Acta Protozool. (2003) 42: 331 - 343

ACTA
PROTOZOOLOGICA

Pseudodidymium cryptomastigophorum gen. n., sp. n., a *Hyperamoeba* or a Slime Mould? A Combined Study on Morphology and 18S rDNA Sequence Data

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Summary. The *Hyperamoeba*-like amoeboflagellate (Wi7/2-PE) has been isolated from a hydrotherapy pool inside a hospital at Wildbach/ Germany. Based on combined morphological and molecularbiological data we conclude that this isolate can neither be assigned to the genus *Hyperamoeba* nor to any of the myxogastrean slime moulds, which are the closest relatives of *Hyperamoeba*. We thus considered describing this isolate within a new genus, *Pseudodidymium*, as a new species, *Pseudodidymium cryptomastigophorum*. As observed by phase contrast microscopy the gross morphology and size of the three stages - trophozoite, cyst, and flagellate stage - is comparable to respective characters of *Hyperamoeba* as described by Karpov and Mylnikov (1997). However, in contrast to these characters they have in common with *Hyperamoeba*, the features of the flagellate stage shown by electron microscopy revealed marked differences to the previously described *Hyperamoeba*. In addition to the anterior flagellum responsible for the locomotion a second recurrent flagellum could be observed which is tightly attached to the cell membrane of the flagellate by a desmosome-like connection zone. Both flagellae are anchored to the tapered frontal part of the cell by kinetosomes with accessory structures located at an acute angle to each other. While the frontal flagellum is as long or even longer than the cell body the second one has a length of about one third of the cell and appears barren. This tight attachment of the inactive flagellum is the reason for its invisibility by means of light microscopy. All three stages possess mitochondria with a dense central core as known from *Hyperamoeba*, which are characteristic for myxogastrean slime moulds as well. However, various attempts to induce the formation of fruiting bodies in order to show a putative relationship to certain slime moulds remained unsuccessful. In 18S rDNA sequencing our isolate did not show highest identity to the only hitherto sequenced strain of *Hyperamoeba* sp., but, as also a undescribed strain of *Hyperamoeba*, to a strain of *Didymium nigripes*, a myxogastrean slime mould. However, this strain of *Didymium* shows an insertion, which our isolate does not have. In conclusion, it is neither possible to assign this *Hyperamoeba*-like isolate to the genus *Hyperamoeba* nor to the genus *Didymium* as it differs fundamentally from both genera in several aspects.

Key words: amoeboflagellates, *Hyperamoeba*, *Pseudodidymium cryptomastigophorum* sp. n., slime moulds, 18S rDNA, ultrastructure.

INTRODUCTION

The amoeboflagellate *Hyperamoeba flagellata* is a rarely isolated species. It was first isolated from an infusion of horse faeces and described by Alexeieff in 1923. In 1997 Karpov and Mylnikov redescribed a strain of this species by means of light and electron microscopy, which they had isolated from a pond in Russia. Later 9 strains of this species were isolated from 200 human faecal specimens from which a single isolate was investigated morphologically and on the molecular level (Zaman *et al.* 1999). The molecular investigation of the 18S rDNA showed that this *Hyperamoeba* isolate was closely related to the plasmodial slime mould *Physarum*

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polycephalum although it did not develop a plasmodium, which, however, is characteristic for *Physarum*. Since 1988 we isolated and collected 9 *Hyperamoeba*-like strains from different aquatic and terrestrial sources. As the genetic distance of our isolate Wi7/2-PE from the only hitherto sequenced and described *Hyperamoeba* (Zaman *et al*. 1999) was remarkably high we selected this strain as object for the present morphologic investigation in combination with studies on the molecular level as basis for a possible description of a novel species.

MATERIALS AND METHODS

Organism. The strain Wi7/2-PE was isolated from water samples of a physiotherapy bath of a hospital at Wildbad in Germany and maintained on NN-agar according to Page (1988) seeded with *Enterobacter cloacae* as food bacteria. With the aim to carry out the flagellate transformation test the amoebae from a 3-5 days old culture were suspended either in distilled water or in As-solution (amoeba-saline) according to Page (1988) and the transformation process was observed. Within 30 min 80-90 % of the amoebae had transformed to fully developed flagellate stages. In order to induce the formation of fruiting bodies amoebae from optimal cultures were transferred to agar plates prepared with either quarter strength Emerson-yps-medium (Schuster, personal comm.) or with sterilized oat flakes as they were made use of in the cultivation of plasmodia of *Physarum.*

Isolation of DNA. For isolation of DNA six parallel cultures were installed. Whole-cell DNA was isolated from actively growing (dividing) cultures. The trophozoites (~10^6 cells) were harvested from the plate cultures with a sterile cotton tipped applicator and washed 3x in sterile 0.9% NaCl by centrifugation 500 *g* for 7 min. For isolation of DNA a modified UNSET-procedure (Hugo *et al.* 1992) was used. Briefly, the cell-pellet was resuspended in 500 µl of UNSET-lysis buffer, overlaid with 500 µl phenol-chloroform-isoamylalcohol (PCI) and shaken gently for 5 h. After several rounds of PCI-extraction with an incubation period of 10 min the DNA was precipitated in alcohol at -20°C overnight. After centrifugation at 12000 *g* for 30 min at 4°C the DNA-pellet was washed in 70% ethanol, air-dried and resuspended in 30 µl of sterile double-distilled water.

PCR and sequencing. The 18S rDNA was amplified using the SSU1 and SSU2 primers (Gast *et al.* 1996), complementary to the strongly conserved ends of the eukaryotic 18S rRNA gene. Thirty PCR-cycles were run with 94°C 1 min, 52°C 2 min, 72°C 3 min. The amplification of the 18S rDNA was visualised with ethidium-bromide in an agarose-gel electrophoresis and the amplification product was purified using the GFX™ PCR-purification kit (Amersham, Pharmacia). The amplified 18S rDNA was sequenced stepwise by direct sequencing from the PCR-product and subsequent construction of complementary internal primers. Sequences were obtained from both strands. The sequencing PCR was run with 96°C 10 s, 50°C 5 s and 60°C 4 min using the ABI PRISM® BigDye sequencing kit (Applied Biosystems). Sequencing was carried out in a 310 ABI PRISM® automated sequencer (Applied Biosystems).

The obtained sequence fragments were aligned using the ClustalX program (Thompson *et al.* 1997) and processed with the GeneDoc sequence editor (Nicholas *et al.* 1997). The complete 18S rDNA sequence was compared to the ones of published strains using BLAST search (Altschul *et al.* 1990).

Cluster analysis. In order to proof the relatedness of our isolate to *Didymium* despite the *Hyperamoeba* morphology observed by light microscopy, we performed a cluster analysis for the myxogastrean slime moulds including other myxogastrean slime mould species. The cluster analysis was carried out using the PHYLIP (Felsenstein 1989) package. Primer sites, unique gaps, and inserts were rejected from the analysis. For evaluation of the statistic significance 1000 replicates of the nucleotide sequence alignment were generated using the SEQBOOT application. Distance matrices were calculated using DNADIST. Bootstrapped matrices were analyzed with the Neighbor joining application and a Kimura two parameter correction. A consensus tree was build from the resulting trees using CONSENSE and prepared as figure with the TREEVIEW (Page 1996) application. A strain of *Vannella anglica* (GenBank Ref. No. AF099101), a species presumed to be rather closely related to, but to branch clearly not within the slime moulds, possibly at the very base of the amoebozoa (Bolivar *et al.* 2001, Cavalier-Smith and Chao 2003), was used as an outgroup.

However, as the relationship between the dictyostelid and the myxogastrid slime moulds has not been wholly elucidated yet (Hendriks *et al.* 1991, Hinkle and Sogin 1993), a second, more large scale cluster analysis was performed in order to analyse the position of the genus *Hyperamoeba* within the amoebozoa. The phylogenetic position of *Hyperamoeba* was inferred with the maximum likelihood (ML) method using *Trypanosoma* and *Leishmania* (GenBank Ref. No. AF416562 and AF303938) as an outgroup. The choice of the outgroup was a critical matter. As in the past years it has become questionable, whether the diplomonads and parabasalids are actually early branching lineages and their phylogenetic position on the whole is rather unclear, we decided to use *Trypanosoma* and *Leishmania* as outgroup, as this has already been suggested by Dacks *et al*. (2002). With this outgroup long branch attraction, which can obscure other relationships, seems to play a minor role. Although also *Trypanosoma* might be a long branch, *Leishmania* seems to reduce its effects (Dacks *et al.* 2002). Data were also analyzed with NJ and maximum parsimony. Bootstrap values were processed with 100 (ML) and 1000 (NJ and maximum parsimony) replicates, respectively.

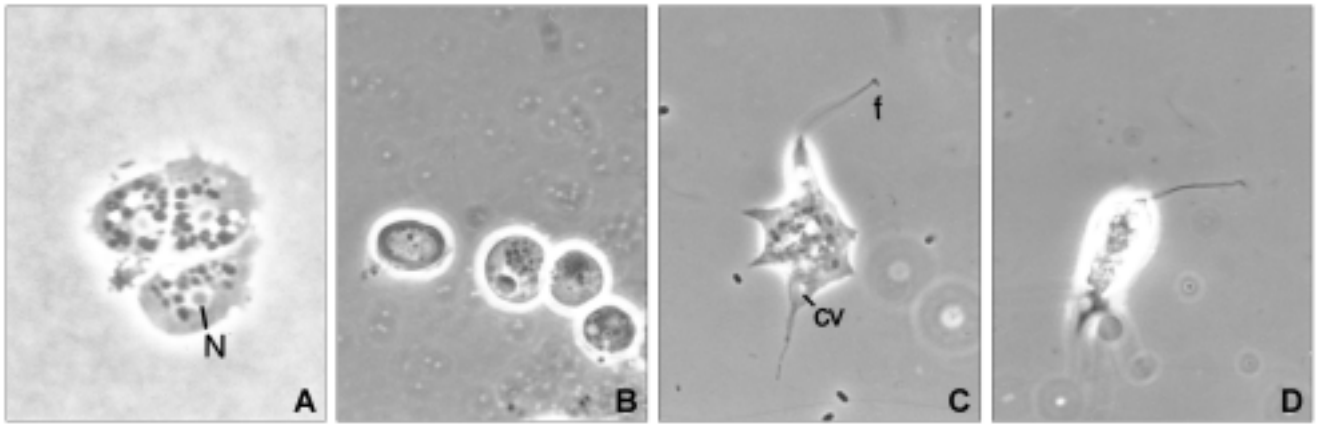
Sequence data reported in this paper are available at GenBank under the following accession number: AY207466.

The strain Wi7/2-PE was deposited as a typestrain at the "Culture Collection of Algae and Protozoa" at Ambleside, Cumbria under the CCAP-nr.: 1573/1.

Electron microscopy. Amoebae or flagellates obtained after transformation test mentioned above were harvested and centrifuged for 10 min at 2000 rpm. Pellets were fixed for 1 h with 3% glutaraldehyde, transferred to 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide and embedded in Spurr resin. Sections were stained with uranyl acetate and Reynold's lead citrate and examined using a Zeiss EM 10a electron microscope.

RESULTS

The strain Wi7/2-PE had been isolated from water samples from a physiotherapeutic bath in a hospital at Wildbad in Germany in the year 1988. The sample of



Figs 1 A-D. A - three trophozoites of Wi7/2-PE with short blunt pseudopodia. N - nucleus. Phase contrast, x1200; **B** - group of three cysts and one encysting stage (right side); **C** - transitory stage from trophozoite to flagellate stage with one frontal flagellum (f) still attached to the substratum, an uroidal filopodium visible. cv - contractile vacuole; **D** - mature flagellate with frontal flagellum and characteristic elongated body shape. Phase contrast, x1000.

100 ml was filtered through a membrane filter (Sartorius), which was then placed upside down onto NN-agar plates according to Page (1988) seeded with *Enterobacter cloacae* as food bacteria. After 48 h it was removed and the mixed population of amoebae was investigated. Since that time the strain Wi7/2-PE was maintained on the same agar plates, as it was not possible to establish axenic cultures of these amoeboflagellates. In order to exclude that the *Hyperamoeba*-like protists were not only stages of slime moulds with similar flagellate stages actively growing amoebae of Wi7/2-PE were transferred to media known to induce the formation of fruiting bodies of a number of true slime moulds. Neither the transfer to Emerson-yps-medium (Schuster, personal communication) nor to plates prepared with oat flakes made use of in cultivating *Physarum* plasmodia induced any fruiting bodies of the present strain.

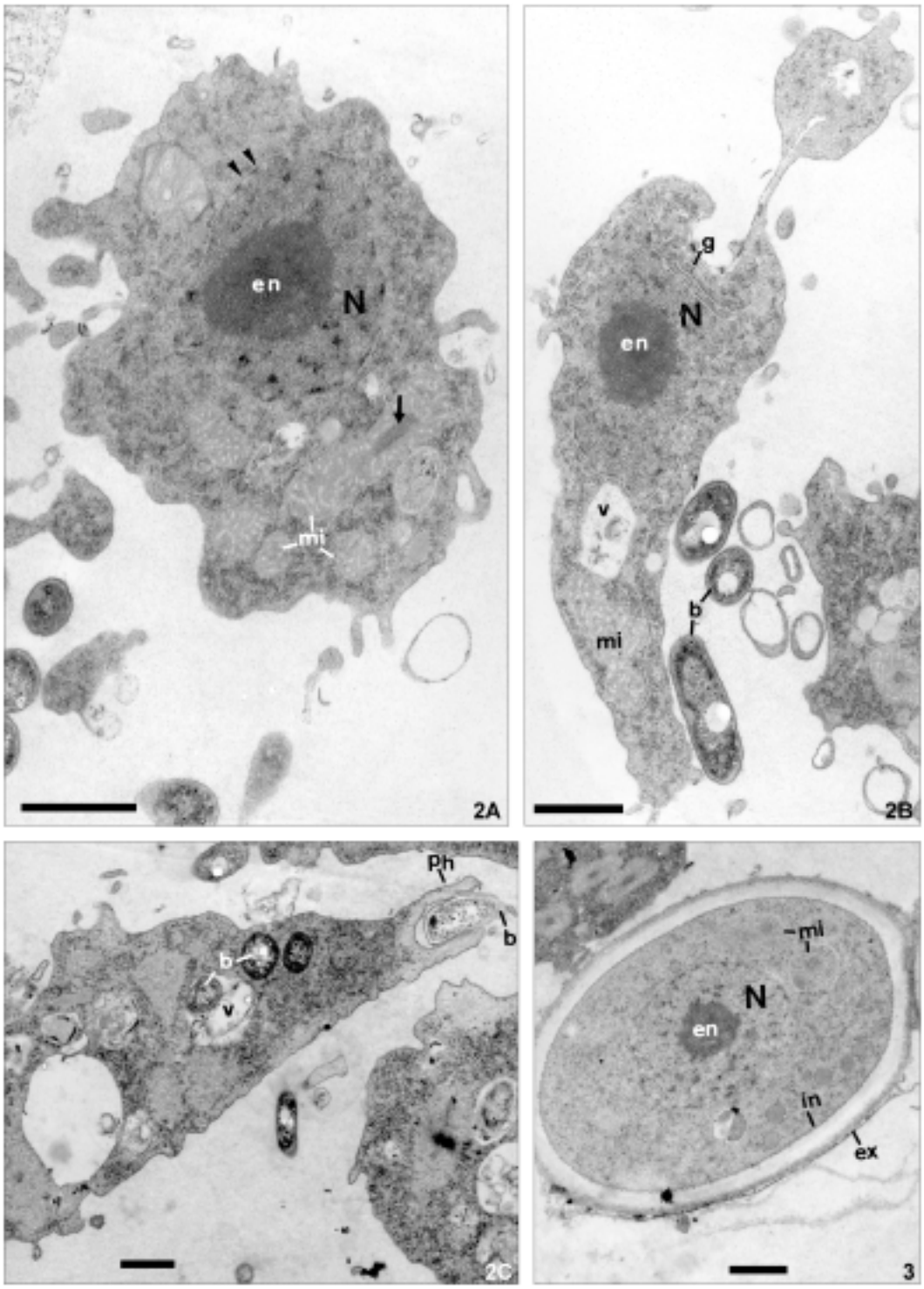
Observations under phase contrast. The novel species is represented by three main stages comprising the trophozoite (Fig. 1A), the flagellate stage (Figs 1C, D) and the cyst (Fig. 1B). Only the uninucleate trophozoite ingests bacteria as food and multiplies by binary fission - in contrast to the flagellates, which represent a temporary stage without feeding and dividing.

The amoebae have a variable size from 15 to 18 µm. They produce blunt filopodia emanating from a frontal hyaline zone. Under phase contrast the nucleus, the contractile vacuole, and some ingested food bacteria can be observed. They sometimes exhibit a tendency to form small aggregates as shown in Fig. 1A. If transferred from the agar surface of the NN-agar plate into Assolution or distilled water a high percentage of the amoebae transform into the flagellated stage with a long single frontal flagellum within 30 min. The flagellate has an elongated shape looking somewhat like a sausage moving actively by gyration in the direction of the flagellum. The flagellate is 15-20 µm long - its fully developed flagellum is as long or even a little bit longer than the cell body. The anterior end of the cell is tapered whereas the posterior end is stouter and somewhat rounded. After retransfer of the flagellates to the agar surface of a fresh agar plate seeded with *Enterobacter cloacae* they attach to the substratum by their cell body and become amoeboid again slowly incorporate their anterior flagellae. Within a period of 8-12 min the retransformation to normal trophozoites is completed.

The inconspicuous cysts have a round or oval outline and range in their size from 6 to 9 µm. They cannot be distinguished from *Hartmannella* cysts by light microscopy.

Electron microscopy

Trophozoite. The investigation by electron microscopy showed trophozoites with an irregular shape containing a nucleus with a diameter of 2.3 µm, a prominent nucleolus of 1.15 µm in diameter, and heterochromatin granules scattered throughout the karyoplasm (Figs 2A, C). The nucleus is separated from the cytoplasm by a nuclear envelope composed of 2 membranes that can be best observed in Figs 2A and 2B. Conspicuous mitochondria with tubular cristae are characterized by a



Figs 2 A-C. A - Wi7/2-PE-trophozoite with large nucleus (N) and prominent nucleolus or endosome (en) and heterochromatin granules. Irregular shape of the amoeba with subpseudopodia. Cytoplasm - containing ribosomes and glycogen-like granules - with conspicuous mitochondria containing tubular cristae (mi) characterized by the central electrondense core (arrow) typical for most slime moulds. Arrowheads - nuclear membrane; **B -** dividing of a trophozoite: Golgi zone (g) is located adjacent to the prominent nucleus (N). en - nucleolus, mi - mitochondrium, v - food vacoule; **C** - trophozoite of Wi7/2-PE ingesting *Enterobacter cloacae* as food bacterium (b) by forming a "food cup" that is surrounded by a hyaline zone produced by a meshwork of actin microfilaments (Ph). Food vacuoles (v) contain some bacteria too. Scale bars 1.0 µm.

Fig. 3. Cyst of strain Wi7/2-PE: the cyst wall is composed of a prominent ectocyst (ex - exine) separated by a wide empty space from the less pronounced endocyst (in - intine) attached closely to the cell membrane of the enclosed amoeba, mi - mitochondrium, N - nucleus with nucleolus (en). Scale bar 1.0 µm.

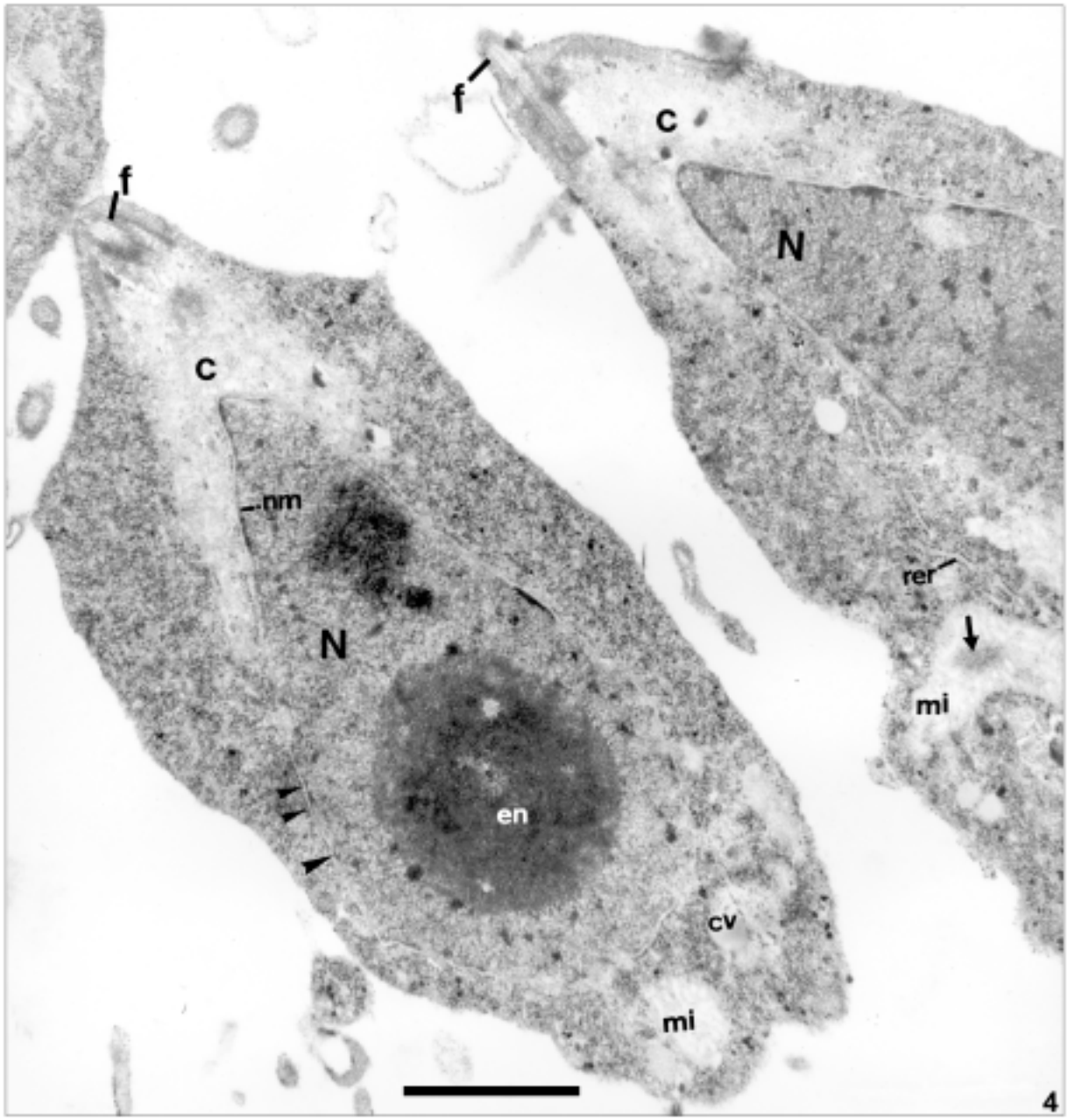
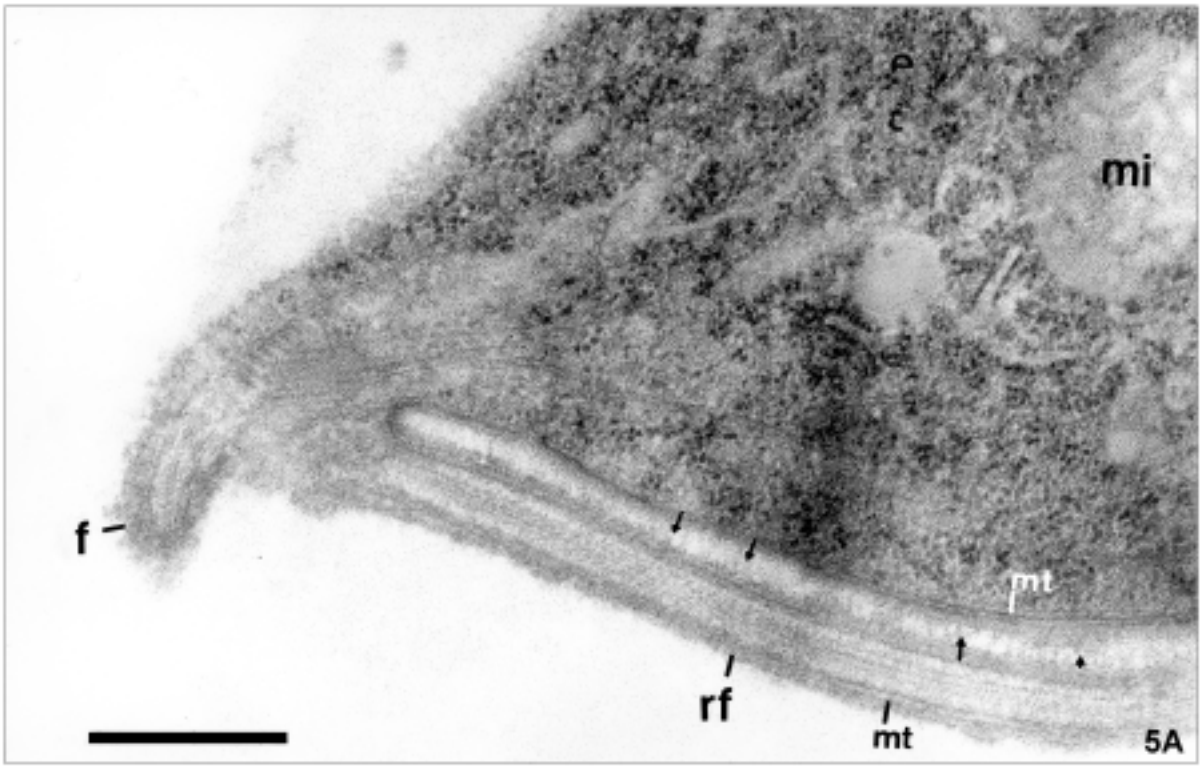


Fig. 4. Two flagellate stages of Wi7/2-PE with the anterior flagellum (f) discernible at the frontal tip of the cells. Nuclei (N) of both flagellates are tapering off in direction to the flagellar bases. They are enveloped by an electron lucent area representing "microtubular cones" (c). Nucleus of the left flagellate with most prominent nucleous (en), cv - contractile vacuole visible at the posterior end. arrow - electrondense core of mitochondrium, mi - mitochondrium, nm - nuclear membrane, rer - rough endoplasmatic reticulum. Scale bar 1.0 µm.

central electron dense core, which is located along the longitudinal axis of the mitochondrium as well known from flagellate stages of various myxogastrien slime moulds. The relative high optical density of the cytoplasm results from numerous ribosomes and glycogen granules. In addition it contains empty vacuoles or others filled with living bacteria and their remnants as a result of intracellular digestion (Figs 2B, C). One trophozoite in Fig. 2C exhibited food cup formation in order to ingest bacteria as prey. A putative dividing stage is presented in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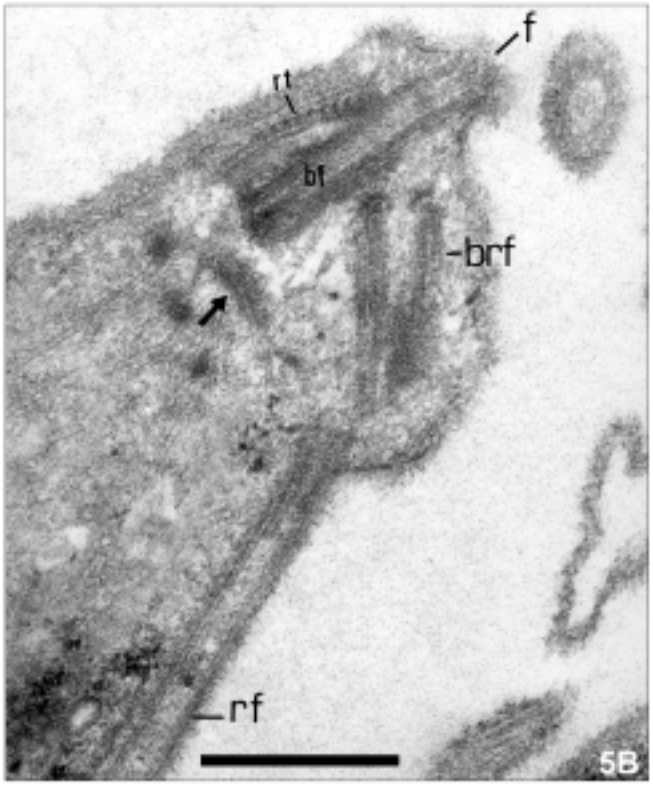
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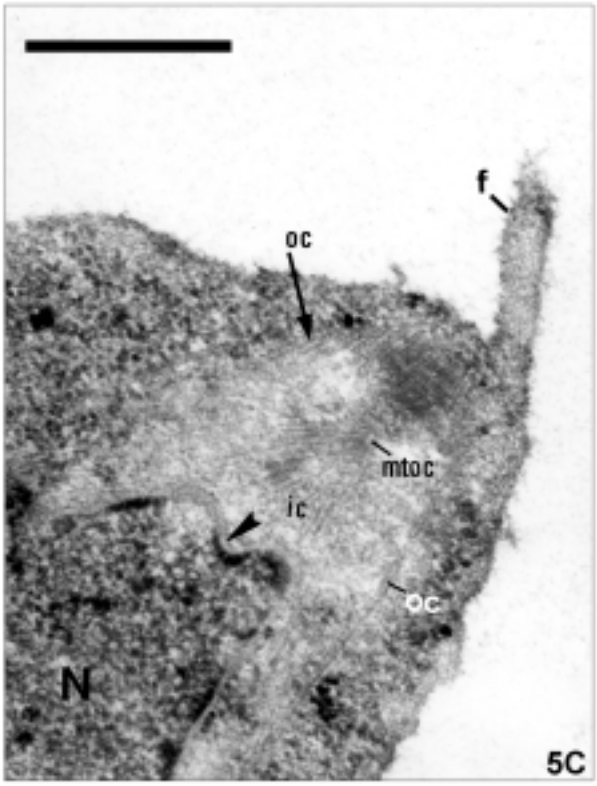
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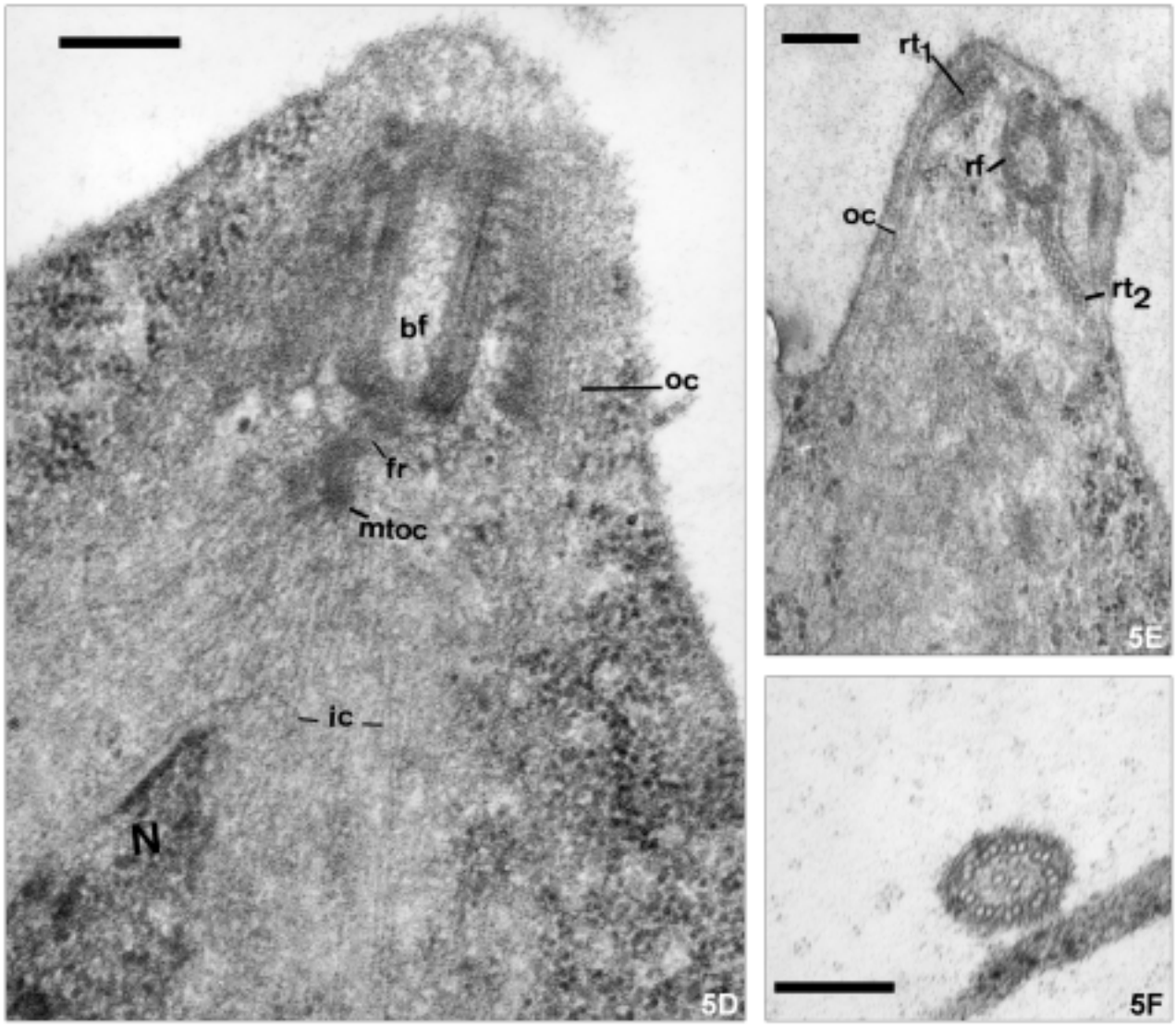

Figs 5 A-F. A - frontal part of a flagellate showing one frontal flagellum (f) and a second recurrent flagellate (rf) attached closely to the cell membrane of the flagellate stage by a desmosome-like connection containing fibrous filaments (arrows). mt - microtubules within the flagellum and beneath the cell membrane opposite to the recurrent flagellum (rf); mi - mitochondrium. **B** - a section through the anterior tip of a flagellate demonstrates both: the kinetosome of the frontal flagellum (bf) and of the recurring flagellum (brf) as well. The second flagellum (rf) appears tightly attached to the cell membrane. Arrow indicates the "microtubular organizing center" (mtoc) from which the inner cone of microtubules originates. rt - rootlet; **C** - anterior tip of a flagellate showing the microtubular organizing center (mtoc) with the origin of the inner cone (ic) surrounding the anterior part of the nucleus (N) that appears beaked (arrowhead) depending on the section plane. Microtubules forming the outer cone (oc) (arrow) are discernible too. f - flagellum; **D** - flagellar apparatus showing the kinetosom of the frontal flagellum (bf) surrounded by microtubules of the outer cone (oc). The kinetosome is connected with the microtubular organizing center (mtoc) by the "fibrillar rootlet" (fr) of the flagellar basal body. The inner microtubular cone (ic) arises from "mtoc". N - nucleus; **E** - anterior tip of the flagellate stage (Wi7/2-PE) reveals the position of the rootlets in relation to kinetosomes: the basal body of the recurrent flagellate (rf) cut obliquely is connected with its rootlet (rt_2) consisting of a double row of microtubules whereas the rootlet of the frontal flagellum (rt_1) consists of only a single row of microtubules. oc - microtubules of the outer cone; **F** - cross section through an anterior flagellum showing the characteristic 9 + 2 pattern of microtubules. Scale bars 0.5 µm (A-D), 0.2 µm (E, F).

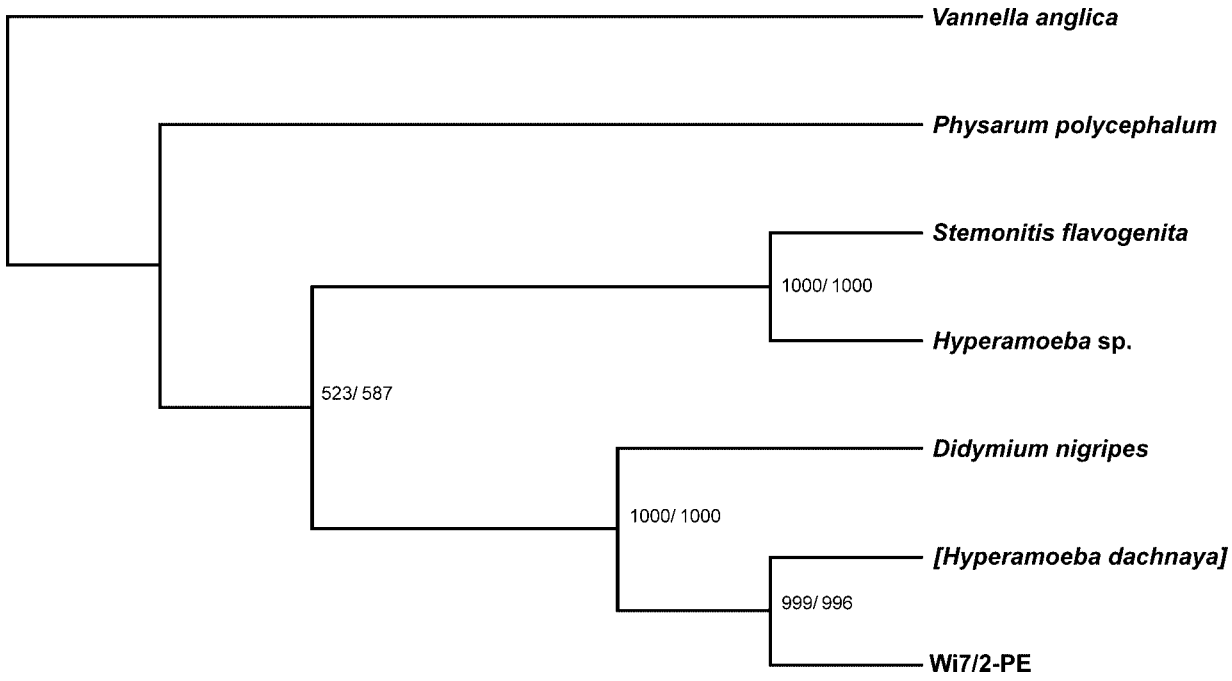

Fig. 6. 18S rDNA neighbour-joining cluster analysis of our isolate Wi7/2-PE, the *Hyperamoeba* sp. strain (GenBank accession number: AF093247), and several myxogastrean slime moulds using a strain of *Vannella anglica* (GenBank accession number AF099101) as outgroup. The still undescribed strain "*Hyperamoeba dachnaya"* was added to the alignment and used in brackets. The numbers at the nodes represent bootstrap values based on 1000 replicates. Bootstrap values of the maximum parsimony analyses are given behind the slash. Our isolate Wi7/2-PE clusters unambiguously with the "*Hyperamoeba dachnaya*" isolate together forming a cluster with the myxogastrean genus *Didymium* (strain *D. nigripes* GenBank accession number AF239230), while the described *Hyperamoeba* sp. strain forms a cluster with the myxogastrean genus *Stemonitis* (strain *S. flavogenita* GenBank accession number AF239229).

Fig. 2B with two daughter cells still connected by a thread-like cytoplasmic bridge. The lower division partner reveals also a conspicuous Golgi apparatus (dictyosome).

Cyst. Most cysts have a round or oval shape with a varying size from 6 to 9 µm (Fig. 3). The cyst wall is composed of a prominent ectocyst (0.1-0.2 µm thick) separated by a wide electron translucent space filled with thin fibrillar material of 0.28-0.38 µm from the delicate endocyst attached tightly to the enclosed amoeba. Within the latter the nucleus with nucleolus and persisting mitochondria are visible. Food vacuoles could not be observed but some lipid granules instead.

Flagellate stage. As the flagellate stages reveal more morphologic details than the trophozoites and cysts together increased attention has to be paid to this important developmental stage in the life cycle of these amoeboflagellates. As recognized by phase contrast microscopy the trophozoites transform *via* transitory stages to an elongated sausage-like form with a single visible flagellum emanating from the frontal tapered end of the cell (Figs 4, 5A-C). The flagellum is anchored by a basal body (kinetosome) with accessory structures in the anterior cytoplasm of the flagellate (Figs 4, 5B, D, E). Mature flagellates of Wi7/2-PE - as can be observed within a period of 30 min after suspension of the trophozoites in As-solution - had a defined sausage-like shape with a smooth and stable outline. Two sections of them can be recognized in Fig. 4, each of them with one anterior flagellum. The prominent nuclei of both are tapering off in direction to the basal bodies of the flagellum. They are enveloped by conical arrays of microtubules corresponding to the electron lucent area in Fig. 4. Mitochondria of the tubular cristae type showing a dense central core characteristic for certain slime moulds can be observed within the flagellates as well. A contractile vacuole - as seen by phase contrast - is located at the posterior end of the cell.

Since only one frontal flagellum can be recognized by means of phase contrast microscopy it was surprising to notice that a second recurrent flagellum was found, which was attached to the cell membrane of the flagel-

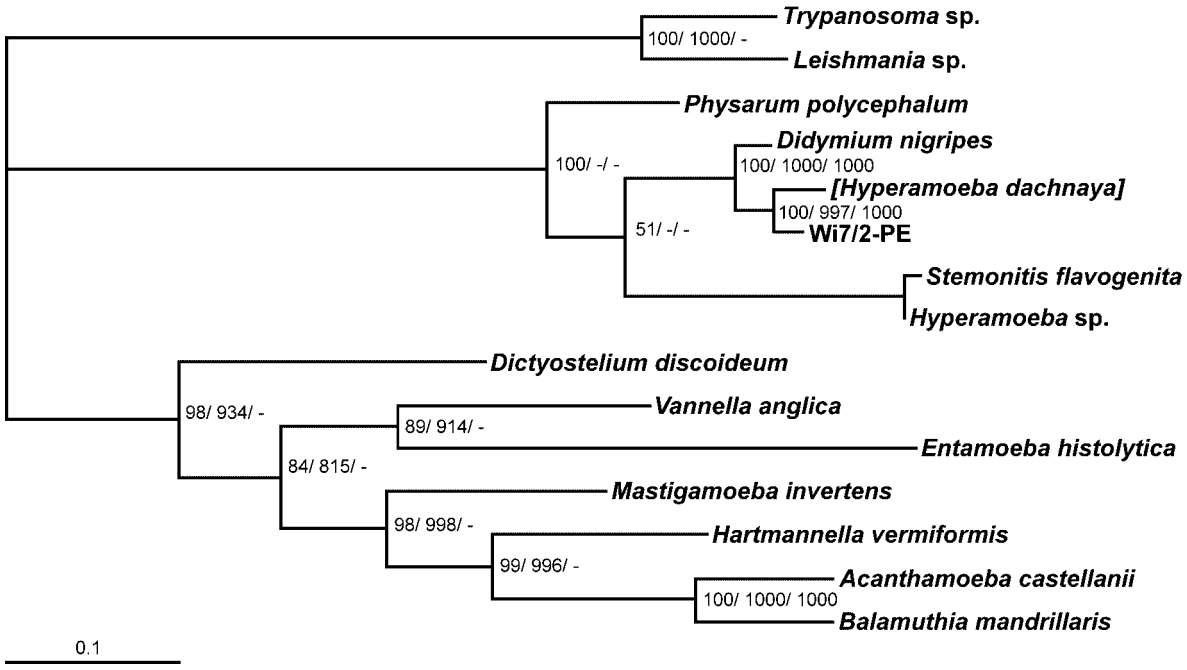

Fig. 7. 18S rDNA maximum likelihood analysis of the position of *Hyperamoeba* within the amoebozoa using *Leishmania* (GenBank accession number AF303938) and *Trypanosoma* (AF416562) as outgroup. Bootstrap values were calculated based on 100 (ML) and 1000 (distance and maximum parsimony) replicates, respectively. The bootstrap values are shown at the nodes (ML/ Distance/ P). The scale bar indicates the mean number of substitutions per site.

late stage by a desmosome-like connection zone containing fibrous filaments (Fig. 5A). Still more tightly attached appears the recurrent flagellum in Fig. 5B. This second hidden flagellum has a length of about one third of the cell body. The kinetosomes of both flagellae are shown in Fig. 5B, where they are located at an acute angel of about 45° to each other with the microtubular rootlet of the anterior flagellum. The organisation of the outer and inner microtubular cone both of which surround the frontal part of the nucleus like a double basket is discernible at Figs 5B-D. The inner cone emanates from the microtubular organisation center (mtoc), which is connected with the kinetosome of the frontal flagellum by the fibrillar rootlet (Fig. 5D). The outer basket is supposed to originate from the microtubular rootlet (Fig. 5E) as well known from *Hyperamoeba* (Karpov and Mylnikov 1997). The tip of the nucleus may appear beaked in some cases depending on the section plane. Rarely the rootlets of both basal bodies can be found in one section (Fig. 5E). The rootlet of the anterior flagellum is composed of a single row of microtubules whereas the rootlet of the second recurrent flagellum is composed of two rows of 7-8 microtubules each. Also a very short single row of 3-4 MT can be recognized in connection with this kinetosome.

Investigation on the molecular level

The 18S rRNA gene of the Wi7/2-PE strain of the *Hyperamoeba*-like amoeboflagellate shows a length of 1876 bp (with primer sites excluded), with a G+C content of 52.72%. It exhibits the highest sequence similarity, namely 94.78% to a strain of *Hyperamoeba dachnaya*, which has been submitted to the GenBank during the preparation of this manuscript, but which has not been described yet. The strain with the second highest sequence similarity (93.96% identity) is a strain of *Didymium nigripes* (GenBank AF239230). However, the strain of *Didymium* shows an insertion of 654 bp length after the 1726th bp. The sequence identity to the only published and described strain of *Hyperamoeba* is only 80.25%!

This closer relatedness of our isolate (and also of the hitherto undescribed "*Hyperamoeba dachnaya*") to *Didymium* than to the initially sequenced strain of

Hyperamoeba was proven by cluster analysis. As shown in Fig. 6 the strain Wi7/2-PE (together with "*Hyperamoeba dachnaya*") clusters with *Didymium*, while the described strain of *Hyperamoeba* clusters with *Stemonitis*. Groupings remained also in the same order when maximum parsimony was used and both analyses revealed high bootstrap values for these groupings.

However, the distinction from *Didymium* is clear, by the insertion this strain of *Didymium* has (all the other strains of the Myxogastria used in this study do not show any insert) and also when the insertion is excluded the identity is only 93.96%.

Altogether, the investigation on the molecular level corroborates the morphological data indicating that it is neither possible to assign this *Hyperamoeba*-like isolate to the genus *Hyperamoeba* nor to the genus *Didymium* as it differs fundamentally from both genera in several aspects. We thus considered to describe this isolate within a new genus, *Pseudodidymium*, as a new species, *Pseudodidymium cryptomastigophorum.*

In order to assess, whether these groupings are constant also in a more large scale investigation and also to reveal the position of *Hyperamoeba* within the amoebozoa a second cluster analysis was performed including several genera of the amoebozoa and using *Trypanosoma* and *Leishmania* as an outgroup. As shown in Fig. 7 the groupings of the different *Hyperamoeba* strains remained in the same order and these groupings also reveal high bootstrap values. However, whether the branch including *Didymium*, the "*Hyperamoeba dachnaya*" strain and our isolate clusters with the *Stemonitits* group or rather with *Physarum* is not well resolved. Interestingly, in this analysis *Dictyostelium* did not cluster together with the myxogastrid slime moulds, but together with *Mastigamoeba* and the lobosean amoebae.

DISCUSSION

As the isolate Wi7/2-PE, which had been isolated from a hydrotherapy pool of a hospital at Wildbach/ Germany could neither be assigned to the genus *Hyperamoeba* nor to any of the myxogastrean slime moulds, which are the closest relatives of *Hyperamoeba*, we considered to describe this isolate within a new genus, *Pseudodidymium*, as a new species, *Pseudodidymium cryptomastigophorum*.

In addition to the distinct genetic distance shown on the molecular level attention was focused to morphological differences on the ultrastructural level. The overall morphology of trophozoites, cysts, and flagellates is comparable to the features of *Hyperamoeba flagellata* (Karpov and Mylnikov 1997, Zaman *et al.* 1999) on one side and to myxogastrean slime mould stages on the other side.

Characteristic for both groups is the nucleus with a prominent nucleolus and dispersed particles of heterochromatine. Within the flagellate stage the frontal part of the nucleus is enveloped by a double conal array of MT emanating from the rootlets and from the "mtoc" respectively, which are located in relation to the basal bodies. Very important are the mitochondria of both vegetative stages exhibiting a dense central core that can be found within *Hyperamoeba* and also within slime moulds where this unique trait is confined to Myxogastrea in contrast to Protostelidae and the cellular slime moulds such as *Dictyostelium* where it is lacking (Schuster 1965, Aldrich 1968, Karpov and Mylnikov 1997).

Concerning solely the morphological similarities mentioned it becomes already evident that *Hyperamoeba* and the presently described new species are distinctly related to Myxogastrea with the main exception that they are unable to form fruiting bodies in the course of their life cycle. After stressing the common features of the organisms mentioned attention has now to be paid to differences between those species in question.

From the three stages differences in cyst shape can be observed at a first glimpse. The *Hyperamoeba* isolated by Zaman *et al.* (1999) from human faeces formed cysts with ectocysts, which greatly varied in thickness and contained clumps of Gram-negative bacteria whereas the ectocyst of our strain was of constant thickness and devoid of bacteria. It is comparable to the also uninuclear cysts of *Didymium nigripes* with inconspicuous ecto- and endocyst (Schuster 1965). One of the most conspicuous traits of Wi7/2-PE is the nucleus of the flagellates tapering off in frontal direction with its tip eventually beaked in some cases - in contrast to *Hyperamoeba* with a round at least moderately pointed nucleus within the flagellate stage (Karpov *et al.*1997) The elongated nucleus of Wi7/2-PE resembles the nucleus of the flagellated stages of *Stemonitis* (Ishigami 1977), *Physarum* (Wright *et al.* 1979), and *Didymium* (Schuster 1965) - the tip of the latter becoming blunt in older stages. However, the most striking difference of Wi7/2-PE from *Hyperamoeba* described so far is the

presence of a second recurrent flagellum in addition to the long frontal flagellum! *Hyperamoeba flagellata* was shown to possess only one functional frontal flagellum with its kinetosome and a second barren kinetosome being located with an acute angle in relation to the first one.

The recurrent flagellum of Wi7/2-PE does not contribute to the swimming locomotion, the force of which is generated solely by the long frontal flagellum. The tight attachment of the useless short flagellum to the cell surface by a desmosome-like connection is the reason why the fagellates appear ostensibly uniflagellate when examined under phase contrast. The possession of such a short second flagellum has Wi7/2-PE in common with various flagellate stages of Myxogastria such as *Didymium nigripes* (Schuster 1965), *Stemonitis* (Ishigami 1977), and *Physarum* (Aldrich 1968; Wright 1979). But in contrast to the situation in Wi7/2-PE these recurrent flagellae can be distinguished as a free however useless trailing flagellum of the flagellate stages in these slime moulds.

Those remarkable differences can be recognized without going into details of the very complex morphology of the flagellar apparatus described as far as possible from the micrographs. The organisation of the flagellar apparatus of Wi7/2-PE corresponds well to the general scheme of these structures at *Hyperamoeba* as presented by Karpov and Mylnikov (1997) - with one exception: The long one of the two rootlets anchoring the second kinetosome (rt2 in Fig. 5E) is composed of a single row of MT of two closely apposed layers of MT with a basal layer of fibrillar material in the case of our isolate resembling the three layered rootlet shown in *Physarum* flagellates (Wright *et al*. 1979).

We think that, as a result of the morphological examination alone a description of Wi7/2-PE as a novel species *Pseudodidymium cryptomastigophorum* is justified. The name *Pseudodidymium* refers to its relatedness to *Didymium* and the species name *cryptomastigophorum* was chosen because of its hidden second flagellum.

By comparison with *Hyperamoeba* on one side and the slime moulds on the other it becomes evident that this isolate has greatest morphologic affinities to the myxogastrean slime moulds. Since no formation of fruiting bodies could be observed by methods of induction mentioned above the discussion is open to the question whether it is a precursor protist or a secondarily derived form of a slime mould such as *Didymium* for instance, which has lost its capability to produce fruiting bodies. Similar considerations for the relationship of *Hyperamoeba* and slime moulds are known from Cavallier-Smith and Chao (1998).

In molecular biological investigations our strain showed highest affinity to "*Hyperamoeba dachnaya*" (GenBank AY062881). However, this strain has not been described yet, and both of these strains show significant higher sequence identity to *Didymium* (93.96% in case of Wi7/2-PE) than to the originally published *Hyperamoeba* strain (80.25%). As also shown in a more large scale analysis including various other amoebozoa, all three *Hyperamoeba* isolates constantly form a cluster with the myxogastrid slime moulds, however, our data indicate that the genus *Hyperamoeba* is not monophyletic. In fact the *Hyperamoeba* isolates are dispersed among the myxogastrid slime moulds.

Interestingly, *Dictyostelium* did not cluster with the myxogastrid slime moulds to form a monophyletic mycetozoan branch, but clustered together with *Mastigamoeba* and the lobosean amoebozoa. A close relationship of *Dictyostelium discoideum*, *Mastigamoeba balamuthi* and *Entamoeba histolytica* has already been observed by other authors (Arisue *et al.* 2002, Bapteste *et al.* 2002).

The phylogenetic position of the mycetozoa and whether they form a monophyletic group is still not wholly elucidated. Baldauf and Doolittle (1997) found evidence for a monophyletic group Mycetozoa standing within the crown of the eukaryote tree. Karpov (1997) assumed that the myxomycetes might be closely related to the cercomonads. And indeed these two groups share quite a few morphological features. However, some very recent papers indicate that the cercomonads might rather be related to the foraminifera, than to the slime moulds (Keeling 2001, Archibald *et al.* 2003). And finally Cavalier-Smith and Chao (2003) proposed to remove the amoebozoa including also the mycetozoa from the opisthokont branch and to place them rather at the base of the bikont clade. There is and has been a lot of regrouping within the protozoa and certainly the last word has not yet been spoken. In the case of the myxogastrid slime moulds it is clear that a lot more data are needed to place them correctly and to solve their relationship to the other mycetozoa. However, it was not the intention of our study to solve the phylogeny of the mycetozoa, but to analyse the position of *Hyperamoeba*

within the amoebozoa and to prove our findings that the genus *Hyperamoeba* at the present state seems to be polyphyletic.

Altogether, we think that it is neither possible to assign the *Hyperamoeba*-like isolate Wi7/2-PE to the genus *Hyperamoeba* nor to the genus *Didymium* as it differs fundamentally from both genera in several aspects, and that it is thus justified to describe it as a new species *Pseudodidymium cryptomastigophorum*, within a new genus.

The possible medical significance of *Hyperamoeba*-like strains is not yet known. The amoeboflagellate isolated from human nasal mucosa by Mascaro *et al.* (1986) was similar to the amoeboflagellates - described and mentioned here - by morphologic terms only. It was highly virulent in mice when introduced into the sinus cavity. Some mice were killed after invasion into the brain tissue - others developed chronic infections. All mice investigated had amoebae in their lungs. The *Hyperamoeba* strains isolated from human feces in Karatschi were shown to induce ulceration of the skin in mice after subcutaneous injection of the amoeboflagellates and were considered therefore to have a pathogenic potential (Zaman *et al*. 1999). These data from experimental animals suggest performing corresponding pathogenicity tests in future with cell cultures and eventually by inoculation tests with suited experimental animals.

***Pseudodidymium cryptomastigorum* gen. n., sp. n.**

Diagnosis: amoeboflagellate with three stages in its life cycle: trophozoite, flagellate stage and cyst. The uninucleate trophozoites of varying size and shape (15-18 µm). Blunt filopodia emanate from a frontal hyaline cytoplasm margin in locomotion. Vesicular nucleus (1.8-2.2 µm in diameter) is rounded or slightly oblong with a central rounded nucleolus (0.8-1.2 µm). Heterochromatin granules are scattered throughout the karyoplasm. Mitochondria with tubular cristae exhibit a central electron dense core well known from myxogastrean slime moulds. A Golgi apparatus is observable. The sausage like shaped flagellated stage shows a variable length of 15-20 µm. The single anterior flagellum is a long or ever a bit longer than cell body. The second recurrent flagellum has about one third of the cell length, is closely connected with the surface of the flagellate, and has no function. The prominent nucleus is tapering off in frontal direction and is partially surrounded by an outer and inner cone of mt emanating from the rootlet of the anterior flagellum and the mtoc, respectively. The rootlet of the anterior kinetosome consists of a single row of mt whereas that of the recurrent flagellum is composed of two rows of 7-8 mt each. The mitochondria are comparable to those of the trophozoite especially with the dense core and correspond as well to mitochondria from myxogastrea. The inconspicuous cyst has a double cyst wall separated by an empty space and has a diameter of 6-9 µm. The 18S rRNA gene of Wi7/2-PE shows a length of 1876 bp (with primer sites excluded), with a G+C content of 52.72%.

Differential diagnosis: *P. cryptomastigophorum* has morphological similarities to *Hyperamoeba flagellata* on one side and to myxogastrean slime moulds on the other. It differs from *Hyperamoeba* by its recurrent flagellum always present in the flagellate stage and by the possession of the distinct double row of mt in contrast to a single row described for *Hyperamoeba*. The possession of mitochondria with tubular cristae and the distinct electron dense core indicate - as in the case of *Hyperamoeba* - the relatedness to myxogastrean slime moulds. Protostelidae and cellular slime moulds do not have these mitochondrial traits. Also *Cercomonas* has normal mitochondria without that dense core indicating no close relationship with our isolate. The close relationship to Myxogastrea is supported by the pear shaped nucleus of the flagellate stage that tapers off in frontal direction as has been described from *Stemonitis Physarum,* and *Didymium*. In addition these myxogastrea have flagellate stages (zoospores) with a second recurrent but trailing flagellum. Despite these morphological similarities with slime moulds the novel species is characterized by its incapability to form fruiting bodies pertaining to these organisms. Wi7/2-PE exhibits 94.78% sequence similarity to a strain of "*Hyperamoeba dachnaya*", which has, however, not been described yet and both of these strains show significant higher sequence identity to *Didymium* (93.96%) than to the originally published *Hyperamoeba* strain (80.25%). This strain of *Didymium*, however, shows an insertion of 654 bp length after the 1726th bp, which Wi7/2-PE does not have.

Acknowledgement. We thank Gerhild Gmeiner (Laboratory for Electron Microscopy, CIFAFMS, Koblenz; Head: B. Hauröder) for excellent technical assistance.

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Received on 28th January, 2003; revised version on 1st July, 2003; accepted on 8th July, 2003