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Protistology

The ultrastructure of *Procryptobia sorokini* (Zhukov) comb.nov. and rootlet homology in kinetoplastids

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Summary

The ultrastructure of a marine, free-living, heterotrophic kinetoplastid *Procryptobia sorokini*, was investigated with special attention to the cytoskeletal structures. The flagellates have a typical eukinetoplastidal mitochondrion, 2 heterodynamic flagella and all other kinetoplastid characters. The basal body of the anterior flagellum nucleates one dorsal microtubular rootlet, and the basal body of recurrent flagellum gives rise to the ventral rootlet. The cytostome-cytopharyngeal complex is supported by 3 microtubular bands. One band (mtr) is derived from the dorsal rootlet, and other two arise from the preoral crest. The recurrent flagellum adheres to the cell surface, with specialised contacts of the "macula adherens" type similar to those found in trypanosomes. This species described earlier as *Bodo sorokini* (Zhukov, 1975) is transeferred into the genus *Procryptobia* Vickerman, 1978, because of adherent recurrent flagellum and body shape characters. This is the first strong evidence that biflagellated kinetoplastids have 2 microtubular rootlets, which are homologous with those in all investigated species of bodonids and cryptobiids. These kinetoplastids contain the most complete set of these structures. Their rootlets have both the same orientations and a similar number of microtubules in different kinetoplastids. Their derivatives occur in all kinetoplastids with the exception of trypanosomatids, where the reduction of the posterior flagellum leads to the complete reduction of the ventral rootlet and structures associated with it.

Key words: *Procryptobia sorokini*, kinetoplastids, bodonids, cryptobiids, ultrastructure, rootlet homology

Introduction

Free-living kinetoplastids are a main and permanent component of aquatic ecosystems, having great significance in food webs at the level of the so-called 'microbial loop' (Arndt et al., 2000). In spite of the broad diversity and frequent occurrence of free-living kinetoplastids, their contribution to the fauna and to their taxonomy are still poorly understood. In addition many well known species and genera require reinvestigation by modern methods, particularly some doubtful genera like *Pleuromonas, Cruzella, Phanerobia,*

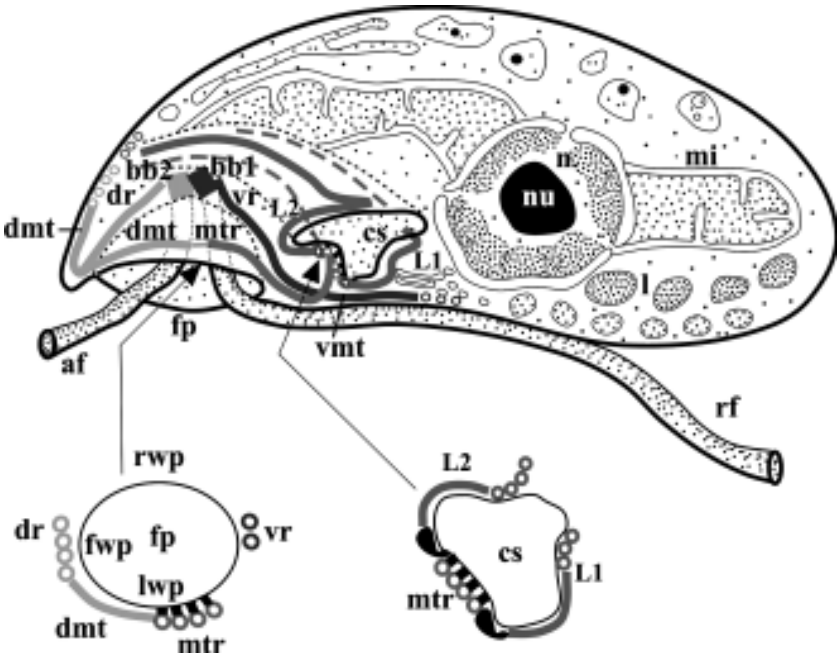


Fig. 1. Scheme of *Procryptobia sorokini* ultrathin organisation. Abbreviations: af – anterior flagellum, bb1 – basal body of posterior flagellum, bb2 – basal body of anterior flagellum, cs – cytostome, dmt – dorsal microtutbules – secondary microtubules of dr, dr – dorsal rootlet, fp – flagellar pocket, fwp – front (anterior) wall of flagellar pocket, l – lipid globules, L1 and L2 – left and right (respectively) cytostomal bands, lwp – left wall of flagellar pocket, mi - mitochondrion, mtr – band of reinforsed microtubules, n – nucleus, nu – nucleolus, rf – recurrent flagellum, rwp – right wall of flagellar pocket, vmt – ventral microtubules – secondary microtubules of vr, vr – ventral rootlet.

Parabodo and *Procryptobia,* whose type species have poor light microscopic descriptions (Patterson and Zöllfel, 1991; Vickerman, 1991). At the same time, even the well known, valid, genus *Bodo*, is not clearly designated. According to its description, it includes all free-living kinetoplastids with a more-or-less oval body and an apical rostrum, two free heterodynamic flagella, and a single kinetoplast (Vickerman, 1976; Zhukov, 1991). However, these characters are shared by many other kinetoplastids. For this reason, the genus *Bodo* has been considered a 'dumping ground' for many bodonid species which cannot be affiliated with the other well-recognised genera like *Rhynchobodo* or *Rhynchomonas*. This work attempts to clarify the taxonomy for such species.

Bodo sorokini was described by Zhukov (1975) from a sample collected from Lake Faro (Sicily) near Messina. The principal character of this bodonid is the adherence of its recurrent flagellum to the cell surface, a character typical of the genus *Procryptobia*. We describe here for the first time the ultrastructure of *Bodo sorokini* and transfer it to the genus *Procryptobia* Vickerman, 1978.

Material and Methods

Several clonal cultures of *Bodo sorokini* were established from coastal samples collected from the White sea (depth 0.5 m, salinity 12‰) near the Marine Biological Station of the Zoological Institute, Russian Academy of Sciences (Kartesh) in May, 1986 and maintained at the culture collection of the protistology group of the Institute of Biology of Inland Waters RAS (Borok). This study is based on observations on clone B-69.

The flagellates were grown on the artificial marine Schmalz-Pratt's medium (Goryatcheva, 1971) containing NaCl-28.15 g/l, KCl-0.67 g/l, $MgCl_2 \cdot 6H_2O$ -5.51 g/l, $MgSO_4 \cdot 7H_2O$ - 6.92 g/l, $CaCl_2 \cdot H_2O$ - 1.45 g/l, KNO_3 - 0.1 g/l, $K_2HPO_4 \cdot 3H_2O$ - 0.01 g/l with a final salinity of 35‰. Cultures were periodically fed with the bacteria *Klebsiella aerogenes*.

An MBR-3 microscope (LOMO, Russia), and a Peraval-Interphako microscope (Zeiss, Germany) were used for LM.

For electron microscopy, the flagellates were transferred into a medium with a lower salinity (16‰) and cultured for a further 2-3 weeks before fixation. An aliquot of 1 ml of culture medium was mixed with 1 ml

of a solution containing 4% glutaraldehyde, 0.05M cacodylate buffer and 0.24M sucrose. After fixation for 2 H on ice, the pellet was collected by centrifugation and rinsed for 15 min in 0.025M cacodylate buffer with 0.06M sucrose. After postfixation with 1% osmium tetroxide in 0.05M cacodylate buffer with 0.05M sucrose for 1 H at 4°C, the pellet was dehydrated in an alcohol series and embedded in Epon resin. Blocks were serially sectioned with a diamond knife on a Reichert Ultracut ultramicrotome, mounted on formvar-coated slot grids, and stained with uranyl acetate and lead citrate. Sections were viewed on a Philips CM 10 electron microscope operating at 80 kV.

The 3-dimensional reconstruction of the cell of *P. sorokini* (Fig. 1) is based upon the analysis of more than 20 series of sections of the apical part of cells, 6 of which allowed for the reconstruction of the whole cell.

Results

Procryptobia sorokini (Zhukov) Frolov, Karpov et Mylnikov comb. nov.

Diagnosis: marine, unicellular, free-living kinetoplastids with 2 heterodynamic flagella emerging from a subapical flagellar pocket opens on the ventral side of the cell. Cell body oval or pear-shaped and laterally compressed, slightly metabolic when attached to the substrate. Body length: 6.6 - 8.3 (mean 8.1) μm, width: 3.0 - 4.5 (mean 4.2) μm. Anterior flagellum equal in length to cell body often hook-like. Recurrent flagellum 1.5 times longer than cell length mostly in contact with the ventral cell surface. Kinetoplast anterior, nucleus central. Cytostome on the left side of the cell, at the level of the posterior wall of flagellar pocket. Refractive granules present along the ventral side parallel to the recurrent flagellum. Contractile vacuole absent. Swimming slow, with rotation around the longitudinal axis. Movement more common with the left side in contact with the substratum and with concomitant vibration. No cysts seen, and growth possible over a broad salinity range (5 - 40‰).

Type locality: saline lake (a former gulf of the Mediterranean sea) Faro (salinity 30‰) near Messina, Sicily.

This species has subsequently been found in coastal waters of the: Black sea (near Yalta) in 1987, the Baltic sea (Ruegen Island) in 1994-95, and the White sea.

Light microscopy (Figs 2, 3)

Exponentially growing cells are 6.6-8.3 (mean 8.1) μm long, and 3.0-4.5 (mean 4.2) μm wide. Rarely, giant cells (12 μm long) may be found. In old cultures (one month or more) many small cells (appr. 6.0 μm long) or abnormal cells with extra flagella, nuclei and kinetoplasts occur. A contractile vacuole is absent in cells of this clone and in this detail alone they differ from those first described by Zhukov (1975).

The general features of *P. sorokini* (cell shape, flagellar orientation) resemble those of marine and brackish water representatives of *Bodo (B. designis, B. saliens, B. saltans, B. curvifilus, B. cephaloporus and B. cygnus*) (Larsen and Patterson, 1990; Vшrs, 1992; Patterson and Simpson, 1996), but this species is clearly distinguishable from *Bodo spp.* by its more flattened body and the attached nature of its recurrent flagellum along its cell surface. The latter character is not noted in the original diagnosis of the genus *Bodo* (Vickerman, 1976).

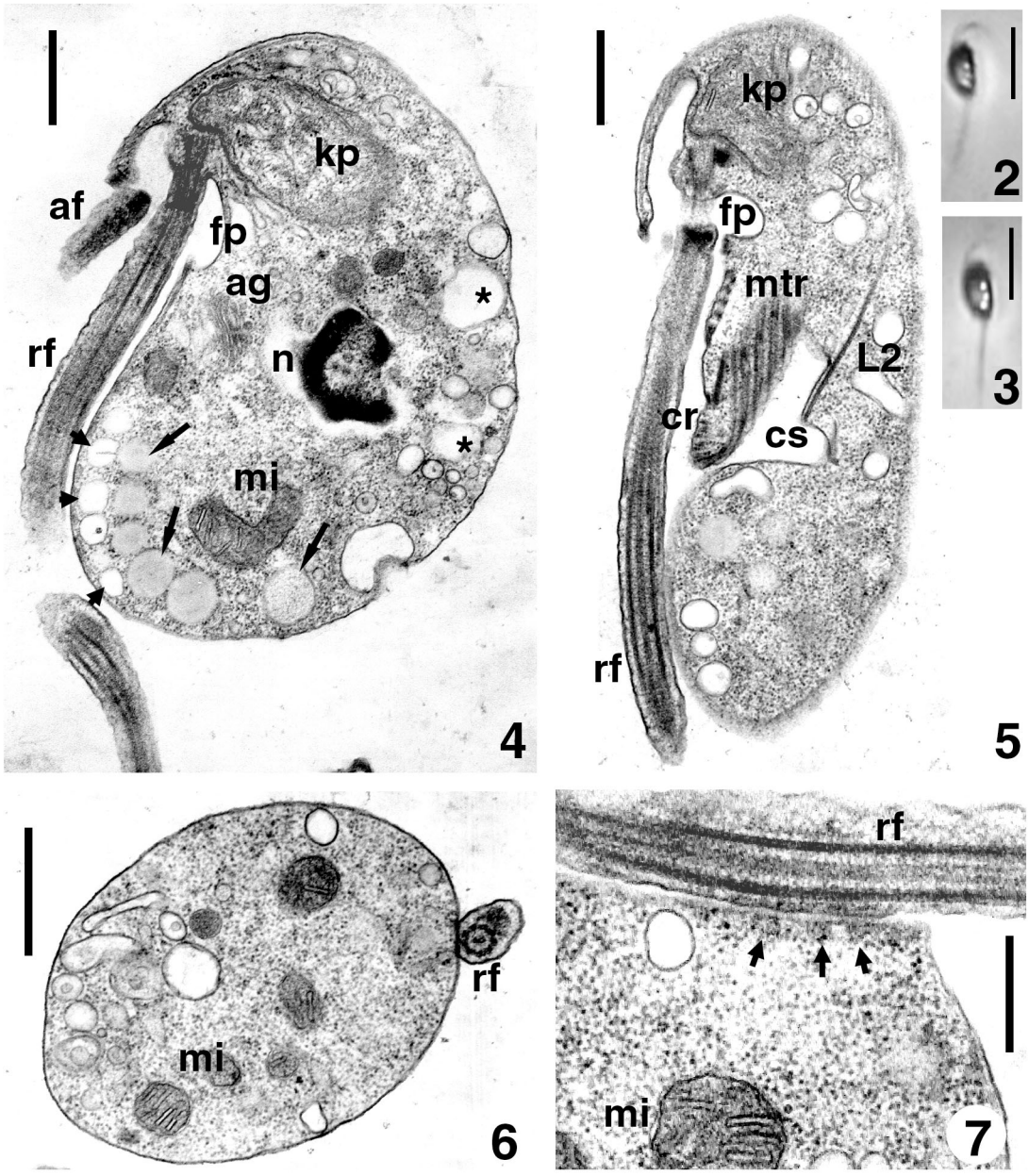
The density in the culture may reach up to 250,000 cells/ml and can stay at this level during rather long time (up to 6 months). Organisms in such cultures move more slowly and settle on the bottom of the Petri dish.

Electron microscopy

General organisation of the cell (Figs 1, 4-32)

The plasmalemma of *P. sorokini* is naked (Figs 4-6) and is underlain by cytoskeletal elements, which do not extend beyond in the anterior part of the body (Fig. 1).

The pear-shaped kinetoplast is typical, with one nucleoid, and is located next to the bottom of flagellar pocket (Figs 4, 5, 8-18). Mitochondrial cristae are plate-like (Figs 7, 9, 10, 18, 31). The Golgi apparatus has 5-7 stacked flat cisternae, and is slightly ventral, between the nucleus and the basal bodies (Figs 4, 16). A cytoplasmic reticulum is scattered throughout the cell, with some concentration around the flagellar pocket (Fig. 4, 12). Many small vesicles (0.1-0.5 μm) with different contents are present at the cell periphery. Two types are arranged in 2 regular rows on the ventral surface of *P. sorokini* (Fig. 4, 32). One of them, beneath the plasmalemma (Fig. 4), is composed of small vesicles (0.10-0.20 μm in diameter) with electron translucent contents (some with tiny inclusions), and the other lies deeper in cytoplasm (Fig. 4), resembling a row of lipid droplets (0.2-0.5 μm in diameter). There are many profiles of vesicles and cisternae in the vicinity of the proximal part of cytopharynx on the dorsal side of the cell. The flagellar pocket is subapical and opens to the ventral cell surface (Figs 1, 4, 5, 14-18). Two smooth flagella emerge from the pocket. One flagellum turns anteriorly and is thus forward-directed, and the other (recurrent) flagellum is posteriorly directed and held close to the ventral cell surface. A ventral groove is absent (Fig. 6). The cytostome opens to the left ventral side of the cell just posterior to the flagellar pocket (Figs 5, 12-25). The cytostome leadűs to the cytopharynx,

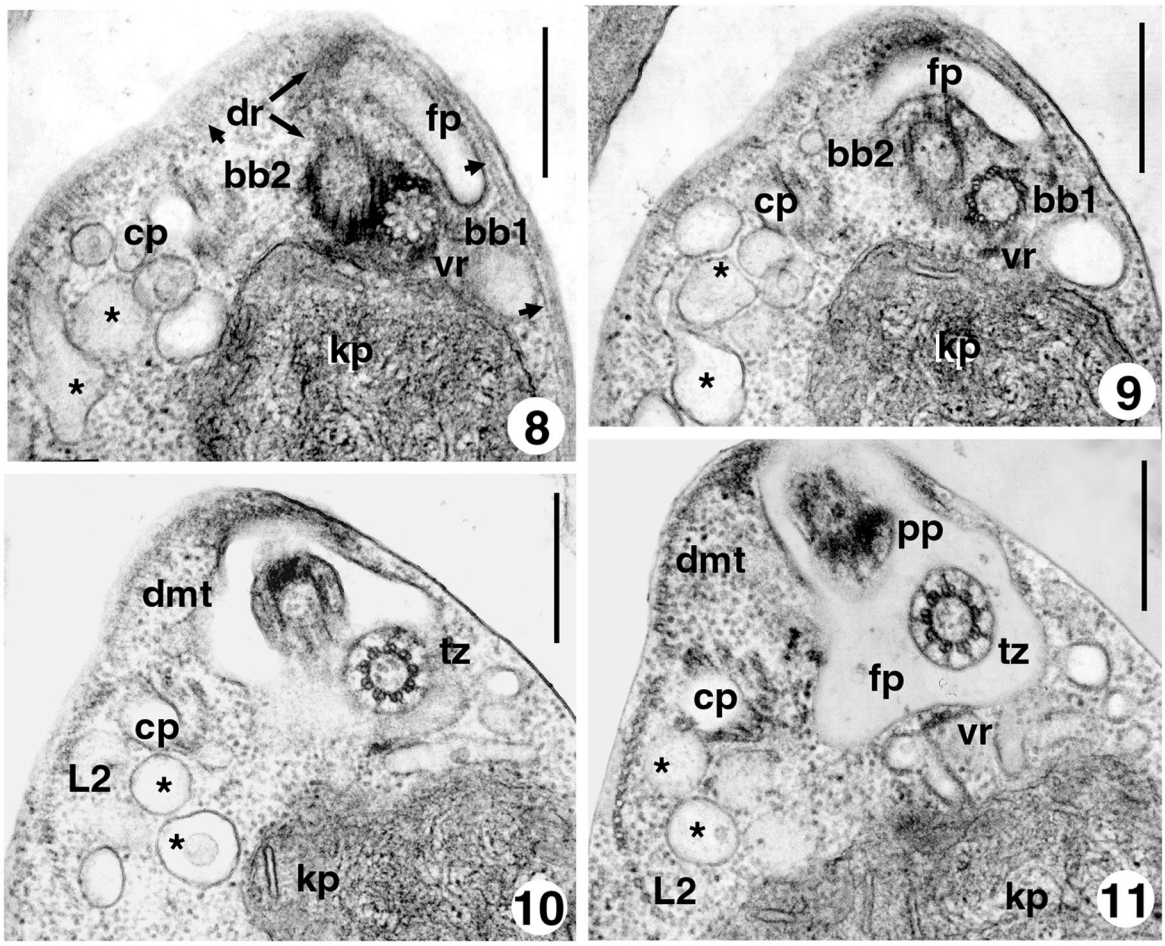


Figs 2-7. Light and electron microscopy of *Procryptobia sorokini*. **2-3** – phase contrast, lateral view. Note hooked anterior flagellum and white dots (refractive granules) at the ventral cell side. **4** – LS through flagellar pocket (fp), kinetoplast (kp), and nucleus (n). Short arrows show small vesicles, long arrows – lipid droplets, asterisks mark the vesicles associated with cytopharynx. **5** – LS through flagellar pocket and cytostome, showing preoral crest. **6** – TS of posterior third part of the cell and recurrent flagellum (rf) behind the nucleus. **7** – LS through the recurrent flagellum adhering to the ventral cell surface. Arrows show desmosome-like contacts. Bars: 10 μm for figs 2-3, 1 μm for figs 4-6, and 0.4 μm for fig. 7.

which passes from the ventral to the dorsal side of the cell, and ends under the dorsal cell surface (Figs 12-15).

Flagellar apparatus

Two basal bodies of normal structure diverge at an angle 35-45° (Figs 8-9, 15-17). Their bases are inserted in the electron dense material, which covers the surface of kinetoplast (Figs 4, 8, 17). The basal body of the anterior flagellum (bb2) is located on the left side of the cell and is orientated in a dorso-ventral plane, while the basal body of the recurrent flagellum (bb1) is located on the right side (Figs 8-9, 15-17).



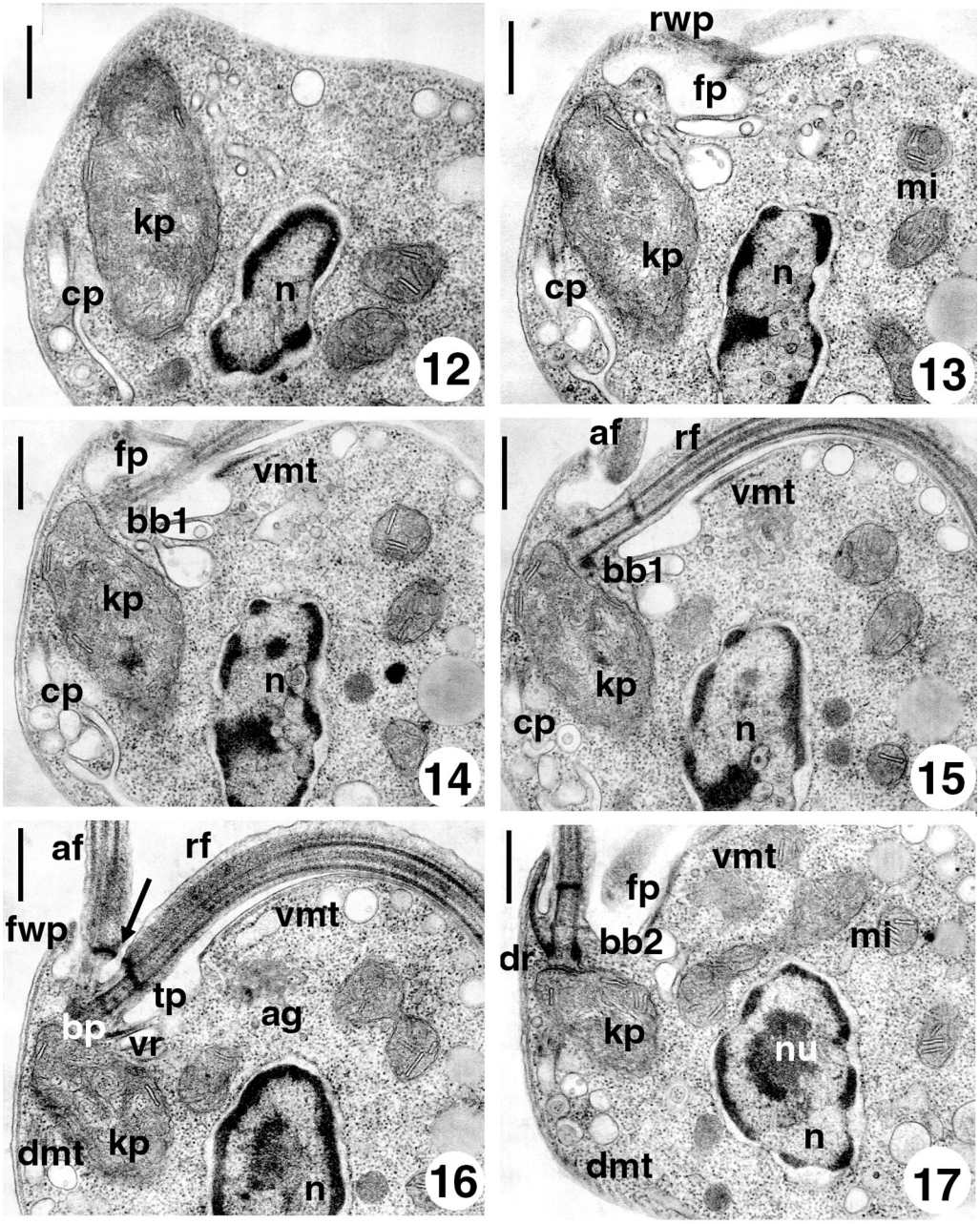
Figs 8-11. Series of dorso-ventral sections through the *Procryptobia sorokini* flagellar pocket, as viewed from the posterior end of the cell. Asterisks mark vesicles associated with cytopharynx. Bars: 1 μm for figs 4-6, and 0.4 μm for fig. 7. Abbreviations: bb1 – basal body of posterior flagellum, bb2 – basal body of anterior flagellum, cp – cytopharynx, pp – paraxial plate, tz – transition zone. Other abbreviations are the same as in previous figures.

The flagellar transition zone is slightly longer, than the basal body. It is limited by transverse and terminal plates (Figs 15-17), and the space between these plates is filled with rather thick fibrillar threads (Figs 10, 11, 15-17). The terminal plate is continuous with the paraxial plate, from which the paraxial rod emanates (Figs 11, 16).

The paraxial plates of the flagella face each other, and the flagellar surfaces have contact at this point (Fig. 16). The proximal half of the recurrent flagellum adheres to the ventral cell surface by means of equally spaced condensations of fibrillar material reminiscent of desmosomes (Fig. 7). The second (posterior) half of this flagellum is free of the cell (Figs 4, 5).

The rootlet system of *P. sorokini* is comprised of 2 microtubular bands. The basal body 2 nucleates a dorsal rootlet (dr) composed of 4-5 microtubules, which passes along the front (anterior) wall of the flagellar pocket (Figs 8, 11, 16-18). At the anterior apex it gives rise to secondary cytoskeletal microtubules (dmt). The majority of these dorsal microtubules radiate posteriorly under the plasmalemma, but some of them reflex and pass along the left wall of the flagellar pocket (Figs 18, 19), where they nucleate a new set of microtubules (Fig. 18) ("mtr" of Brugerolle et al., 1979), which is connected to the plasmalemma by electron dense material (Figs 26-29).

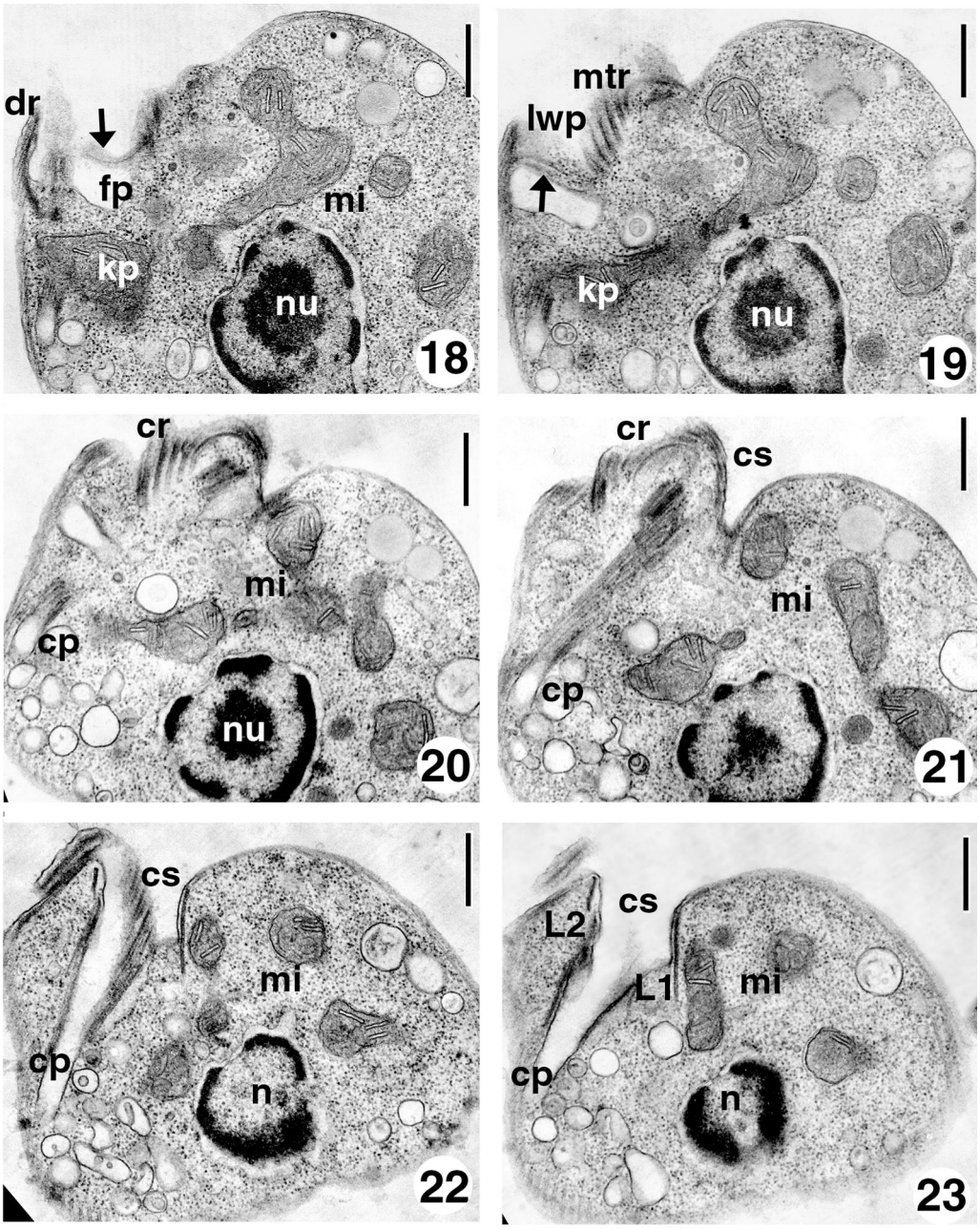
The basal body1 gives rise to a single ventral rootlet (vr) composed of 2-3 microtubules, which passes along the posterior wall of flagellar pocket and nucleates 4-5 submembranal microtubules (vmt), reinforcing the plasmalemma under the recurrent flagellum (Figs 9-11, 14-17).



Figs 12-25. Selected serial sections of *Procryptobia sorokini* orientated from the right side of the cell (12) to its left side (25); ventral side up, dorsal – bottom. Arrow in fig.16 shows the contact between two flagella at the level of paraxial plates. Arrows in figs 18 and 19 show the connection of dorsal microtubules (dmt) with mtr. Bar: 0.4 μm. Abbreviations: bp – basal plate, fwp – front wall of flagellar pocket, lwp – left wall of flagellar pocket, rwp – right wall of flagellar pocket, nu – nucleolus. Other abbreviations are the same as in previous figures.

The cytostome-cytopharyngeal complex

The cytostomal surface is covered with simple hairs. The cytostome leads into the cytopharynx, which invades the left side of the cell (Figs 8-11). Three groups of microtubules support this complex (Fig. 1). One group (mtr) originates at the left wall of the flagellar pocket (Figs 18, 19). It passes up to the distal and posterior side of flagellar pocket, and then reflexes almost through 180° to underlie the inconspicuous preoral crest (Figs 18-21, 26). The reflexed portion of the mtr reinforces the cytopharyngeal wall that faces the flagellar pocket (Figs 27-29). The mtr is associated with fibrillar material, which is connected to the plasmalemma (Figs 26-29).

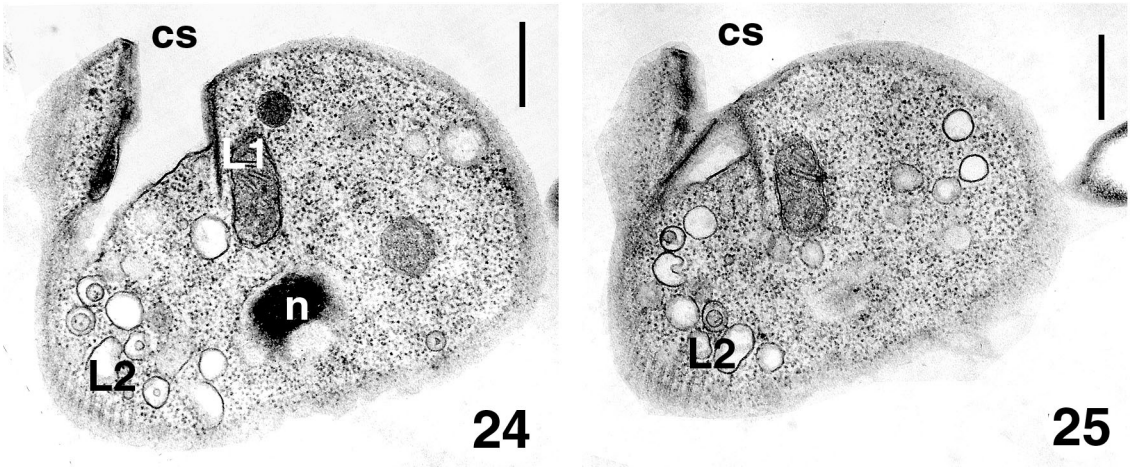


Figs 12-25. *(Continuation).*

Two other groups of microtubules (designated L1 and L2) nucleate on the electron dense material of the preoral crest (Figs 26-29). Each band forms a semicircle on either side of the cytostomal funnel: L1 on the right wall, and L2 on the left one (Figs 27-29). The microtubules of both bands then turn to along the cytopharynx (Figs 1, 5, 23). L1 reaches the proximal part of the cytopharynx, underlying its membrane, and L2 initially follows a similar path, but later turns away from the cytopharyngeal wall (Figs 10, 11, 24, 25). Under the dorsal cell surface, L2 is accompanied by dorsal cytoskeletal microtubules (Figs 10, 11, 24, 25).

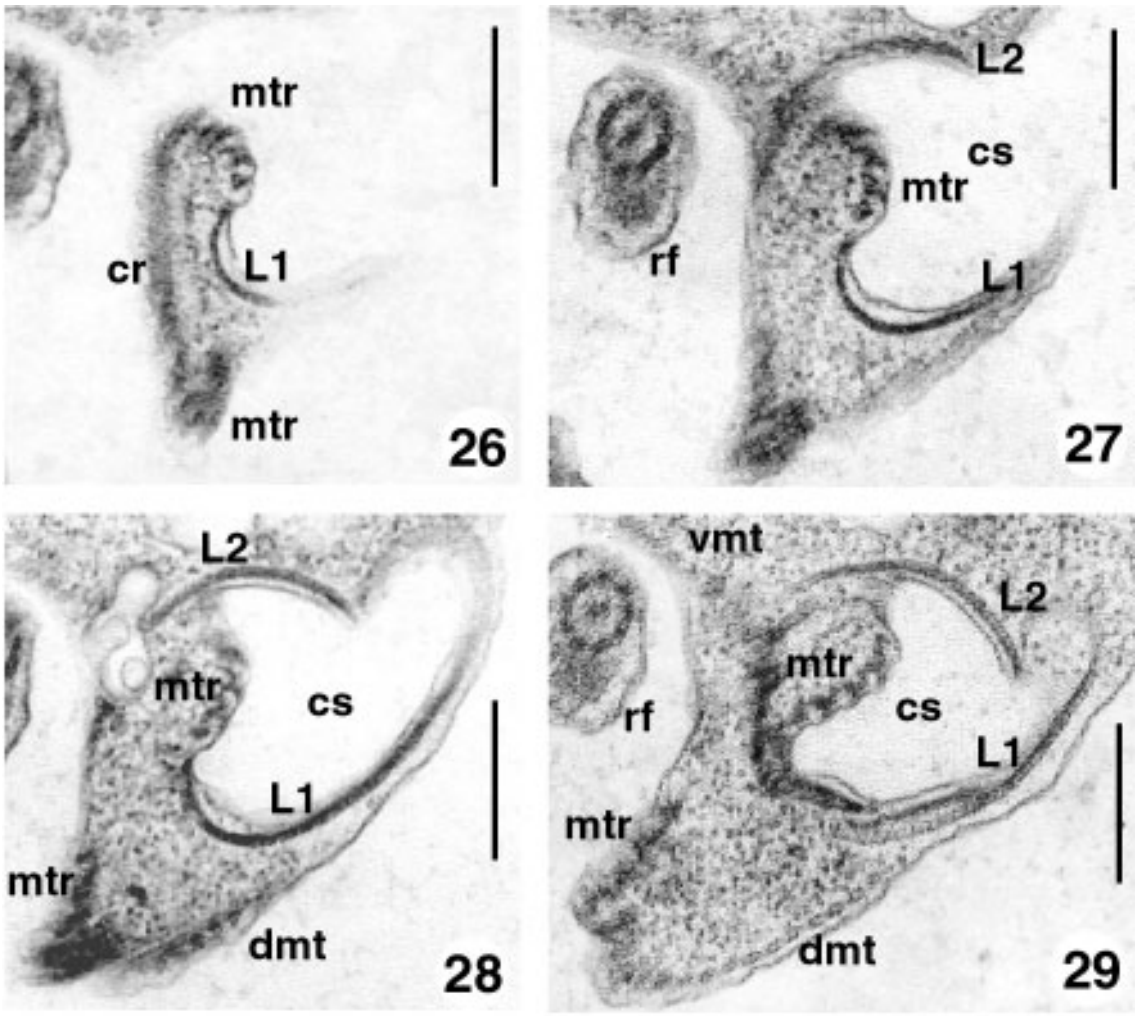
The mitochondrion

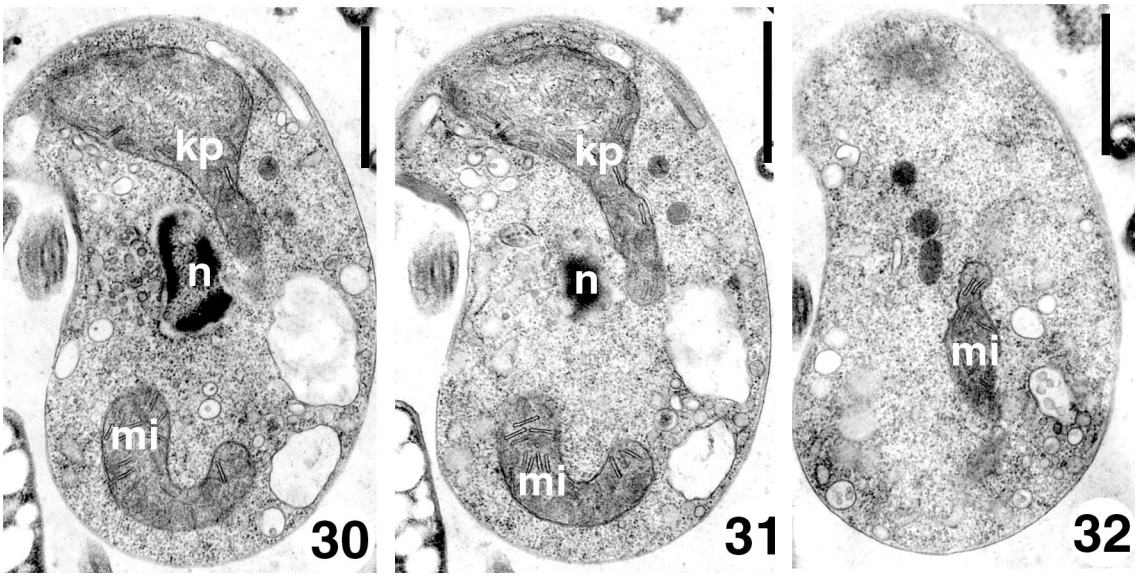
In each non dividing cell of *P. sorokini* there is only a single giant mitochondrion (Figs 30-32). The kinetoplast is located between the nucleus and anterior cell surface (Figs 4, 14, 15). Its ventral surface faces the bottom of the flagellar pocket. The adjoining part



Figs 12-25. *(Continuation).*

of the mitochondrion is sharply narrowed, and its sausage-like "tail" extends along the longitudinal cell axis towards the posterior end of the cell. The end of this tail normally curves toward the ventral side, forming a hook (Figs 4, 30).





Figs 30-31. Series of LS of *Procryptobia sorokini*, showing the kinetoplast-mitochondrion complex. Bar: 1 μm. Abbreviations are the same as in previous figures.

Discussion

Species characters

The adherence of the recurrent flagellum to the cell surface in *P. sorokini* indicates that this organism is similar to cryptobiids, probably belonging to this family. This affiliation is corroborated by recent phylogenetic analysis based on SSU rRNA gene sequences (including the sequence of our strain of *P. sorokini*). It clearly showed that *P. sorokini* belongs to the clade including *Trypanoplasma borreli* and fish *Cryptobia*, which are separated with high bootstrap value from other *Bodo* (Dolezel et al., 2000). Thus, both the morphological and molecular data show that *P. sorokini* is a cryptobiid and therefore it has to be a member of genus *Procryptobia* Vickerman, 1978. The genus *Procryptobia* is easily distinguished from *Cryptobia*, another genus of this family, by the absence of a ventral groove and cortical contractility, and by the presence of obligate phagotrophy (Vickerman, 1978). Unfortunately, all other representatives of *Procryptobia (P. vorax, P. tremulans* and *P. glutinosa*) only have a light micoscopic description, except *P. glutinosa* which was also investigated with SEM (Vickerman, 1978). Such studies do not permit a complete comparison of *Procryptobia* species. However, *P. sorokini* is more similar to *P. vorax*. Both species have close dimensions: *P. vorax* - 9.5 x 4.8 μm (Vickerman, 1978) and *P. sorokini* - 8.1 x 4.3 μm. They also share a line of refractive granules on the ventral side of the cell, which may correspond to the inner ventral row of lipid globules in *P. sorokini*. Nevertheless, there are specific LM characters of *P. sorokini*: it doesn't produce cysts, lives in marine water and does not possess a contractile vacuole. This set of characters is considered sufficient for its recognition as a valid species.

General ultrastructure

In general, the ultrastructure of *P. sorokini* is similar to those of other bodonids and cryptobiids (Brooker, 1971; Burzel, 1975; Eyden, 1977; Vickerman, 1977; Brugerolle, 1985; Brugerolle et al., 1979; Hitchen, 1974; Karpov and Zhukov, 1983; Mylnikov, 1986; Breunig et al., 1993; Frolov et al., 1997). However, it differs from the others by the absence of: a contractile vacuole, mastigonemes, a microtubular prism, extrusomes, and striated fibrillar rootlets. In other words, *P. sorokini* has a typical kinetoplastid organisation with the minimal set of organelles. At the same time, it has some peculiarities with respect to the absolute orientation of organelles within the cell.

The position of cytostome-cytopharynx in this species is unusual for bodonids and cryptobiids. In the majority of investigated biflagellated kinetoplastids it is located more anteriorly relative to the flagellar pocket (Brugerolle et al., 1979; Vickerman, 1991; Frolov and Karpov, 1995).

× **Figs 26-29.** Series of TS through *Procryptobia sorokini* preoral crest (cr), showing the origin of cytostomal bands L1 and L2. Bar: 0.4 μm. Abbreviations are the same as in previous figures.

The cytostome in *P. sorokini* is located on the left side of the cell just posterior to the flagellar pocket. This may be connected with the feeding behaviour of the cell, because it always glides along the substrate by its left side. The dorso-ventral orientation of the cytopharynx is also unusual, as in the majority of other bodonids and cryptobiids this structure passes in anterior-posterior direction (Brugerolle et al., 1979; Frolov and Karpov, 1995). The relatively short cytopharynx of *P. sorokini* has, nevertheless, a complete set of supporting elements connected with microtubular rootlets.

Rootlet homology

According to the traditional view based on the pioneering work of Brugerolle et al. (1979), the cytoskeleton of kinetoplastids consists of: dorsal and ventral rootlets, bands of dorsal and ventral submembrane microtubules, and at least 3 microtubular bands, supporting the cytopharynx. The dorsal rootlet originates from bb2 and nucleates the most prominent dorsal microtubular band. The ventral rootlet originates from bb1 and is associated with a small microtubular band that reinforces the ventral cell surface. The band of reinforcing microtubules (mtr) connects the flagellar and cytostomal apparatuses. According to Brugerolle et al. (1979), the mtr passes near the basal bodies, but these authors never considered it as a rootlet. However, Kivic and Walne's (1984) publication on the phylogenetic relationships between kinetoplastids and euglenids based on morphological data, led to recognition of the mtr as a rootlet by many authors (Farmer and Triemer, 1988; Cavalier-Smith, 1993; Simpson, 1997; etc.). Kinetoplastids have already been recognised to have only two microtubular rootlets (Frolov and Karpov, 1995), but this work conclusively demonstrates this. It is clear (Figs 18-22), that the mtr originates at the wall of the flagellar pocket and is not connected with the basal bodies.

The presence of two microtubular bands (L1 and L2) also has been shown by Brugerolle et al. (1979), but their origin was not clear at that time. The bands L1 and L2 arise from the crest, surround the cytostome from right and left sides respectively, and then turn at almost right angles along the cytopharynx wall.

Thus, in general, all elements of the oral cytoskeleton of biflagellated kinetoplastids are associated only with the dorsal rootlet of bb2. The ventral rootlet and its derivations do not take part in the formation of the cytostome-cytopharyngeal skeleton.

Uniflagellated kinetoplastids (trypanosomatids) with a cytostome, also have this dorsal rootlet with an associated set of microtubules comprising dorsal rootlet, mtr, L1 and L2 (the latter components may be partly reduced – see: Frolov and Karpov, 1995). The recurrent flagellum is totally reduced in these kinetoplastids and all cytoskeletal elements, that normally associate with it are absent.

In general, our data clearly demonstrate the homology of the main cytoskeletal elements in kinetoplastids. Biflagellated kinetoplastids contain the most complete set of these structures. They have dorsal and ventral microtubular rootlets emanating from bb2 and bb1 respectively. Both rootlets have the same orientation and comprise a similar number of microtubules in different species. The dorsal rootlet is normally composed of 3-5 microtubules while the ventral one has 2-3 microtubules (Frolov and Karpov, 1995). Their derivatives occur in all investigated kinetoplastids with exception of the trypanosomatids, where the reduction of the posterior flagellum leads to the complete reduction of the ventral rootlet and its derivatives.

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