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The flagellar apparatus structure of *Apusomonas proboscidea* and apusomonad relationships

Sergey A. Karpov

Biological Faculty, Herzen State Pedagogical University, St. Petersburg, Russia

Summary

Apusomonads represent one of the most enigmatic groups among free-living heterotrophic flagellates, as in spite of having knowledge on their morphology and molecular phylogeny we still cannot find their nearest relatives. The cytoskeleton, particularly the flagellar apparatus, is regarded as an evolutionary conservative structure and often helps to show the relationships and taxonomic position of a group. The flagellar apparatus structure of *Apusomonas proboscidea* was investigated by serial sections and a 3D reconstruction is presented here for the first time. *Ap. proboscidea* has 2 smooth heterocont flagella without paraxonemal rods and with short simple transition zones. Both kinetosomes have a thin cylinder and a cartwheel structure. The kinetosome of the anterior flagellum associates with a broad right microtubular root (RM), a dorsal left root of 2 microtubules and with a striated fibrillar root. The kinetosome of the posterior flagellum associates with a rhizostyle (RH). Both kinetosomes are connected to a multilayered fibrillar structure (MFS), located at the apical part of proboscis. This MFS initiates RH and RM. Each kinetosome has prominent lateral feet, which fuse apart from them, and form a thick electron dense axis. The common features of the apusomonad cytoskeleton have been established and compared with the flagellar apparatuses of other protists. Some cytoskeletal elements of apusomonads were found in other protists, but there are no protists with the same set of roots. The most similar groups in this respect are cryptomonads and myxogastrids; *Multicilia* and excavates have fewer common features. This description of common cytoskeletal characters for apusomonads confirms their monophyly, as revealed by molecular data, but still shows their isolated position among the eukaryotes.

Key words: *Apusomonas proboscidea*, apusomonads, flagellar apparatus, phylogeny, taxonomy, 3D reconstruction.

Introduction

Apusomonads represent a small group of free-living heterotrophic flagellates with two heterocont flagella. They are widespread throughout the world and very common in marine, fresh water and soil samples (Scheckenbach et al., 2006), yet may be overlooked because of their small size and low density. At the same time, some of them (*Apusomonas*) have very distinctive characters (e.g. long anterior proboscis, definite localization of nucleus and contractile vacuole, a slow gliding movement upon the substratum), and can be easily distinguished from other protists.

The Order Apusomonadida Karpov and Mylnikov, 1989 was established of the basis of their distinctive morphology and biological peculiarities (Vickerman et al., 1974; Karpov and Zhukov, 1984; Karpoff and Zhukov, 1986; Mylnikov, 1989); and

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originally included two genera and three species (Karpov and Mylnikov, 1989). At present the Order Apusomonadida includes a Family Apusomonadidea Karpov and Mylnikov, 1989, two genera: *Apusomonas* (=*Rostromonas* Karpov and Zhukov, 1980) with two species, and *Amastigomonas* (=*Thecamonas* Larsen and Patterson, 1990) with 11 species (Mylnikov, 1989; Molina and Nerad, 1991).

The majority of apusomonads have a proboscis, or mastigophore at the anterior end of the body. The flattened body has convex dorsal side and flat ventral one with a ventral groove restricted by longitudinal margin folds. The ventral groove used to produce short pseudopodia for food (bacteria) capture. The cell is covered by a plasmalemma with dense glycocalyx and underlined by thin layer of dense epiplasm. The smooth heterocont flagella have kinetosomes with prominent microtubular roots. Peripheral endoplasmic reticulum separates ectoplasm from the endoplasm. The mitochondria have tubular cristae, and a Golgi dictyosome is located at the anterior part of the cell and is associated with an electron dense body and one of the roots. Some species have in their life cycle a plasmodial stage which is the result of merging of individual specimens (Karpov and Mylnikov, 1989). They do not form cysts, yet can survive drying out (Vickerman et al., 1974; Karpov and Mylnikov, 1989). Sexual reproduction is unknown, asexual reproduction is by binary fission.

The first molecular trees with *Apusomonas proboscidea* shown its very separate position from the other eukaryotes (Cavalier-Smith and Chao, 1995). Molecular data are known at present for *Apusomonas proboscidea*, *Amastigomonas debruynei*, *Am. mutabilis* and *Am. bermudensis*, and still illustrates their isolated position from the other large groups of eukaryotes (Cavalier-Smith and Chao, 1995, 2003; Adl et al., 2005; Scheckenbach et al., 2006), which is reflected in the recent classifications by referring apusomonads as Eukaryota incertae sedis group (Karpov, 2001; Adl et al., 2005). Cavalier-Smith (2002) even established a new phylum Apusozoa for these two genera plus *Ancyromonas* and spironemids. The most recent and very informative study of apusomonad molecular phylogenies based on six gene analysis of *Apusomonas* showed their relationships to either amoebozoans, or opisthokonts (Kim et al., 2006).

As far as both the general ultrastructure and molecular phylogeny demonstrate the peculiarities of apusomonads and do not show their sister group unambiguously, it takes to study their representatives in more details. The flagellar apparatus is one of the conservative structure in many taxa of protists (Karpov, 2000; Simpson, 2003), therefore its study with transmission electron microscopy (TEM) and the reconstruction of flagellar root system of *Apusomonas proboscidea* is very important and may help to clarify its relationships.

Material and Methods

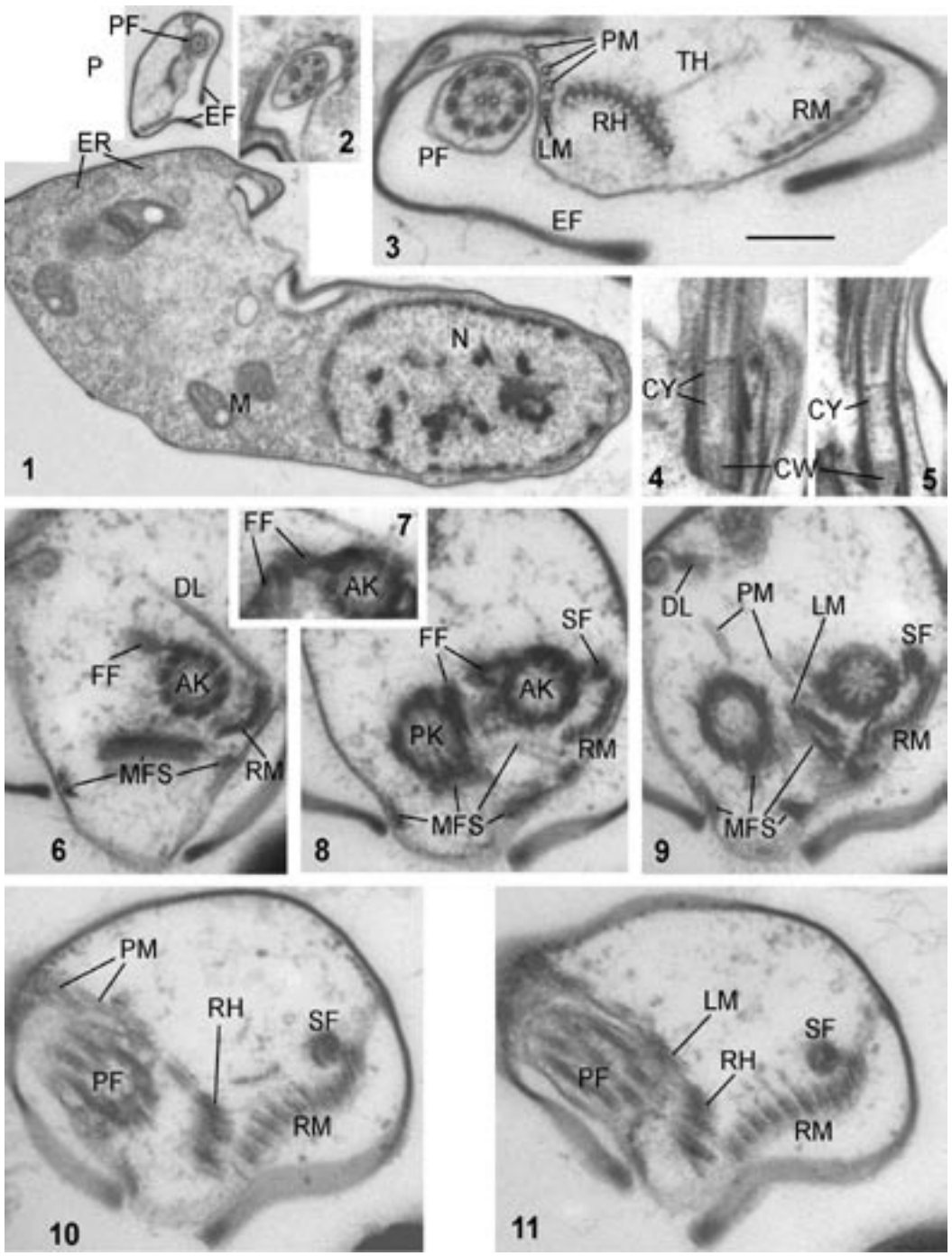
The strain El-4 of *Apusomonas proboscidea* was received from the culture collection of the Institute for Biology of Inland Waters RAS (Borok, Russia) and fixed for TEM in 1984 using the methods for ultrathin sections described earlier (Karpov and Zhukov, 1984). Particular attention for the kinetosomes and root structures was paid. The 3D reconstruction of flagellar apparatus was done on the basis of serial ultrathin sections of 7 cells. Importantly, the 18S rRNA gene of the same strain of *Ap. proboscidea* has already been sequenced, and is deposited in Genbank under the accession number DQ207567 (Scheckenbach et al., 2006).

Results

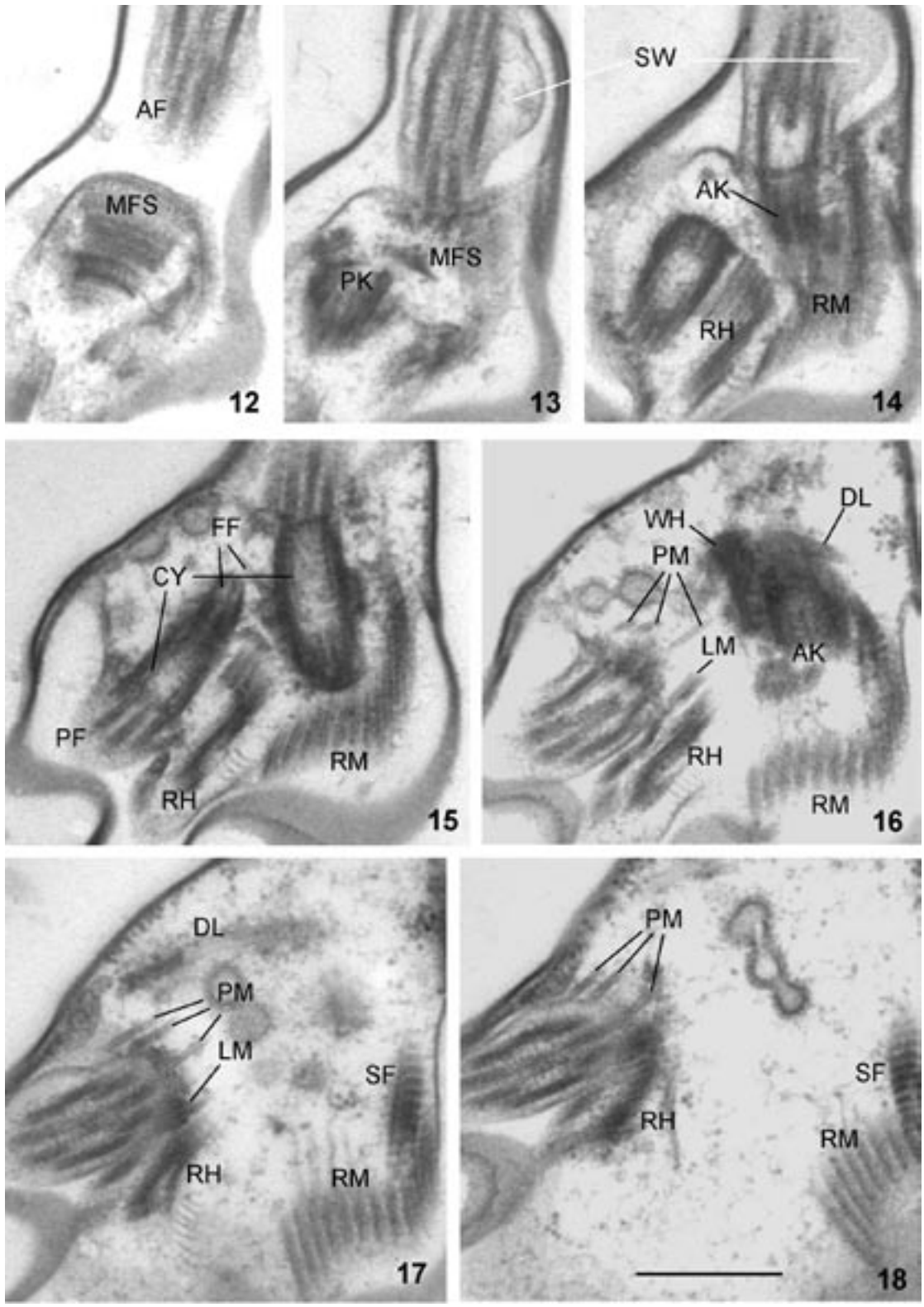
Apusomonas proboscidea has an anterior proboscis built from two portions: the anterior thin part contains the short anterior flagellum surrounded by the margin folds, which form a sleeve; the posterior portion is thick, also restricted by margin folds, and contains the cytoplasm with microtubular roots and the long posterior flagellum passing back along its left side (Figs 1, 3). Thus, both flagella lie in the ventral groove and are oriented in opposite directions: the anterior is directed forward and posterior backward. The anterior flagellum is much more active, serving as a motile one, and always beats to the left. The posterior recurrent flagellum passively trails under the cell. Both flagella are smooth without any mastigonemes and scales, and end with acronemes in which a reduced number of microtubules can be seen (Fig. 2). The axoneme of each flagellum has the usual microtubular set (9+2) without paraxonemal structures. In some cells we found a swelling just above the transversal plate, but it has no any peculiar structures inside (Figs 13, 14), and looks like a result of flagellar bending.

The transition zones of both flagella are identical and simple: the transversal plate crosses the axoneme at the plasma membrane level, two central microtubules attach to the center of this plate (Figs 4, 5). No additional structures worth noting were found in the transition zone.

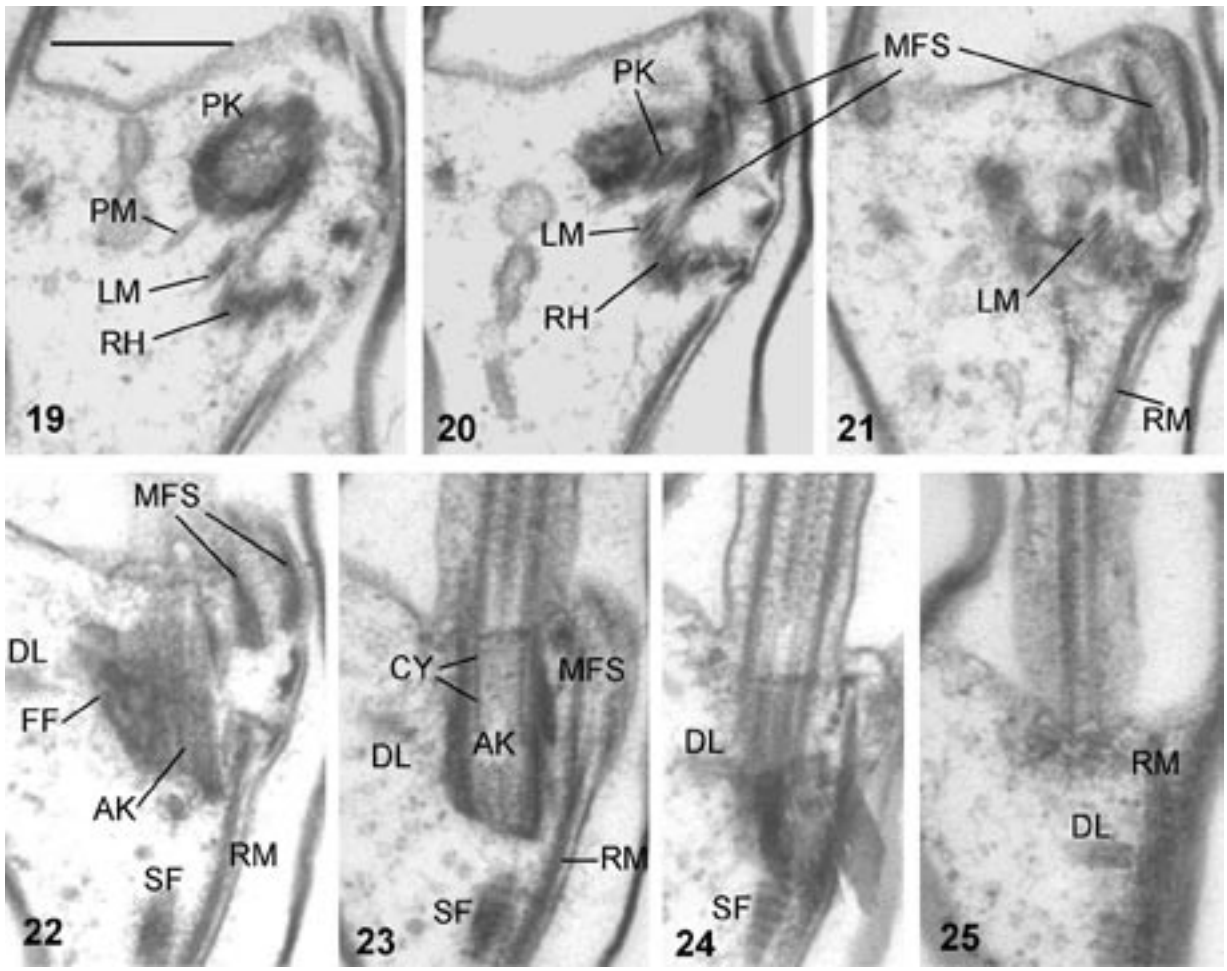
The kinetosomes lie close to each other at a very obtuse angle (almost antiparallel) but in different



Figs 1-11. Ultrathin structure of *Apusomonas proboscidea*. 1 – general view of the cell, 2 – acronema of posterior flagellum, 3 – TS of the thick part of proboscis (view from base to tip of proboscis), 4 – LS of anterior flagellum, 5 – LS of posterior flagellum, 6-11 – consecutive TSs of flagellar apparatus from anterior to posterior. 6-11 - view from base to tip of flagellum and ventral side of the cell faces to the bottom. *Abbreviations*: AK-anterior kinetosome; CY-cylinder inside kinetosomes; CW-cartwheel structure; DL-dorsal left microtubular root; EF-edge folds; ER-peripheral endoplasmic reticulum; FF-fibrillar feet of kinetosomes; LM-left microtubular root of 2 microtubules splitting from RH; MFS-multilayered fibrillar structure, initiating right microtubular root (RM) and rhizostyle (RH); N-nucleus; P-thick part of proboscis; PF-posterior flagellum; PK-posterior kinetosome; PM-posterior singlets of microtubules accompanying posterior flagellum; SF-striated fibrillar root associated with RM; TH-threads of rhizostyle. Scale bar (μm): 1 – 1.25; 2, 4 – 0.4; 3 – 0.25, 5 – 0.6; 6-11 – 0.3.



Figs 12-18. Consecutive LSs of *Apusomonas proboscidea* flagellar apparatus from ventral (12) to dorsal side of the cell. View from dorsal, anterior end to the top. *Abbreviations*: AF-anterior flagellum; SW-swelling of the anterior flagellum; WH-wheel – result of feet fusion. Other abbreviations as in figs 1-11. Scale bar (μm): 0.5.



Figs 19-25. Consecutive LSs of *Apusomonas proboscidea* flagellar apparatus from the left to (19) the right side of the cell. View from dorsal, anterior end to the top. *Abbreviations* as in figs 1-18. Scale bar (μm): 0.5.

planes (Fig. 8). The distal half of each kinetosome contains a hollow thin walled cylinder (Figs 4, 15). In cross sections it looks like a fiber connected by short bridges to A-tubules of the peripheral triplets. The proximal part of each kinetosome has a well developed cartwheel structure (Figs 4, 5).

Since no significant differences between the kinetosomes were found, and there are no data on their maturity cycle (which means that we cannot numerate them) they are designated here as anterior and posterior kinetosomes in reference to their corresponding flagella. The posterior kinetosome (PK) is more ventral with its distal end oriented slightly left and dorsal, while the anterior kinetosome (AK) lies almost parallel to the longitudinal axis of proboscis, with some sections showing the distal end turned to the left and ventral (Figs 8, 9, 15, 28, 29).

Both kinetosomes are connected to each other by several fibrillar connectors. The most peculiar is the multilayered fibrillar structure (MFS) (see below). Another conspicuous connector is formed by the lateral left feet of AK and dorsal right feet of PK, which fuse at some distance from both kinetosomes and form a thick electron dense structure that runs approximately parallel to the AK (Figs 6-8, 15, 16).

Almost all of the roots run posteriorly from the kinetosomes and support the thick part of the proboscis, which contains no organelles. The main roots are normally clearly visible in cross sections (Figs 1, 3). The most prominent is a rhizostyle (RH), a broad microtubular band passing along the proboscis' axis deep into the cell up to the nucleus. It is S-shaped in cross section, and its microtubules are slightly spaced from each other and connected by bridges. Each microtubule has ventral and dorsal ridges and the latter are united by a common plate at the dorsal convex side (Fig. 3). At its middle part it has long perpendicular fibrillar threads passing into the cytoplasm

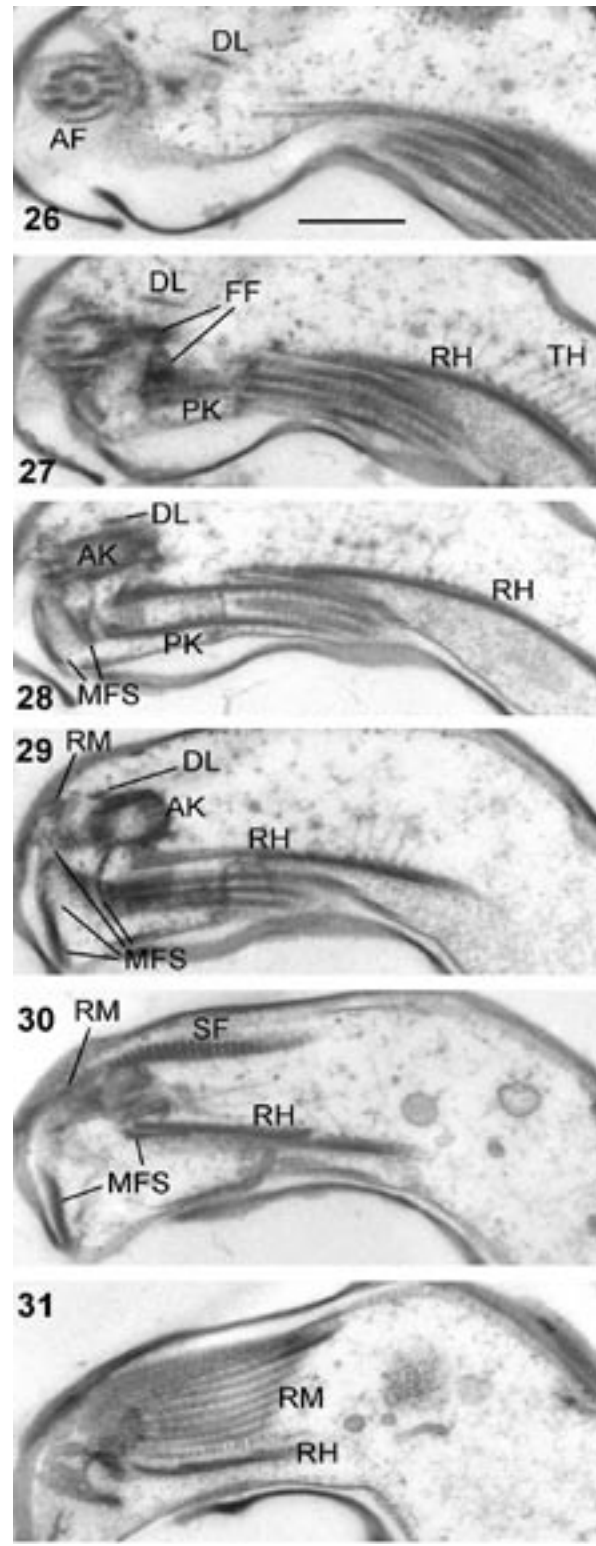
from the convex side (Figs 15, 27). The rhizostyle originates in between the kinetosomes in connection with MFS and contains from 9 to 12 microtubules, depending on the plane of section (Figs 3, 6-14, 20, 31). The pair of rhizostyle microtubules closest to the PK splits from the others very close to their origin to form the posterior left root (LM), which follows the posterior flagellum underlying the plasmalemma of the left and ventral side of the proboscis (Figs 3, 14-16, 19-21). The LM is accompanied by three more dorsal single microtubules (PM), which also underline the plasmalemma of the left side of proboscis and pass alongside the posterior flagellum (Figs 3, 9, 16-19). Two of them (the most dorsal) certainly originate from the PK and the third one seems to originate as an additional microtubule in the cytoplasm (Figs 15, 16).

A broad root (RM) consisting of 11 microtubules at its origin passes from the AK in connection with the MFS and supports the right side of proboscis (Figs 6, 8-11, 12-18, 23). This right microtubular root envelopes the AK from the right and dorsal side, and runs posteriorly to the base of proboscis, where the number of microtubules gradually reduces to five. From its inner side approximately at the middle of the right side of the AK a rather thick (up to 90 nm) striated fibrillar root (SF) appears (Figs 6-11, 30). It has striation approximately 50 nm in period and passes along the inner side of RM for a rather long distance (Fig. 30).

One more root of two microtubules, the dorsal left root (DL), originates from near the anterior kinetosome at a point between the RM and the SF origin and passes to the left, forming a dorsal arc almost perpendicular to the longitudinal axis of the proboscis, and then turns slightly posteriorly (Figs 6-9, 16, 17, 22-29).

A thick part of proboscis terminates with the broad and thick multilayered fibrillar structure (MFS) located on its ventral side (Figs 6-9, 12-14, 20-23, 28-30). This prominent structure has right and left fibrillar wings supporting the proboscis' shape. It is connected to the proximal end of posterior kinetosome and is associated with the distal part of anterior kinetosome by the right wing (Figs 13, 14, 22, 23, 28, 29). The MFS nucleates two main roots: RH and RM (Figs 6-9, 12-14, 19-23). There are more minor fibrillar bridges between kinetosomes and also between AK and RM (Figs 9, 16, 21, 28).

To summarize, the AK associates with two microtubular roots (RM and DL), and with a striated fibrillar one (SF); the PK associates with one microtubular root (RH, which then splits to form the LM of two microtubules) and gives rise for two other posterior



Figs 26-31. Consecutive sagittal LSs of proboscis' thick part of *Apusomonas proboscidea* from left (26) to the right side of the cell. Lateral view. Anterior end to the left. *Abbreviations* as in figs 1-18. Scale bar (μm): 0.5.

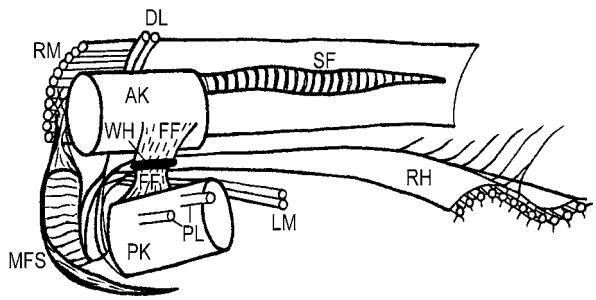


Fig. 32. General scheme of the flagellar apparatus of *Apusomonas proboscidea*. View from the left side; anterior to the left, dorsal to the top. *Abbreviations* as in figs 1-18.

dorsal microtubules (PM). These and other cytoskeletal elements of *Ap. proboscidea* are represented in Figure 32.

Discussion

The detailed description of apusomonad flagellar apparatus is presented here for the first time, therefore it is difficult to compare it with that of other apusomonads to establish the common characters for all of them. The most complete set of apusomonad cytoskeletal elements was published in the review by Karpov and Mylnikov (1989). The authors did not use serial sections and gave preliminary microtubule counts for the main microtubular roots in *Ap. proboscidea*: 4 (left posterior microtubules) — 9-12 (rhizostyle) — 5-9 (right microtubular root); *Amastigomonas caudata*: 3-5 — 8-10 — 5, and in *Amastigomonas* sp.: 3 — 7-9 — 0 (the RM seems to be reduced because of short proboscis). The root system of *Amastigomonas bermudensis* has the organization 4 – 12-16 – 10-12 (Molina and Nerad, 1991). The homology of RH and RM is obvious for both genera, and particularly the association of rhizostyle with an electron dense body. The homology of the left roots is not clear, and it has to be confirmed by further detailed study of *Amastigomonas* species.

The MFS and its association with RM and RH, LM splitting from RH, the presence of DL and SF, and the kinetosomal feet fusion have been reported here from apusomonads for the first time.

A cylinder inside the kinetosomes has been reported from *Amastigomonas* sp., *Am. caudata*, and *Ap. proboscidea* (Karpov and Mylnikov, 1989; Mylnikov, 1989). Prominent fused feet for the kinetosomes are visible in *Am. bermudensis* (Molina and Nerad, 1991), as well as *Amastigomonas* sp., *Am. caudata* and *Ap. proboscidea*. Interestingly, the latter character is present in cercomonads where the feet give rise to a short microtubular root, sometimes with secondary microtubules (Karpov et al., 2006). In *Ap. proboscidea* derivates of that fusion were not found.

Earlier the rhizostyle was referred to as MLS (multilayered structure) as it has at least two layers on the longitudinal sections (Karpov, 1988; Karpov and Mylnikov, 1989; Molina and Nerad, 1991), but its resemblance to cryptomonad rhizostyle is so striking, that I propose to call it the rhizostyle too.

Thus, the common features of apusomonad flagellar apparatus are: 2 smooth heterocont flagella with simple and short transition zone, and a thin cylinder under the transversal plate in the distal part of kinetosome; the RH connected to electron dense body and dictyosome. At present, we have to add the RM/DL association, MFS and feet fusion, which seems to have been overlooked in previous investigations. The SF is not a common character as it was not found in *Amastigomonas*.

To find the position of apusomonads among the other eukaryotes we have to start from molecular phylogeny. The recent 18S rRNA gene sequence data of three *Apusomonas proboscidea* strains (which also include our strain) and eight *Amastigomonas* strains showed, that they compose a monophyletic group with high bootstrap value (Scheckenbach et al., 2006). It means, that we can use a molecular phylogeny of any apusomonad to find their relatives. The first molecular tree with *Apusomonas* suggested a close relationship to opisthokonts (Cavalier-Smith and Chao, 1995). Later studies the ssu rRNA gene of this group did not show any sister groups, besides the *Ancyromonas* (Cavalier-Smith and Chao, 2003). Cavalier-Smith created the phylum Apusozoa including these two groups plus spironemids (Cavalier-Smith, 2002), therefore we have to compare the apusomonads with *Ancyromonas* and spironemids first.

Ancyromonas and spironemids are similar to apusomonads in respect of common cell characters: distinctive coverings with dense glycocalyx and epiplasm, smooth flagella and flagellar pockets (Foissner et al., 1988; Mylnikov, 1990). Both the *Ancyromonas* and spironemids have at least three microtubular roots, what is not in general a special character of these three groups. But all of them have a root composed by spaced microtubules connected to each other with fibrillar bridges (RH in apusomonads, L1 in *Ancyromonas*, and M1 in *Hemimastix*), which is rare among the protists. All representatives of these three groups have short flagellar transition zone, but *Hemimastix* has a thin walled cylinder above the transversal plate, and *Ancyromonas* seems to have a

similar cylinder. *Apusomonas*' cylinder locates under the transversal plate.

According to the recent molecular phylogeny based on the analysis of six genes the most likely relationships of *Apusomonas* are to the Opisthokonta clade and to the Amoebozoa clade (Kim et al., 2006). The root systems of opisthokonts (chytrid zoospores, choanoflagellates and choanocytes of sponges) have some common and peculiar characters, like the radial microtubules of flagellar kinetosome (Barr, 1981, Karpov, 1981; Karpov and Efremova, 1994; Maldonado, 2004), which are very different from those of apusomonads. But there is a thin cylinder in the distal part of kinetosome in both the choanoflagellates and apusomonads (Karpov and Zhukov, 1984; Karpov, 1985).

At the same time, there are more similarities of apusomonad cytoskeleton to the flagellated amoebozoans (*Multicilia*, myxomycetes) and Excavata.

The microtubular roots association (RM/DL) can be found in *Multicilia* (Mikrjukov and Mylnikov, 1998), whose root system resembles that of the myxogastrid zoospores. Each single kinetosome of *Multicilia* has descending microtubular cone enveloping the kinetosome from three sides, and the lateral band of four microtubules which originates from one side of the cone perpendicular to the latter. This microtubular root association is well known for zoospores of myxomycetes: r2 represent the microtubular cone and r3 accounting from 2 to 5 microtubules passes perpendicular to it (Wright et al., 1979; Spiegel, 1981). According to our data on three myxogastrid species (Karpov et al., 2003) the most prominent structure of their flagellar apparatus is the fibrillar sheet composed by five to eight strips, which cover the kinetosome from three sides. From the upper strip, the microtubules of the flagellar cone (r2) in the myxogastrids descend deep into the cell. *Multicilia* also has those strips (discs by Mikrjukov and Mylnikov's terminology) initiating the microtubules of flagellar cone.

Recent investigations of excavates (see Simpson, 2003 for review) revealed the same root association (F/R4) in *Malawimonas, Trimastix, Carpediemonas* and in oxymonads (as Pelta/R4).

It means that the microtubular roots association (RM/DL, r2/r3, F/R4 etc.) occurs in several distantly related taxa of eukaryotes, therefore this character seems can not indicate phylogenetic relationships.

The MFS structure should be more useful, as it occurs in myxogastrids and protostelids only. A broad MFS and its association with RH and RM are reminiscent of the posterior parakinetosomal structure initiating the r4 and r2 of myxogastrids (Spiegel, 1981; Karpov et al., 2003). The RM is similar to r2 in location and association with DL, which is also similar to r3 of myxogastrids. The r3 origin in myxogastrids is connected with the posterior parakinetosomal structure, and we can propose the same for *Apusomonas*, as there are fibrillar bridges between MFS and DL, and the latter also passes perpendicular to the RM as r3 to r2 of myxogastrids. The differences concern the orientation of some elements: r3 continues ventrally (not dorsally as for the DL in apusomonads), and a parakinetosomal structure is attached to the right side of the PK (not to the proximal end of the PK as in apusomonads).

Another conspicuous character with quite restricted occurrence is a rhizostyle, which is very similar to that structure of cryptomonads: the microtubules form a broad ribbon that is curved in cross section has fibrillar protrusions and runs from the region between the kinetosomes towards the nucleus (Roberts, 1984; Karpov, 1988, 1990). Some heterolobosean also have R1 or R2 roots, which resemble the rhizostyle of apusomonads (Simpson, 2003).

The cryptomonads have a rather conservative root system, which, in its most complete form includes a rhizostyle, 4 lateral microtubular roots and 2 fibrillar roots (Roberts et al., 1981; Roberts, 1984; Karpov, 1990). One fibrillar root has striations and is associated with a microtubular root, like the SR and LR in *Apusomonas*. In cryptomonads, however, this complex passes laterally and does not produce an additional microtubular root similar to the DL of *Apusomonas*, and cryptomonads also do not possess the MFS. Of course, the cryptomonads have several very distinctive and different characters that are absent from apusomonads: anisocont flagella with peculiar mastigonemes, a characteristic flagellar transition zone, mitochondria with lamellar cristae, and ejectosomes. At the same time we do not know other protists with such a conspicuous rhizostyle and striated fibrillar root/microtubular root association.

Unfortunately, the kinetosome development cycle is not known for *Apusomonas*, and therefore we cannot discuss a homology of the microtubular roots with confidence.

Thus, we found in apusomonads some cytoskeletal elements which occur in other protists, but there are no protists with the same set of roots. The most similar groups in this respect are cryptomonads and myxogastrids, but they are very different in many other morphological characters. Description of several cytoskeletal characters that are common for all apusomonads studied previously confirms their monophyly, but still indicates their isolated evolutionary position among the other eukaryotes. Regarding the

molecular phylogeny (Kim et al., 2006) our data support the apusomonad relation to Amoebozoa rather than to Opisthokonta.

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Address for correspondence. Sergey A. Karpov. Biological faculty of Herzen Pedagogical State University, Moika 48, St. Petersburg, 191180. Russia. E-mail: sakarpov4@gmail.com