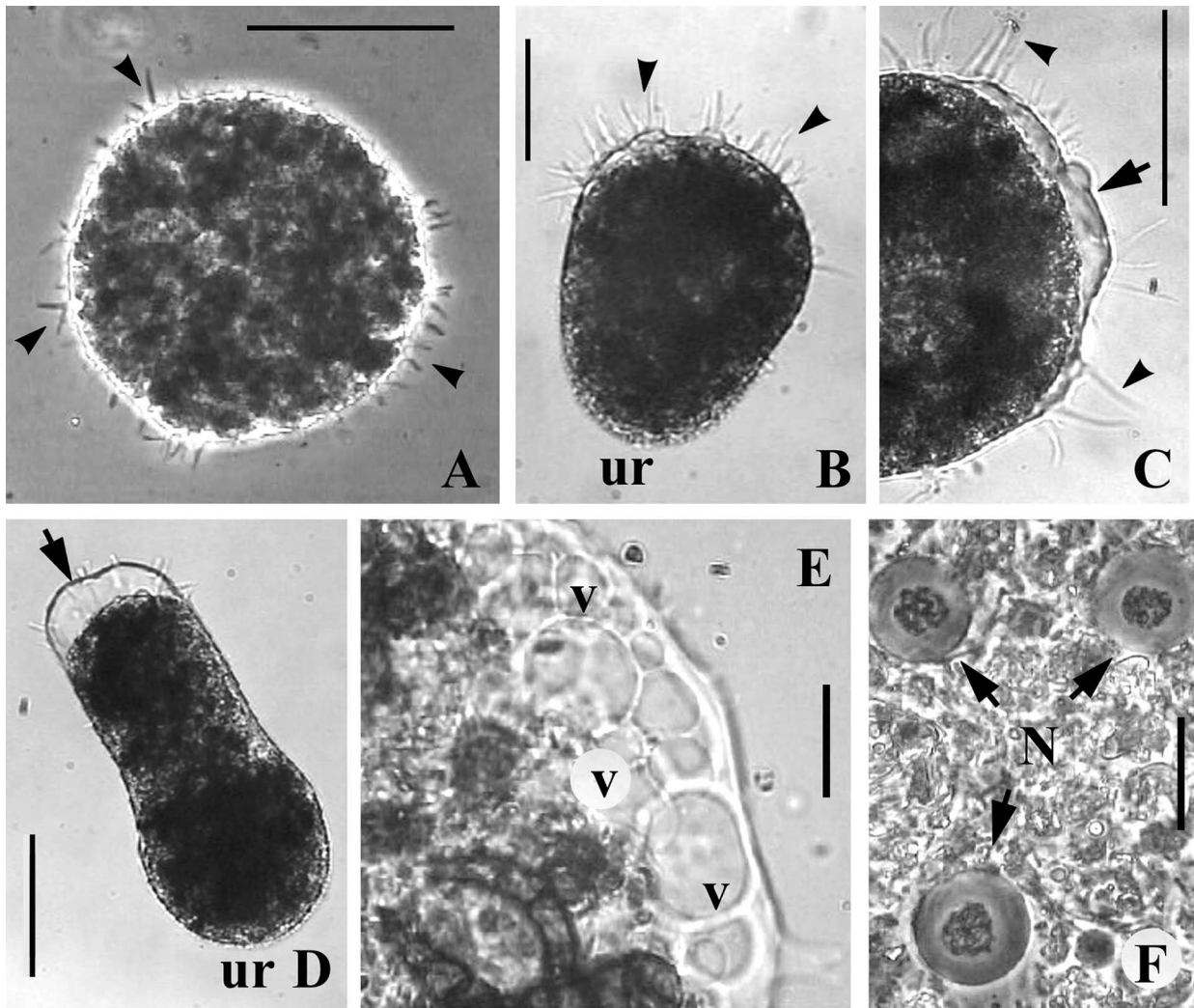
Fig. 3. Photomicrographs of multinucleate stages of *P. gruberi*. A - a young multinucleate amoeba, arrows point to short finger-shaped pseudopodia on the cell surface; B - a multinucleate amoeba starting directed locomotion, arrowheads point to pseudopodia at the anterior cell pole; C - anterior end of the same cell after a short time, a arrow points to a forming hyaline lobopodium, arrowheads point to finger-shaped pseudopodia; D - fully formed locomotive form, arrow points to the hyaline lobopodium with secondary pseudopodia; E - multinucleate cell, slightly pressed with a cover slip, with vacuoles at the uroid zone; F - nuclei in the cytoplasm of a multinucleate cell (under a slight pressure of cover slip). A, F - phase contrast. *Abbreviations*: v - vacuoles, other abbreviations as in Fig. 2. Scale bars: A-D - 100 μm; E-F - 20 μm.

(A, B) shows the plasmotomy rosette and a young individual that was formed in such a rosette. Interestingly, *P. gruberi* rosettes are of rare occurrence in the nature. We recorded them only 4 times: thrice in July (2001, 2003 and 2004; a pond in the Lyady Village, the Pskov region) and once in January (2003; the Lake Osinovskoye, the Sosnovo Village, the Leningrad region). The rosettes comprised from 5 to 8 individuals in the process of their formation. Since natural disintegration of rosettes into separate cells was never observed, we could not count the exact number of nuclei in these amoebae. The diameter of rosettes made of 8 individuals was 400-450 μm.

In the course of *P. gruberi* plasmotomy, the parent cell usually buds out smaller cells with varying numbers of nuclei (Fig. 4, C-F). Such divisions were frequently observed, both in amoebae taken from the nature and in those maintained in the laboratory. Here is a description of one of our observations of *P. gruberi* individuals from the Lake Osinovskoe. A parent
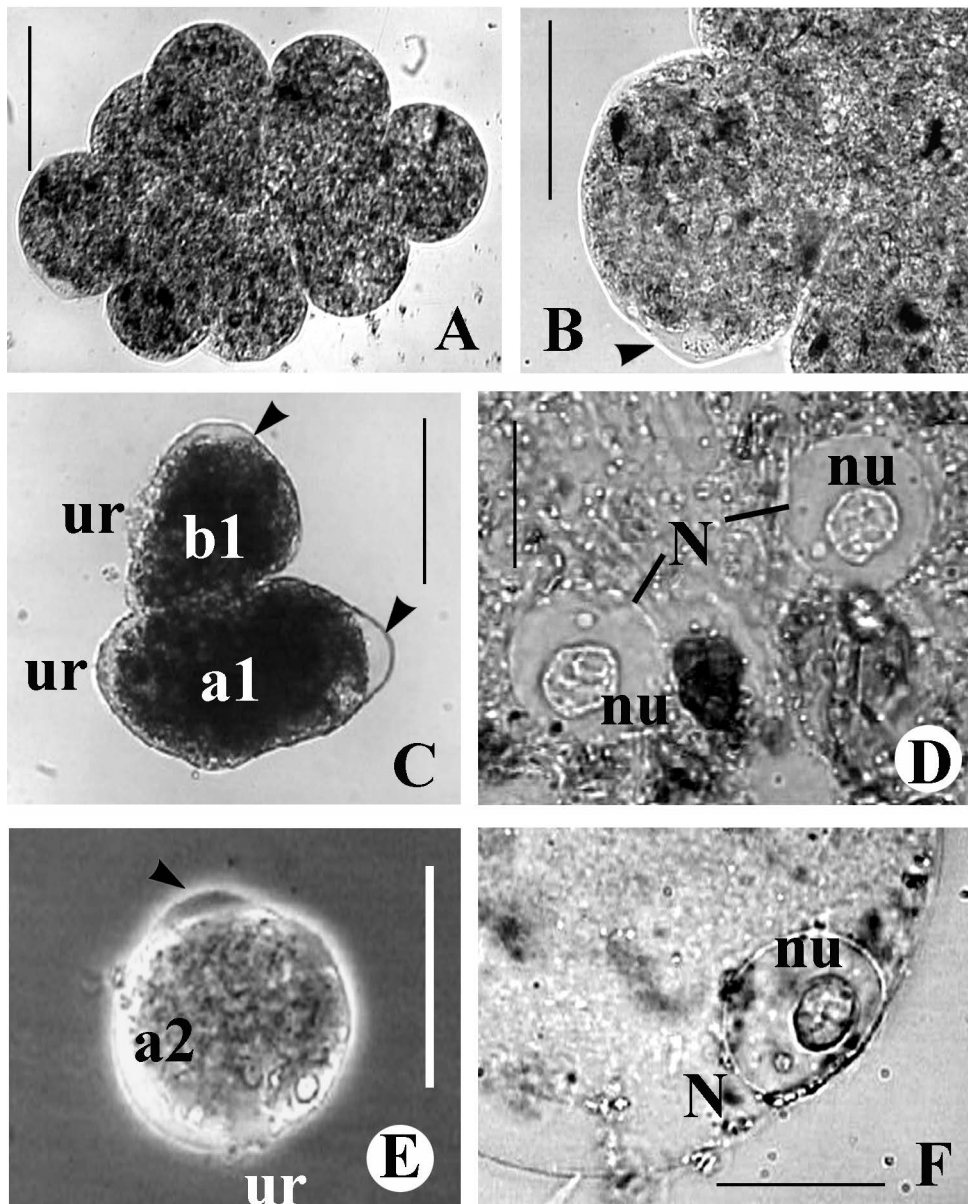
Fig. 4. Photomicrographs of plasmotomy in *P. gruberi*. A - plasmotomy rosette with 8 forming individuals; B - a cell forming in the plasmotomy rosette, an arrowhead points to the hyaline lobopodium at the anterior end; C - F - asynchronous plasmotomy. C - cells a1 and b1 formed during plasmotomy (for explanation see the text), arrowheads point to the hyaline lobopodium at the anterior pole; D - two of 10 nuclei of cell a1 (under a slight cover slip pressure); E - cell a2, uninucleate product of cell a1 division (for explanation see the text), arrowhead points to the hyaline lobopodium at the anterior pole; F - the only nucleus of cell a2 (under a slight cover slip pressure). E - phase contrast. *Abbreviations* as in Fig. 1. Scale bars: A - 150 μm; B - 50 μm; C - 150 μm; E - 80 μm; D, F - 20 μm.

individual with 16 nuclei was seen to divide into two cells of unequal size (Fig. 4, C, D). The larger of these cells (cell a1) contained 10 nuclei, whereas the smaller one had only 6 nuclei (cell b1). Within the next 24 h cell a1 budded to give rise to one uninucleate cell (a2) (Fig. 4, E, F), and cell b1 divided into two cells of almost the same size: b2 with 2 nuclei and b2 with 4 nuclei.

ELECTRON MICROSCOPY

The external surface of the plasma membrane of *P. gruberi* bears a pronounced layer of poorly structured filamentous-like glycocalyx 30-50 nm thick (Fig. 5, A, B). In the posterior cell part, small food particles, mainly bacteria, are often seen pasted to the glycocalyx
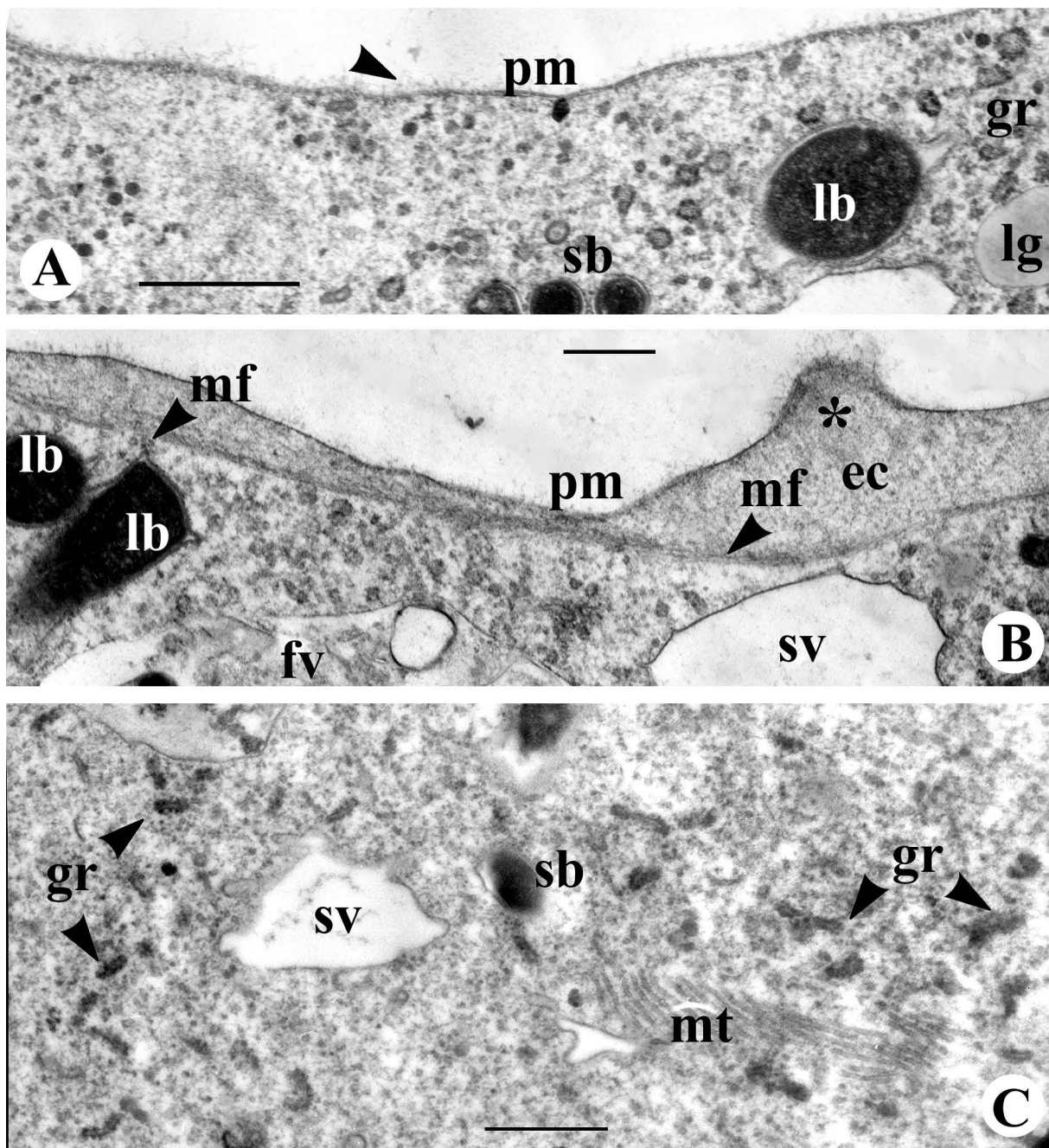
Fig. 5. Fine structure of *P. gruberi*. A - non-contracted area of the cell surface, arrowhead points to glycocalyx at the surface of the plasma membrane; B - cell surface area in the point of contraction and pseudopodium formation (*asterisk*), arrowheads point to microfilaments at the border of ecto- and endoplasm; C - endoplasm. *Abbreviations*: ec - ectoplasm; fv - food vacuoles; gr - granular endoplasmic reticulum; lb - large rod-shaped prokaryotic cytobionts; lg - lipid granules; mf - microfilaments; mt - microtubules; pm - plasma membrane; sb - small rod-shaped prokaryotic cytobionts; sv - structural vacuoles. Scale bars: A-C - 0.5 μm.

of uroid villi (Fig. 6, A). The distinct ectoplasm layer is present at the uroid area (Fig. 6, A), and it involved, at a given moment, in transformation associated with movement or feeding (Fig. 5, B). There is almost none such a layer in other cell parts (Fig. 5, A, C). A distinct microfilamentous network is present in the ectoplasm
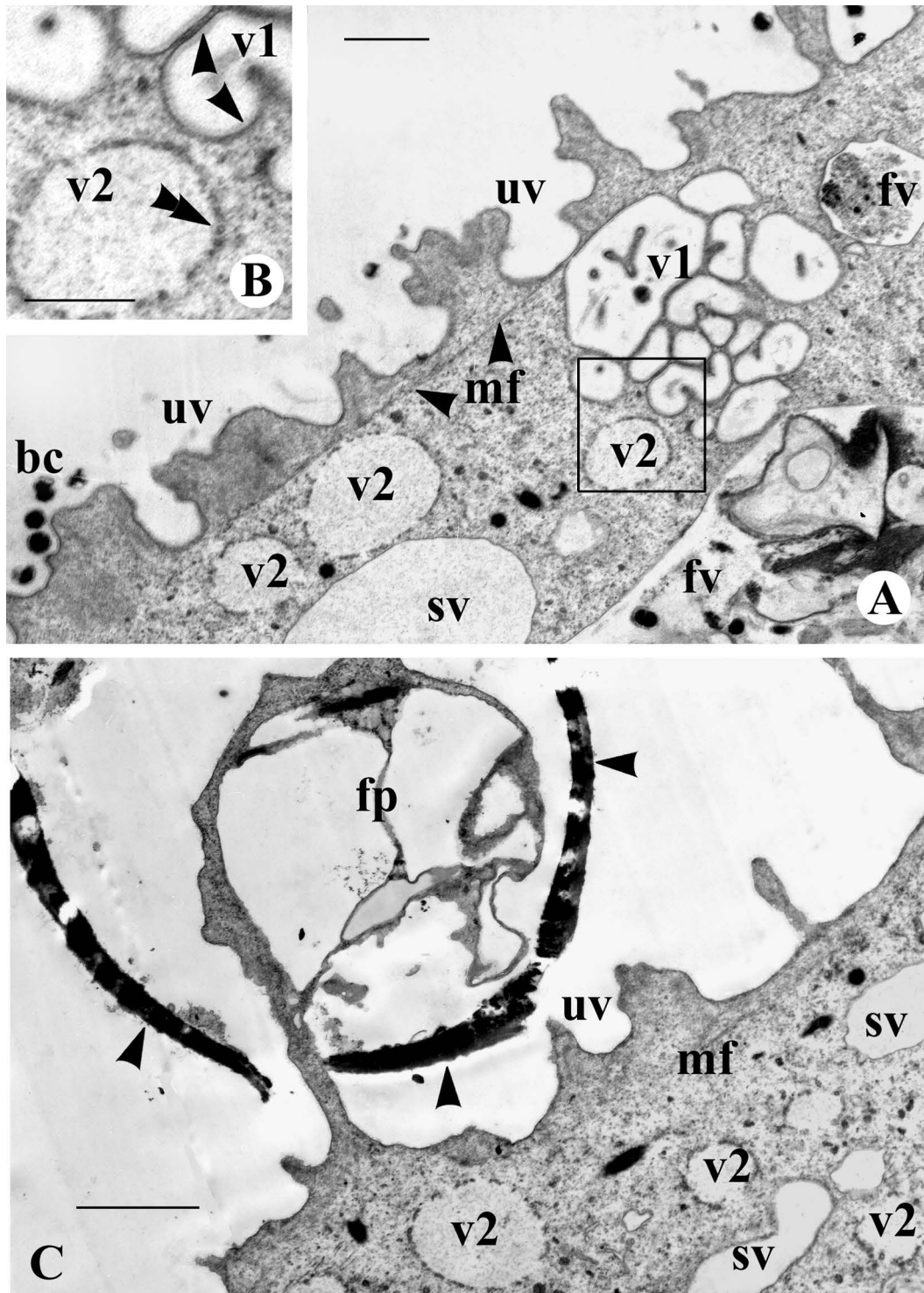
Fig. 6. Fine structure of *P. gruberi* (continued). A-C - the uroid zone. A, B - vacuole formation in the uroid zone (for explanations see the text); B - enlargement of the boxed area in A; arrowheads point to glycocalyx at the inner surface of phagosome membranes, a double arrowhead points to vesicles integrating into the vacuole membrane; C - food pseudopodium penetrating the shell of an unidentified microorganism. *Abbreviations*: bc - bacteria pasted to the glycocalyx; fp - food pseudopodium; uv - uroid zone villi; v1 - phagosomes; v2 - stages of secondary lysosome formation. Other abbreviations as in Fig. 5. Scale bars: A, C - 1.0 μm; B - 0.5 μm.

and in the hyaline protrusions (Fig. 5, B; 6, A, C). At the border of ecto- and endoplasm, long dense bundles of ordered microfilaments are situated (Fig. 5, B).

The endoplasm of *P. gruberi* contains numerous tubules and cisternae of the granular endoplasmic reticulum (GER), vacuoles, prokaryotic endocytobionts, single microtubules and their associations, various inclusions and nuclei (Fig. 5, C). GER run through the whole cytoplasm, their profiles being rather evenly scattered in the cell.

A considerable part of *P. gruberi* cell volume is occupied with large vacuoles of various types. The vacuolar system is most variable in the uroid zone (Fig. 6), where primary phagosomes are formed. The internal membrane surface of the latter retains a layer of glycocalyx (Fig. 6, A, B). Phagocytosis occurs in sites between the uroid villi or at the expanded tips of special food pseudopodial protrusions (Fig. 6, C). The organizatio of large vacuoles of another types appears to be associated with the formation of secondary lysosomes. The membrane of these vacuoles integrates numerous fine vesicles, containing certain material of average and high electron density (Fig. 6, A-C). At low TEM magnifications, the contours of these vacuoles look like a dashed line. Mature secondary lysosomes or digestive vacuoles are surrounded with a simple cytoplasmic membrane and differ from other vacuoles in their contents, where various food inclusions can be seen (Fig. 6, A). Digestive vacuoles occupy most of the cell space. Depending on quantity of food particles engulfed, their size varies considerably (from less than 1 μm to tens and even hundreds of microns).

In the cytoplasm of *P. gruberi* two kinds of prokaryotic endocytobionts were found (Fig. 7, A-D), differing in size and shape. Relatively small electron-dense rod-shaped bacteria (Fig. 7, C, D) are approximately 0.18 μm in diameter and 1.5-2.0 μm in length. Larger bacteria (Fig. 7, A, B) are 2.5-3.5 μm long, with a diameter of 0.4-0.6 μ m. Their characteristic longitudinal cleft is often as deep as a half of bacterium diameter (Fig. 7, A, B). At sections, endocytobionts of both types are more or less evenly scattered throughout the cytoplasm. Their aggregations are formed mainly close to the nuclei, however, endocytobionts are unable to penetrate into the perinuclear zone armed with microtubules (Fig. 10, A, C). Sometimes small groups of bacteria of both types are also seen in the cytoplasm at some distance from the nuclei. In these cases all bacteria in aggregations are oriented in one direction, along the bundles of cytoplasmic microtubules (Fig. 7, B, D).

In young uninucleate amoebae, the number of flagella per cell is considerably less than in more mature and multinucleate forms. This observation is based upon the frequency of occurrence of the flagellar apparatus fragments, derived from viewing the same number of cell sections of these stages. This index is almost an order of magnitude lower in young uninucleate *P. gruberi*. The organization of the flagellar apparatus may also differ considerably in *P. gruberi* individuals of different age (compare Fig. 8 and Fig. 9). Thus, in young uninucleate amoebae undulipodia may be absent (Fig. 8, E, F). In this case their cytoplasm contains an aflagellar kinetosomes with a full set of cytoplasmic microtubular derivatives. Alternatively, the cell surface of uninucleate *P. gruberi* may bear peculiar "bud" undulipodia rather varying in size (Fig. 8, A-D). So, if no undulipodia are present a small cytoplasmic knob containing short microtubules of a transition zone is formed directly above the kinetosome (Fig. 8, A, B). This knob is located at the bottom or on the lateral wall of special cytoplasmic pocket, or tunnel. Sometimes the undulipodian length does not exceed 1.0-1.5 μm (Fig. 8, C, D). Such undulipodia are filled with electron-dense material, which forms a so-called dense column masking microtubules of axoneme. In this case the basal part of undulipodia is also located in the cytoplasmic pocket (Fig. 8, C, D).

Undulipodia in mature uninucleate and multinucleate individuals of *P. gruberi* are 10-15 μm long (Fig. 9, A, B). They have a non-standard set of microtubules (Fig. 9, H), varying even in different flagella of the same cell. The following axoneme microtubular patterns were found: $(8\times2)+1$, $(7\times2+1)+1\times2$; $(8\times2)+1\times2+1$ (arrangement formulae of axoneme peripheral microtubules are given in brackets).

Uninucleate and multinucleate forms display similar organization of their rootlet apparatus (Figs 8, A-F; 9, A-G) equipped with a long kinetosome (1.2 μm) in all life cycle stages of *P. gruberi* (Figs 8, C- E; 9, A-C). Two sets of microtubular derivatives are associated with the basal body: radial and basal microtubular bundles. Radial microtubules start from the lateral surface of kinetosome to form 15-17 rows visible at longitudinal sections (Figs 8, A-F; 9, A-D, F, G). The bases of microtubules are submerged in the electron-dense material forming a "muff" around the kinetosome (Figs 8, E, F; 9 B, G). Up to 15 such microtubules can be seen on each cross-section of the basal body (Figs 8, F; 9, F). Thus, altogether over 250 radial microtubules form an umbrella-like structure around the longitudinal axis of the kinetosome. The microtubules are often united in groups from beginning to end.

Microtubular bundles proceeding from the bottom of the basal body into the cytoplasm are further referred to as basal bundles (Figs 8, E; 9, A-E). Commonly there are 2 bundles per kinetosome (Fig. 9, C), the number of microtubules in them reaching 50-60 (Fig. 9, E). In some cases we managed to trace microtubules of the basal bundles deep into the cytoplasm, down to 13 μm from the cell surface.
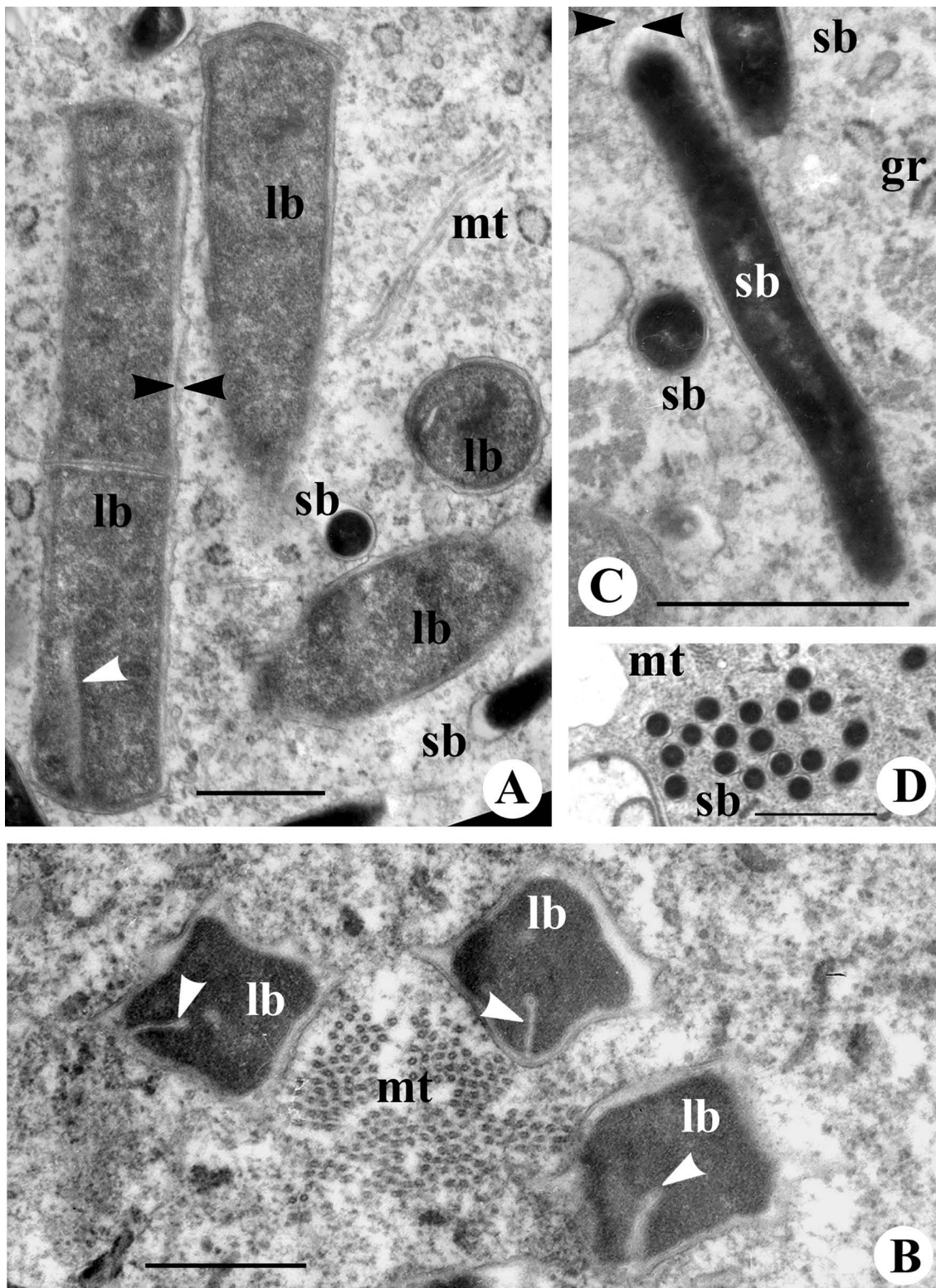
Fig. 7. Fine structure of *P. gruberi* (continued). A - D - cytoplasmic prokaryotic endobionts of *P. gruberi*. A, B - large rod-shaped bacteria with a pronounced longitudinal cleft (white arrowhead), black arrowheads point to two membranes of cytobiontophorous vacuole; C, D - small rod-shaped bacteria, black arrows point to two membranes of cytobiontophorous vacuole. *Abbreviations* as in Fig. 5 and 6. Scale bars: A-C - 0.5 µm; D - 1.0 µm.

In *P. gruberi*, the transition zone of flagella (Fig. 9, D, G) is much shorter than the basal body and is located at the border of the cell surface. We failed to establish the spatial arrangement of peripheral microtubules in this area, because the internal space of the transition zone was filled with electron-dense material. However, at some cross-sections of the transition zone, the structure corresponding to the transition cylinder was revealed (Fig. 9, G).

During interphase, each vesicular nucleus of *P. gruberi* is surrounded with a typical double-membrane envelope, which is perforated by pore complexes (from 30 to 45 per 1 μm^2). The external and internal diameters of these pore complexes are about 110 and 70 nm, respectively. The cytoplasm adjacent to the nuclear membrane usually contains numerous microtubules separately arranged at random or forming compact bundles. The latter microtubules can be traced up to 10 μm into the cytoplasm.

The karyoplasm contains chromatin fibrils about 10 nm thick and blocks of dense chromatin of 0.5 μm in diameter. A compact nucleolus is generally present in the centre of each nucleus. The nucleolar domain mostly contains a granular component of high electron density, with large chromatin blocks up to 1 μm in diameter scattered in it.

Discussion

Pelomyxa gruberi seems to represent one of the most common pelomyxoid species in the water bodies of the North-West Russia. Its developmental stages are found living inside silt lumps, the prevailing cytoplasm colour being very similar to that of the surrounding detritus particles, mineral grains and immobile plant matter. It is probably due to the "cryptic" life style that this amoeboid protist was not described as a separate species earlier. In the *P. gruberi* life cycle, three clear developmental stages can be recognised: (1) uninucleate cells, (2) multinucleate cells, and (3) fission multinucleate cells which divide unequally or form division rosettes during the course of plasmotomy. The life cycle of *P. gruberi* is definitely different from that of *P. palustris*. Whereas the life cycle of the former species lacks the cyst stage, the life cycle of the latter species does not involve any pronounced uninucleate stage (Whatley and Chapman-Andresen, 1990). The life cycles of all *Pelomyxa* species studied so far are usually completed within a year period. However, the life cycle of *P. gruberi* is not seasonally synchronized, as this is usually the case in *P. palustris, P. binucleata, P. corona,* and *P. prima* (Whatley and Chapman-Andresen, 1990; Frolov et al., 2004; 2005a, 2005b). At the same time, *P. gruberi* is unique in that its micropopulations, even those situated close enough to each other in a small lake, develop

asynchronously. Nevertheless, development of individuals within the given micropopulation is almost synchronous.

Domination of uninucleate forms, whose development usually lasts 6-7 months, is a characteristic feature of the life cycle of *P. gruberi*. Uninucleate forms have been also described in *P. binucleata* and *P. corona* (Frolov et al., 2004, 2005b), but their development in both these species takes only 2-3 months. In *P. binucleata*, most of the life cycle stages are represented by binucleate forms, and in *P. corona*, by multinucleate forms. Uninucleate forms are absent in the life cycle of *P. palustris* (Whatley and Chapman-Andresen, 1990). Interestingly enough, the *P. gruberi* cells reach maximum size in the uninucleate phase of their life cycle, and the growth almost completely stops after transition to the multinucleate phase.

Structural organization of *P. gruberi* nuclei in the interphase and during mitosis will be studied in detail elsewhere. In this work, we describe only the general events which take place during formation of multinucleate amoebae. As the cell growth progresses, the volume of the nucleus increases, and so does the size of its central nucleolus. Having reached the maximum size (45-52 μm in diameter), the nuclei of *P. gruberi* begin to divide mitotically. Nuclei of the same cell divide very rapidly in a more or less synchronous manner, while the interphase is rather long.

The mature multinucleate individuals, sampled from different localities in the northern part of the distribution area investigated (the Leningrad region), display with rare exceptions up to 16 nuclei per cell, formed as a result of four consecutive divisions. The multinucleate protists distributed in the southern part of the area studied (the Pskov region), had mostly 32 nuclei per cell as a result of an additional fifth division in the *P. gruberi* life cycle. Differences in the number of divisions between the "northern" and the "southern" populations of *P. gruberi* are unlikely to be associated directly with the temperature regime of the environment, since they were observed irrespectively of the season. On the contrary, no differences in the number of nuclei have been noted in two other *Pelomyxa* species, *P. palustris* (Whatley and Chapman-Andresen, 1990) and *P. binucleata* (Frolov et al., 2005b), sampled from different temperature zones.

Some morphological characteristics of *P. gruberi* suggest affinities with other *Pelomyxa* and related *Mastigamoeba* species. For example, multinucleate and multiflagellate forms of *P. gruberi* appear to be most common for *Pelomyxa* life cycle (Seravin and Goodkov, 1987; Griffin, 1988; Goodkov, 1989; Goodkov and Seravin, 1991; Goodkov et al., 2004). The prokaryotic endocytobionts of *P. gruberi*, large rod-shaped bacteria with a pronounced longitudinal cleft and smaller Gram-
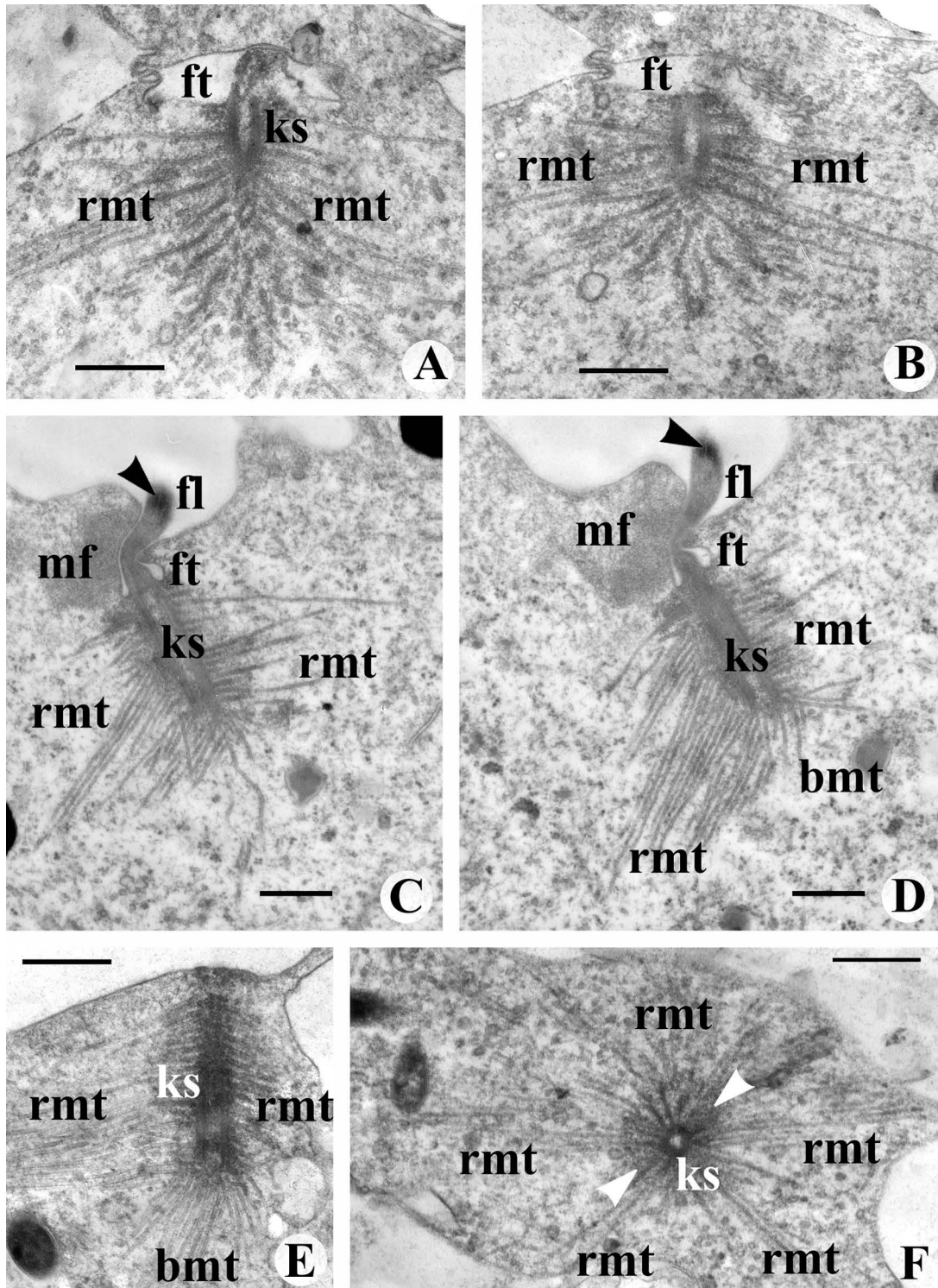
Fig. 8. Fine structure of *P. gruberi* (continued). A - E - structure of the flagellar apparatus in uninucleate stages. A, B - serial sections showing formation of the flagellar knob ("bud") in the wall of the flagellar pocket; C, D - serial sections showing emergence of a weakly developed undulipodium from the flagellar pocket. Arrowheads point to dense column. E - longitudinal section through an aflagellar basal body. F - transverse section through an aflagellar basal body, arrowheads point to the muff of electron-dense material into which the bases of radial microtubules are submerged. *Abbreviations*: bmt - bundles of basal microtubules; fl - undulipodia; ft - flagellar pocket, or tunnel; ks - flagellar basal body; rmt - radial microtubules. Scale bars: A-F - 0.5 μm.
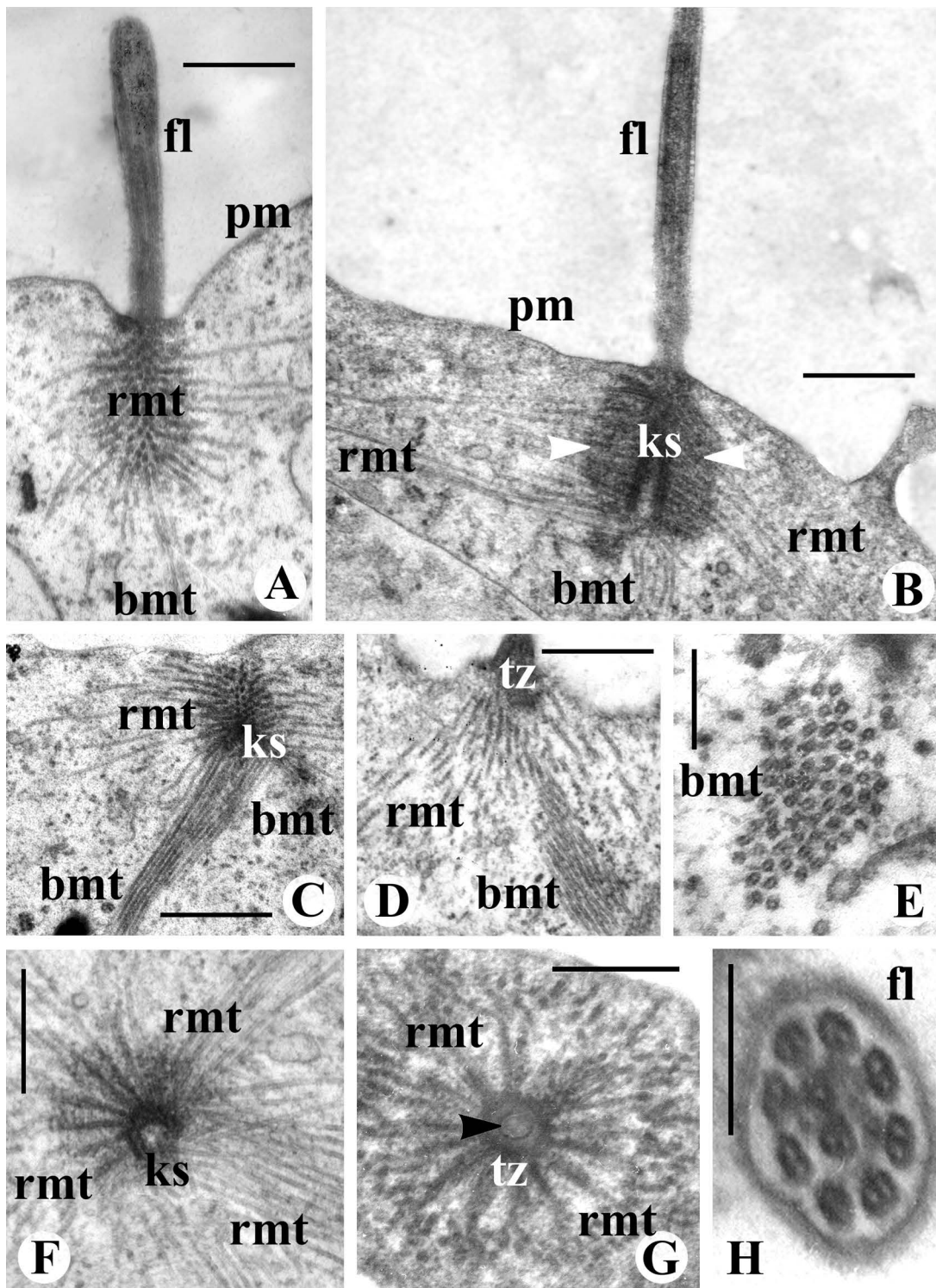
Fig. 9. Fine structure of *P. gruberi* (continued). A - H - the flagellar apparatus in multinucleate stages. A - D - transverse sections of various parts of flagellar apparatus; E - transverse section of a basal bundle of microtubules; F - transverse section of a basal body; G - transverse section through the transition zone of the flagellum close to the cell surface, an arrowhead points to the transition cylinder; H - transverse section of flagellum with a non-standard pattern of axonemal microtubules $(8 \times 2) + 1 \times 2 + 1$. *Abbreviations*: tz - transition zone of the flagellum; other abbreviations are the same as in Fig. 5-8. Scale bars: A - D - 0.5 μm; E - 0.20 μm; F, G - 0.4 μm; H - 0.15 μm.

positive rod-shaped bacteria, are also typical of the genus *Pelomyxa* (Daniels et al., 1966; Daniels, 1973; Whatley, 1976; van Bruggen et al., 1988; Whatley and Chapman-Andresen, 1990). It is noteworthy that morphologically similar prokaryotic endocytobionts are as well found in the cytoplasm of mastigamoebids and, in particular, in *Mastigella commutans, M. vitrea, M. nitens* (van Bruggen et al., 1985; Walker et al., 2001). Another typical pelomyxoid characteristic of *P. gruberi* is the occurrence of unusual axonemal structure in non-motile flagella (Seravin and Goodkov, 1987; Griffin, 1988; Goodkov, 1989; Goodkov and Seravin, 1995). Instead of the normal $9 \times 2 + 2 = 20$ axoneme, *P. gruberi* undulipodia have $(8 \times 2) + 1 = 17$, $(7 \times 2 + 1) + 1 \times 2 = 17$, or $(8 \times 2) + 1 \times 2 + 1 = 19$ microtubule patterns. Mastigamoebid species studied so far, with *Mastigina hylae* as an exception, display the normal 9-doublet, 2-singlet axoneme (Walker et al., 2001).

P. gruberi lacks so-called structural vacuoles which may occupy up to 40-75% of the cytoplasm in other *Pelomyxa* species (Fortner, 1934; Daniels et al., 1966; Andresen et al., 1968; Daniels, 1973; Chapman-Andresen and Hamburger, 1981; Goodkov and Seravin, 1991; Whatley and Chapman-Andresen, 1990; Frolov et al., 2004; 2005a, 2005b). In *P. gruberi*, the cytoplasm is vacuolated only in the uroidal zone, however, both primary phagosomes and secondary lysosomes prevail in this tailpiece. As in *P. gruberi*, vacuolation of cytoplasm is also not a characteristic feature in most mastigamoebids. Up to now, "foamy" cytoplasm has been noted only in *Mastigamoeba lacustris* and *M. setosa* (Goldschmidt, 1907; Penard, 1909).

P. gruberi can be distinguished from other *Pelomyxa* species by the absence of glycogen bodies in the cytoplasm (Daniels et al., 1966; Andresen et al., 1968; Daniels, 1973; Whatley and Chapman-Andresen 1990; Frolov et al., 2004; 2005a, 2005b). No glycogen bodies were also found in mastigamoebids (Chavez et al., 1986; Simpson et al., 1997; Walker et al., 2001).

The nuclear structure of *P. gruberi* resembles that of *P. prima* (Frolov et al., 2005a) but clearly differs from that in other pelomixoids demonstrating many similarities with mastigamoebid species studied (Brugerolle, 1982; Chavez et al., 1986; Simpson et al., 1997; Walker et al., 2001). The following structural components may be distinguished in *P. gruberi*: a large centrally located nucleolus and numerous pores. The nuclear matrix forms no distinct nuclear lamina beneath the inner membrane of the nuclear envelope, whereas the outer nuclear membrane lacks any membraneous or fibrillar elements on its cytoplasmic surface. However, the nuclei are surrounded by numerous microtubules, which run separately or are arranged in bundles. The number of perinuclear microtubules in *P. gruberi* is comparable with that in mastigamoebids.

However, the perinuclear microtubules of mastigamoebid species, e.g. *Mastigina hylae*, are typically arranged in a single cone, which connects one of the nuclear poles to the flagellar apparatus of the cell (Brugerolle, 1982, Fig. 10, p. 232). On the contrary, the nucleus-associated microtubules of *P. gruberi* approach the nuclear envelope from different directions and are scattered all over the nuclear surface. It is likely that these microtubules derived from different kinetosomes including aflagellated ones.

Flagellated kinetosomes of *P. gruberi* reach 1.2 μm in length. In most of other pelobionts studied to this level, the average basal body length is only 200-400 nm (Walker et al., 2001, Frolov et al., 2005b). In general, the ultrastructure of *P. gruberi* basal bodies resembles that of mastigamoebids. The latter have a characteristic dense column immediately above the transition zone (Walker et al., 2001). It has been generally accepted that this column is present in mastigamoebids and protostelids only (Walker et al., 2001). Now *P. gruberi* should be added to this list. The dense column is especially well pronounced in flagella of young *P. gruberi* individuals, which also have a cytoplasmic "pocket" from which the flagellum emerges. A similar flagellar canal is present in *Mastigamoeba simplex* (Walker et al., 2001). At the level where the flagellum leaves the cell, a hollow thin-walled cylinder is present in the transition zone. Such a cylinder is also found in all other mastigamoebae species studied (Walker et al., 2001), and in *Pelomyxa binucleata* (Frolov et al., 2005b).

Two microtubular derivatives appear to be associated with each kinetosome of *P. gruberi*: radial rootlet and basal rootlet. The former contains much more microtubules than its counterparts in any other pelobiont species studied, and the latter may be homologous to the nucleus-supporting cone of microtubules of mastigamoebids. However, unlike mastigamoebids (Simpson, et al., 1997; Walker et al., 2001) and *P. prima* (Frolov et al., 2005a), *P. gruberi* lacks the third (lateral) microtubular rootlet.

Our data on the ultrastructure and the development of *P. gruberi*, a new pelobiont species, cast new light upon the genus *Pelomyxa* Greeff 1874. Until very recently, only one question was debated: whether there are *Pelomyxa* species other than *Pelomyxa palustris* (Whatley and Chapman-Andresen, 1990; Brugerolle and Patterson, 2002; Goodkov et al., 2004). However, after a reinvestigation of several "forgotten" *Pelomyxa* species and descriptions of new ones (Frolov et al., 2004; 2005a, 2005b; this article) another question arises: what principles may underlay unification of all these species into the same genus *Pelomyxa* Greeff 1874. Noteworthy, diagnoses of the Pelomyxa genus available in the literature are based almost exclusively upon light-
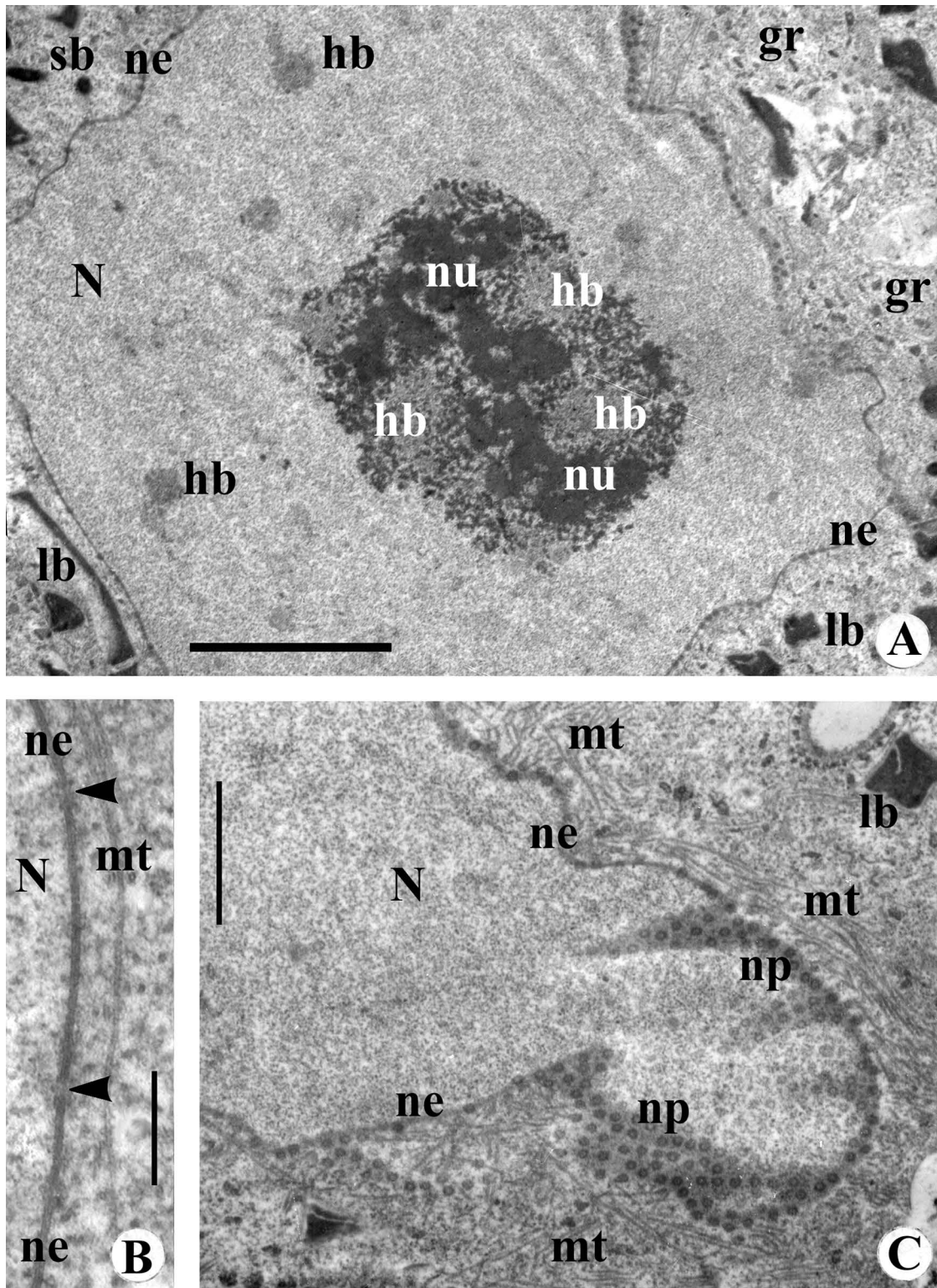
Fig. 10. Fine structure of *P. gruberi* (continued). A - C - the nuclear apparatus. A - general view of the vesicular nucleus. B - nuclear envelope, arrowheads point to nuclear pores; C - microtubules around the nucleus. *Abbreviations*: hb - chromatin blocks; ne - nuclear envelope; np - nuclear pores; other abbreviations as in Fig. 2-9. Scale bars: A - 3.0 μm; B - 0.40 μm; C - 1.0 μm.

microscopic characters obtained from the type species, *P. palustris*. One of the latest circumscriptions of the genus *Pelomyxa*, which includes ultrastructural data, was proposed by G. Brugerolle and D. Patterson (2000). According to these authors, *Pelomyxa* is a free-living amoeboid organisms with a single large pseudopodium and a posterior uroid, the number of nuclei is one to many, flagella are numerous, *basal bodies are connected to a cone of microtubules, cytoplasm contains glycogen bodies, three types of prokaryotic endocytobionts; a complex life cycle with a pronounced seasonal nature includes the cyst stage.* We have italicized those specific characters of *P. palustris* that are not expressed in other *Pelomyxa* species and therefore should not be included in the genus diagnosis. Though the rest of the characters do provide a basis for unification of all the *Pelomyxa* species studied in a single taxon, the taxonomic rank of this taxon is questionable. In our opinion, the polymorphism of its members exceeds the generic level. However, this problem is beyond the scope of the present paper. Moreover, since faunistic studies of pelomyxoid organisms are now intensive as never before, a revision of the genus *Pelomyxa* would be premature. At the same time, even the facts presently available testify, in the very least, that the genus *Pelomyxa* is paraphyletic.

References

Andresen N., Chapman-Andresen C. and Nilsson J.R. 1968. The fine structure of *Pelomyxa palustris*. Compt. Rend. Trav. Labor. Carlsberg, Ser. chim. 36, 285-320.

Brugerolle G. 1982. Caracteres ultrastructuraux d'une mastigamibe: *Mastigina hylae* (Frenzel). Protistologica. 18, 227-235.

Brugerolle G. 1991. Cell organization in free-living amitochondriate heterotrophic flagellates. In: The Biology of Free-living Heterotrophic Flagellates, (Eds D.J. Patterson and J. Larsen). Clarendon Press, Oxford. pp. 133-148.

Brugerolle G. and Patterson D. 2002. Order Pelobiontida Page, 1976. In: An illustrated guide to the protozoa, 2nd ed. Vol. II. (Eds. J. Lee, G. Leedale and P. Bradbury). Allen Press, Lawrence, pp. 1097-1103.

Chapman-Andresen C. 1978. The life cycle of *Pelomyxa palustris*. J. Protozool. 25, 1, 42A.

Chapman-Andresen C. 1982. Identification of *Pelomyxa binucleata* as a stage in the life cycle of *P. palustris*. J. Protozool. 29, 499-500.

Chapman-Andresen C. and Hamburger K. 1981. Respiratory studies of the giant amoeba *Pelomyxa palustris*. J. Protozool. 28, 433-440.

Chavez L.A., Balamuth W. and Gong T. 1986. A light and electron microscopical study of a new, polymorphic free-living amoeba, *Phreatamoeba balamuthi* n. g., n. sp. J. Protozool. 33, 397-404.

Daniels E.W. 1973. Ultrastructure. In: The Biology of Amoeba. (Ed. K.W. Jeon). Acad. Press, New York, London. pp. 125-169.

Daniels E.W., Breyer E.P. and Kudo R.R. 1966. *Pelomyxa palustris* Greeff. II. Its ultrastructure. Z. Zellforsch. 73. 367-383.

Frolov A.O., Chystjakova L.V. and Goodkov A.V. 2004. A new pelobiont protist *Pelomyxa corona* sp. n. (Peloflagellatea, Pelobiontida). Protistology. 3, 233-241.

Frolov A.O., Chistyakova L.V., Malysheva M.N. and Goodkov A.V. 2005a. Light and electron microscopic investigation of *Pelomyxa prima* (Gruber, 1884) (Peloflagellatea, Pelobiontida). Tsitologiya. 47, 89-98 (in Russian with English summary).

Frolov A., Chystjakova L. and Goodkov A. 2005b. A light- and electron-microscopical study of *Pelomyxa binucleata* (Gruber, 1884) (Peloflagellatea, Pelobiontida). Protistology. 4, 57-73.

Goldschmidt R. 1907. Über die Lebensgeschichte der *Mastigella vitrea* n. sp. und *Mastigina setosa* n. sp. Arch. Protistenk. Suppl. 1, 83-168.

Goodkov A.V. 1989. Ultrastructure of the giant amoeba *Pelomyxa palustris*. I. Cytoplasmic microtubules, subcentrioles, and flagella: a comparative morphological analysis of organization. Tsitologiya. 31, 371-379 (in Russian with English summary).

Goodkov A.V. and Seravin L.N. 1991. Ultrastructure of the 'giant amoeba' *Pelomyxa palustris*. III. The vacuolar system; its nature, organization, dynamics and functional significance. Tsitologiya. 33, 17-25 (in Russian with English summary).

Goodkov A.V. and Seravin L.N. 1995. *Pelomyxa palustris*: amoeba, caryoblastean, archezoan, or peloflagellatan? Tsitologiya. 37, 1053-1063.

Goodkov A.V. Chistyakova L.V. Seravin L.N. and Frolov A.O. 2004. The concept of pelobionts (class Peloflagellatea): a brief history and current status. Zool. Zh. 83, 643-654 (in Russian with English summary).

Griffin J.L. 1988. Fine structure and taxonomic position of the giant amoeboid flagellate *Pelomyxa palustris*. J. Protozool. 35, 300-315.

Gruber A. 1884. Studien über Amoben. Zeit. wiss. Zool. 41, 186-225.

Page F.C. 1981. Eugene Penard's slides of Gymn-amoebia: re-examination and taxonomic evaluation. Bull. Brit. Mus. Nat. Hist. (Zool.). 40, 1-32.

Page F.C. 1988. A new key to freshwater and soil gymnamoebae with instructions for culture. Ambleside, Freshwater Biol. Assoc. Sci. Publ.

Page F.C and Siemensma F. G. 1991. Nackte Rhizopoda. Protozoenfauna Band 2. Nackte Rhizopoda und Heliozoea. Gustav Fisher Verlag, Stuttgart, New York.

Penard E. 1902. Faune rhizopodique du bassin du Leman. W. Kundig et Fils, Geneva.

Penard E. 1909. Sur quelques mastigamibes des environs de Geneve. Revue Suisse Zool. 17, 405-439.

Seravin L.N. and Goodkov A.V. 1987. The flagella of the freshwater amoeba *Pelomyxa palustris*. Tsitologiya. 29, 721-724 (in Russian with English summary).

Simpson A.G.B., Bernard C., Fenchel T., and Patterson D.J. 1997. The organisation of *Mastigamoeba schizophrenia* n. sp.: more evidence of ultrastructural idiosyncrasy and simplicity in pelobiont protists. Eur. J. Protistol. 33, 87-98.

van Bruggen J.J.A., van Rens G.L.M., Geertman E.J.M., Zwart K.B., Stumm C.K. and Vogels G.D. 1988. Isolation of methanogenic endosymbiont of the sapropelic amoeba *Pelomyxa palustris* Greeff. J. Protozool. 35. 20-23.

Walker G., Simpson A.G.B., Edgcomb V.P., Sogin M.L. and Patterson D.J. 2001. Ultrastructural identities of *Mastigamoeba punctachora, Mastigamoeba simplex*, and *Mastigella commutans* and assessment of hypotheses of relatedness of the pelobionts (Protista). Eur. J. Protistol. 37, 25-49.

Whatley J.M. 1976. Bacteria and nuclei in *Pelomyxa palustris*: comments on the theory of serial endosymbiosis New Phytol. 76, 111-120.

Whatley J.M. and Chapman-Andresen C. 1990. Phylum Karyoblastea. In: Handbook of protoctista. (Eds. J.O.Corlis, M.Melkonian and D.J.Chapman). Jones and Bartlett Publishers, Boston. pp. 167-185.

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