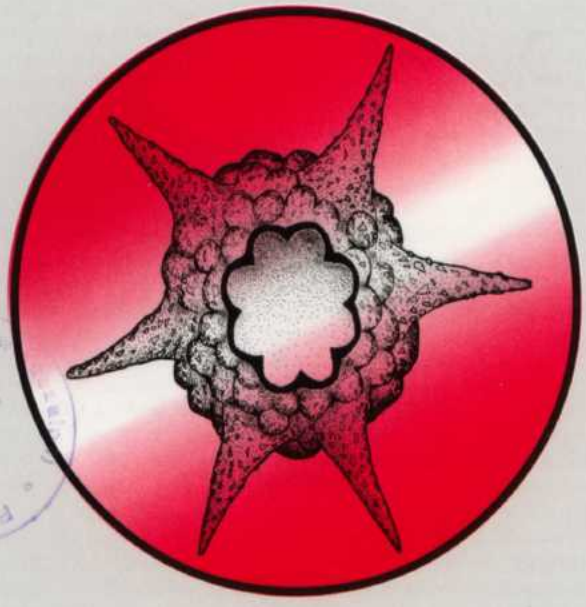
ACTA PROTOZOOLOGICA



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Plasmodium falciparum Pfs25 Gene Promoter has no Polymorphism in Natural Isolates of Eastern India

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Summary. Most of the potential vaccine candidate genes show polymorphism or point mutation, which affects the structure of antigen and thereby, production of antibody to protect the host against further infection. In the present study, we have analyzed the promoter regions of 415 base pair (nucleotide position -722 to -308) of *Pfs25* gene. Previous studies have shown that, removing these important regions drastically affects the promoter activity and Pfs25 protein expression. We for the first time, report that though promoter regions of *Pfs25* gene is AT rich, it doesn't show any polymorphism or point mutation in sequences important for its activity in 155 natural isolates of *Plasmodium falciparum* from hyper endemic malaria transmission regions. Since, *Pfs25* expression is vital for the survival of the parasite in the mosquito vector, a stable promoter sequence of *Pfs25* in all the strains may have fair chances of survival in the mosquito vector. Our finding of a stable promoter sequence in combination with previous reports of limited polymorphism in the coding regions of *Pfs25* indicates that Pfs25 might be a strong candidate for vaccine.

Key words: PCR-SSCP, *Pfs25* promoter, *Plasmodium falciparum,* point mutation, polymorphism.

INTRODUCTION

Malaria is a public health problem in more than 90 countries, inhabited by a total of some 2.5 billion people almost 40% of the world's population. There are 300 to 500 million clinical cases every year and between one and three million deaths, mostly of children, are attributable to this disease. Every 40 seconds a child dies of malaria, resulting in a daily loss of more than 2,000 young lives worldwide (Sachs and Malaney 2002). These estimates render malaria the pre-eminent tropical parasitic disease and one of the top three killers among communicable diseases. Vector control and malaria chemotherapy that were previously effective in controlling and treating malaria are now largely ineffective due to insecticide-resistant mosquitoes and drug-resistant parasites. As alternatives, immunological intervention will be needed to stop the resurgence of malaria, a disease with a devastating impact on the human health. Pfs25 (Kaslow *et al.* 1988, 1989; Kaslow 1990), cysteine-rich 25 kDa surface protein of *Plasmodium falciparum*, is a sexual

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stage specific potential vaccine candidate (Vermeulen *et al.* 1986), which is expressed on the surface of zygote and ookinete forms of the parasite in mosquito vector (Vermeulen *et al.* 1985). Monoclonal antibodies directed against native Pfs25 could block completely the development of *P. falciparum* oocysts in the midgut of the mosquito vector (Charoenvit *et al.* 1997). Thus, this 25-kD protein is a potential vaccine candidate antigen of *P. falciparum*, which may assist in the control of lethal forms of human malaria (Zou *et al.* 2003). Recombinant vaccines based on Pfs25 are in various stages of clinical trials (Barr *et al.* 1991, Kaslow *et al.* 1991). Numerous studies had shown that very limited or no polymorphism occurs in the coding regions of *Pfs25* (Niederwieser *et al.* 2000). Most part of the *Pfs25* coding regions are conserved and shows low titer yet highly potent antibody production in animal models and human hosts (Stowers *et al.* 2000).

Previous studies have demonstrated that promoter region (-722 to -308) of *Pfs25* is very important for the expression of *Pfs25* gene (Dechering *et al.* 1999). In this report, for the first time, we showed that this regulatory region of *Pfs25* gene has no polymorphism in Indian isolates of Orissa.

MATERIALS AND METHODS

Study area and *Plasmodium falciparum* isolates

We have selected Orissa, a state of India as our study area with longitude 30.20.N and latitude 85.54.E and the whole region comes under endemic zone of malaria transmission. With 38% of the India's population Orissa contributes 20% of the *P. falciparum* cases and 40% of malarial deaths of India. Blood samples (n = 155) were collected from *P. falciparum* infected individuals attending malaria clinic at primary health centers of hyper endemic regions of Orissa. Blood was collected from consenting volunteers as 1 ml samples in 0.08M EDTA and stored at -70°C for further use. The Institutional Ethical Committee approved this study.

DNA isolation

DNA was prepared from these samples using a rapid DNA isolation method described earlier by Foley *et al*. (1992), with little modifications. Briefly, 500 µl of ice-cold 5 mM sodium phosphate (pH-8) was added to 20 µl of venipuncture blood and vortexed. After centrifugation for 10 min in a microcentrifuge tube at 4°C, the supernatant was discarded. The pellet was suspended in 100 µl PBS containing 0.01% saponin and washed twice with same buffer by repeating the above steps of vortexing and centrifugation. After washing, the pellet was suspended in 50 µl of sterile water, vortexed and then boiled for 20 min at 100°C. After centrifugation in a micro centrifuge tube at 6,500 g for 10 min at 4°C, the supernatant was collected and used in PCR.

Primer designing and PCR

The sequence of the promoter regions of *Pfs25* gene was taken from GenBank, (Accession number- AF030628, Dechering *et al.* 1999). The sequence of *Pfs25* promoter is AT rich, so the primers were designed in such a manner that it included the regions important for promoter activity and few GC bases for optimum annealing in PCR. Primer sequences, P310F 5'- GAT ACT TCT CTA TGT ACA TAT ATA - 3', P310R 5'- CCA AAC TCT TAA GTA TCA GTA c-3' and P128F - 5'- GTA CTG ATA CTT ATA GAG TTT GGC - 3', P128R - 5'- TGT ATT TAT TTA CAA TGA TTA TAT AAA CGG - 3' were used for the amplification of 310 base pair (bp) (nucleotide position -722 to -413) and 128 bp (nucleotide position -435 to -308) promoter region fragments, respectively. PCR was carried out in 25 µl reactions with 1.5 U of Taq polymerase (Amersham, USA). The cycle parameters for first set of primers (for 310 bp fragment) includes, initial denaturation for 5 min at 94°C followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 50°C and extension at 72°C for 1 min. Final extension was carried out at 72°C for 10 min. The PCR conditions for second set of primers (for amplification of 128 bp fragment) were remained same except the change of annealing temperature from 50°C to 52°C.

Polymerase chain reaction and single strand conformational polymorphism(PCR-SSCP) analysis

PCR- SSCP was done as described earlier by Raj *et al*. (2004a) with little modifications. Briefly, 5 µl (20 ng) of the genomic DNA was amplified for 35 cycles as 20 µl reactions in the presence of [α^{32}P] dATP. The reaction conditions were same as described above for the second set of primers. 2 µl of the labeled PCR-product was mixed with 9 µl of loading dye (98% formamide, 20mM EDTA; 0.05% bromophenol blue; 0.05% xylene cyanol). The samples were denatured at 95ºC for 10 min and immediately chilled on ice for 5 min. 1-2 µl of the above samples were electrophoresed through a non-denaturing 8% polyacrylamide gel containing 3% glycerol. Electrophoresis was performed at 250 V for 18-20 h at 25°C. The gel was then autoradiographed.

RESULTS AND DISCUSSION

The PCR showed a single step amplification of the required fragment even at low parasitaemia of 0.1% (Fig. 1). When same primer was taken for *P. vivax* DNA in PCR, we got the amplification of similar fragment size as obtained in case of *P. falciparum*. The data indicates that there might be a very similar gene in *P. vivax*, which is yet to be reported.

Previous reports have indicated that a change in the important promoter regions of a gene severely affects the gene expression in *P. falciparum* (Horrocks and Lanzer 1999, Osta *et al*. 2002, Porter 2002). But the

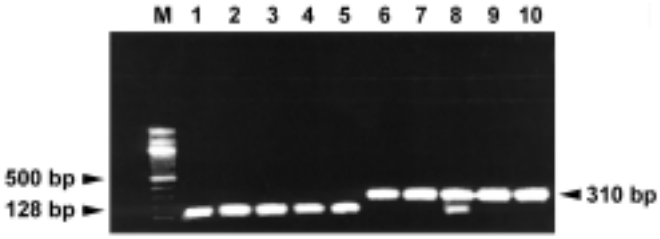

Fig. 1. A representative number of samples (out of 155) showing PCR amplification of promoter regions of 128 bp (Lane 2-5) and 310 bp (Lane 7-10) in *P. falciparum* natural isolates. M, 100 bp ladder. Lane 1 and 6, 3D7 clone of *Plasmodium falciparum.*

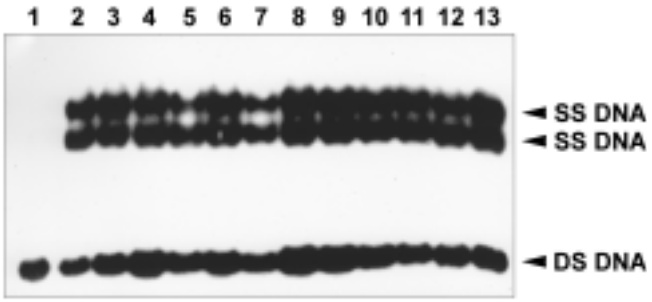

Fig. 2. A representative number of samples (12 out of 155) showing PCR-SSCP of 128 bp PCR amplified fragments. Lane 1: PCR amplified product of clone 3D7 DNA without denaturation showing only double stranded DNA; lane 2: Clone 3D7 DNA denatured with formamide showing both single and double stranded DNA; Lane 3-13: DNA of natural isolates of *Plasmodium falciparum* collected from an hyper endemic region.

PCR - SSCP analysis of the 128 bp important fragment of *Pfs25* promoter (Fig. 2) showed that none of the isolates out of 155 samples analyzed were polymorphic or having any point mutations. Similar results were obtained with 310 bp fragment (Figure not shown). Previous studies has shown that deletion of regions between nucleotide positions -648 to -484 reduces the *Pfs25* promoter activity by more than 16 fold and nucleotide position -484 to -368 completely abolishes *Pfs25* promoter activity (Dechering *et al.* 1999). Both the PCR amplified fragment of 310 bp and 128 bp generated by the two set of primers, contain binding sites for the transcription factor PAF I. To confirm the sequence of PCR amplified products of *Pfs25* promoter regions, we have sequenced the 128 bp and 310 bp fragments by PCR Sequencing Kit (Amersham) from few natural isolates and a 3D7 clone, which was taken as control.

Preliminary analysis of coding regions of *Pfs25* gene containing epidermal growth factor (EGF) like domain showed very limited polymorphism (data not shown). Most of the potential vaccine candidate genes of *P. falciparum* show polymorphism, which affect the vaccine potential by change of antigenic domain in the coding regions (Su and Wellems1994, Hanke *et al.* 1998, Tonhosolo *et al.* 2001, Raj *et al.* 2004b). The absence of polymorphisms or point mutations in the above regions showed that the *Pfs25* gene expression may not affected by the polymorphism of its promoter in natural isolates of *P. falciparum*. This study on promoter regions along with previous work on *Pfs25* coding sequence may be a supporting evidence for stable expression of Pfs25 protein, which is vital for the survival of the parasite in mosquito vector. Probably there is no alternative path exist for the survival of gametocytes after fertilization without expression of *Pfs25* gene. The reports of pervious studies on *Pfs25* shows antibodies to Pfs25 can completely block the transmission of malaria parasites when mixed with infectious blood and fed to mosquitoes through a membrane feeding apparatus (Lobo *et al.* 1999, Zou *et al.* 2003), but at this point of time we can't deny the involvement of other factors involved in the expression of *Pfs25* gene. Numerous studies on polymorphisms of *Pfs25* coding sequence in natural isolate showed that it displays limited or no polymorphisms in natural isolates of different geographical location of the world (Niederwieser *et al*. 2000). Again the effect of limited changes in the coding sequence of *Pfs25* on the vaccine potential of the gene is yet to be studied. Recombinant DNA vaccines by taking various coding regions of the gene may proved to be effective in animal models and human host (Barr *et al.* 1991, Kaslow and Shiloach 1994, Gozar *et al.* 1998). In conclusion, we believe that our study on sequence polymorphism of *Pfs25* promoter regions may provide additional information on Pfs25 to be used as a vaccine in case of *P. falciparum* malaria.

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Ethanol Affects Endocytosis and Proliferation of *Tetrahymena pyriformis* GL and Promotes Encystment

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Summary. Exponentially multiplying cells were exposed to 0.25-2.5 % ethanol (v/v) in the growth (PP) medium. Acute exposure was studied during a 6-h period, i.e. two normal cell generations, whereas chronic exposure was determined after 24 h, or more, in ethanol. The effects of low ethanol concentrations (0.25-1.0 %) were modest as the rate of endocytosis was almost normal and the rate of cell proliferation only slightly decreased. In high concentrations (>1.0 %) the acute effects of ethanol were dose- and time-dependent with respect to a lag period before proliferation resumed at a decreased rate and to the capacity of cells to form food vacuoles. After 24 h in the low ethanol concentrations the cells appeared to have adapted. In the high concentrations, however, chronic effects of ethanol were a much decreased rate of endocytosis and a distinct separation of the cells into two populations: normal sized cells and small mostly round cells. The small round cells were lying at the bottom af the culture dish, motionless or slowly moving with no visible oral structure but with somatic cilia, some cells lacked cilia and had a refractive outline or layered wall structure. These cells looked like precystic stages among which a few resting cysts were seen with pores in the cyst wall. Promotion of encystment in *Tetrahymena pyriformis* is surprising as the phenomenon is not considered a feature of this organism.

Key words: encystment, ethanol, growth, phagocytosis, *Tetrahymena pyriformis* GL-C.

INTRODUCTION

Exposure to ethanol is probably greater than to any other solvent, apart from water (Andrews and Snyder 1986). As a solvent ethanol is used widely for water-insoluble substances in industry and medicine but it is also a component of various intoxicating beverages. The effect of ethanol is dose- and time-dependent and chronic exposure may lead to serious conditions, such as a fatty liver (Andrews and Snyder 1986).

Ethanol is often considered as a “stimulant” but it is in fact an inhibitor (Albert 1981). The general effect of alcohol consumption is well known, it ranges from mild animation over impaired muscular coordination to loss of consciousness related to the level in blood. The mechanism by which alcohol causes these effects is not fully understood as many factors are involved (Andrew and Snyder 1986, Kranzler 1995). Pharmacological and toxi-

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cological effects of ethanol relate to the fact that it acts as both a general anesthetic and a nutrient as it may be metabolized by most organisms. As an anesthetic, ethanol causes a dose-dependent central nervous system depression (Andrews and Snyder 1986) which may be ascribed to lipid changes in the neuronal membrane (Moring and Shoemaker 1995) with displacement of proteins, i.e. an altered membrane functioning. Acute toxicity of ethanol may, however, be decreased by the presence in cells of heat shock proteins (HSP), i.e. stress proteins, which prevent protein degradation (Kampinga 1993) and ethanol is an inducer of HSP synthesis (Lepock *et al.* 1988).

The ciliate *Tetrahymena* is used as a model cell system in cytotoxicology and is highly adaptable to environmental stress (e.g. Nilsson 1989). A common reaction of cells to various stress conditions is induced changes in the membrane lipid composition as seen on exposure to ethanol (e.g. Moring and Shoemaker 1995). In *Tetrahymena*, chronic exposure, i.e. several days, to a high concentration of ethanol (~ 0.35 M) induced distinct changes in the membrane phospholipid composition (Nandini-Kishore *et al.* 1979, Goto *et al.* 1983).

In the present study *T. pyriformis* GL, an amicronucleate species, was exposed to different concentrations of ethanol (44-445 mM). The acute effect of ethanol on endocytosis and cell proliferation was studied during a 6-h period, i.e. 2 normal cell generations, and chronic effects were observed after 24 h and later. That ethanol affected cell proliferation is in agreement with reported findings by Nindini-Kishore *et al.* (1979). However, a most unexpected finding was that high ethanol concentrations promoted encystment, a phenomenon not generally recognized a feature of *T. pyriformis* (Furgason 1940, Corliss 1973).

MATERIALS AND METHODS

Tetrahymena pyriformis GL-C was grown axenically at 28°C in 2% proteose peptone (PP) enriched with 0.1% yeast extract and inorganic salts, pH 7.0 (Plesner *et al.* 1964). Batch cultures were maintained in 7 ml PP medium in test tubes. For experiments 100-ml cultures in 500-ml Fernbach flasks were agitated and aerated. Two cultures in the exponential growth phase (20-40,000 cells/ml) were mixed and divided into 50-ml cultures of which 3 received different concentrations of ethanol (v/v) and the 4th control culture received distilled water in a volume corresponding to that in which ethanol was added to the other cultures. Addition of ethanol did not change the pH of the medium. To reduce loss of the volatile ethanol the experimental cultures were not aerated or agitated although agitation of the cultures had little influence on the data as compared to those obtained without agitation; however, aeration made a great difference. The cultures were followed for 24 h or more.

The cells were also exposed to ethanol under starvation conditions in an inorganic salt (IM) medium (Plesner *et al.* 1964) to which the cells were transferred 1 h before addition of ethanol (3 experiments). Moreover, the cells were exposed to beer (Carlsberg lager, 4.6 % ethanol = 1 M; pH 4.3) after dilution with IM medium to 1.75 % ethanol and adjustment of pH to 7.2 before addition of concentrated cells. Concentrated cells were obtained by spinning down exponentially multiplying cells in a hand centrifuge, removal of the supernatant (PP medium), and refilling to the original volume with diluted beer; the control culture was treated similarly but refilled with PP medium diluted with IM in the same proportion as beer (4 experiments).

The cell density was determined electronically (Coulter Multisizer II, Coulter Electronics Ltd., England). Triplicate 0.5-ml cell samples were withdrawn from the cultures at hourly intervals and fixed in an equal volume of 1% glutaraldehyde in phosphate buffer (pH 7.2). The samples were diluted with 0.45% NaCl prior to counting.

The endocytic capacity was determined by a 10 min exposure of a 2-ml cell sample to 2 ml carmine particles (0.4 mg/ml) suspended in the same medium as that of the cells. At the end of the exposure the cells were fixed in 4 ml 1% buffered glutaraldehyde. After wash in distilled water, the number of labelled vacuoles was counted in 100 cells (Nilsson 1976). The endocytic capacity of the treated cell populations was expressed as the percent of that of control cultures (100%).

RESULTS

Proliferation and endocytosis

Exponentially multiplying cells were exposed to 0.25 - 2.5 % (v/v) ethanol (44 - 445 mM) in the growth, proteose peptone (PP), medium without aeration and agitation to minimize evaporation of the ethanol. The acute effect on cell proliferation is shown in Fig. 1 for a 6-h period, i.e. two normal cell generation times. A dose-dependent reduction of the increase in cell density (compared with the control) was seen after 1 h, i.e. the division of cells most advanced in the cell cycle. Cells in 0.25 and 0.5 % ethanol continued proliferation at slightly decreased rate without a lag period, whereas cells in 1, 1.5, and 1.75 % ethanol exhibited lag periods of 2, 3, and 5 h, respectively, before resuming proliferation at a decreased rate. Cells in 2 and 2.5 % (not shown) ethanol did not proliferate during the 6 h period. After 24 h and dose-dependently, cells in 0.25, 0.5, and 1 % had reached maximum cell density (~ 1,000,000 cells/ml), i.e. doubled 5-6 times, indicating adaptation to ethanol. The cell density in 1.5 and 1.75 % ethanol increased only 2.5 and 1.4 times after 24 h, respectively, i.e. cells in 1.75 % did not complete a full cell doubling. After 24 h in 2 % and

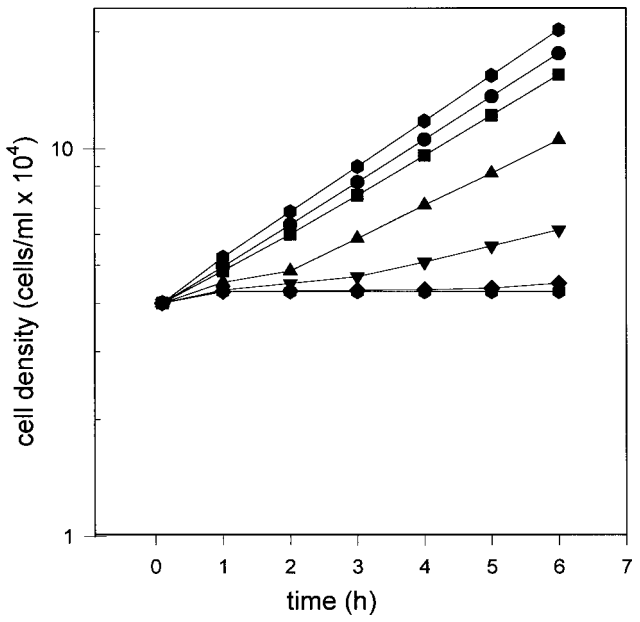


Fig. 1. The dose- and time-dependent effect of ethanol on proliferation of *Tetrahymena* during a 6-h exposure in proteose-peptone medium. Control (top solid hexagon), 0.25 % (solid circle), 0.5 % (solid square), 1 % (solid triangle), 1.5 % (solid upside-down triangle), 1.75 % (solid diamond), and 2 % (lower solid hexagon) ethanol. Means of 4-12 experiments per ethanol concentration. S.D. < 10 %.

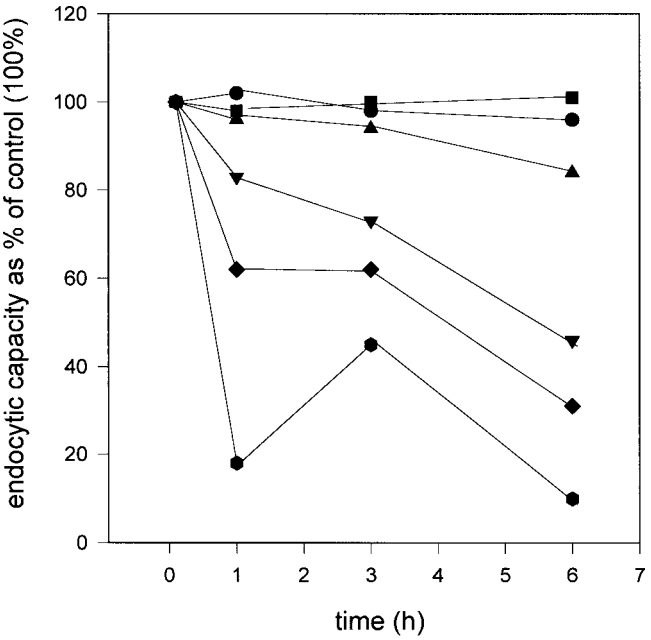


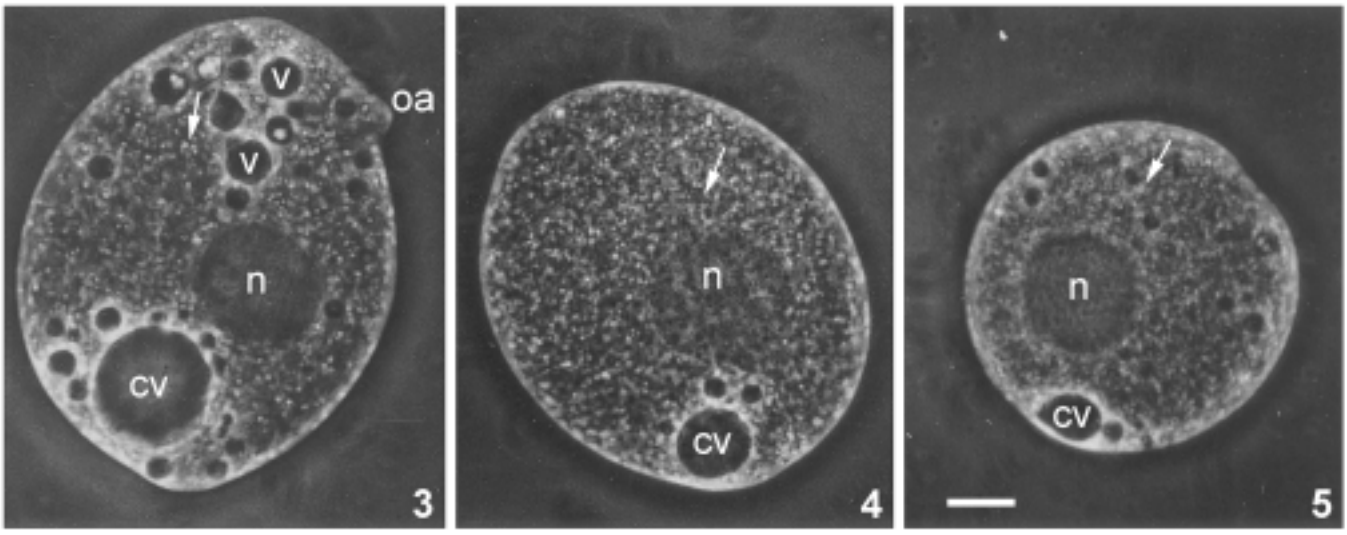
Fig. 2. The dose- and time-dependent effect of ethanol on the capacity of *Tetrahymena* to form food vacuoles (endocytosis) during a 6-h exposure in proteose-peptone medium. The data are expressed as the percent of the capacity of control cells (100 %). Cells in 0.25 % (solid circle), 0.5 % (solid square), 1.0 % (solid triangle), 1.5 % (solid upside-down triangle), 1.75 % (solid diamond), and 2.0 % ethanol (solid hexagon). Means of 4-12 experiments per ethanol concentration. S.D. < 10 %.

2.5 % ethanol the cell density decreased more than 20 % due to cell deaths. Hence, chronic exposure to ethanol revealed cell adaptation in the low concentrations but severely affected cells with deaths in the high concentrations. The highest sublethal concentration (without cell deaths) is therefore 1.75 % (~ 300 mM) ethanol in the PP medium.

The capacity of the cells to form food vacuoles (endocytosis) in the presence of ethanol, is shown in Fig. 2 for a 6-h period as a percentage of that of control cells (100 %). The rate of endocytosis was unaffected in 0.25 and 0.5 % ethanol, slightly decreased in 1 %, and time- and dose-dependently affected in 1.5, 1.75, and 2 % ethanol; the low 1-h values indicate a strong initial reaction to ethanol. Cells in 2.5 % ethanol did not form any food vacuoles, i.e. the rate follows the abscissa. After 24 h the rate of endocytosis increased 10-20 % above the control value (100 %) in 0.25 - 1 % ethanol, whereas it decreased to 11, 3, and 0 % in 1.5, 1.75, and 2 % ethanol, respectively.

The cells were also exposed to 0.5, 1.0, and 1.5 % ethanol in the absence of nutrients in an inorganic salt (IM) medium. The only increase in cell density was division of the cells most advanced in the cell cycle at addition of ethanol; this portion of the cells was affected dose-dependently as also seen after 1 h in PP medium (see Fig. 1). Endocytosis was affected, i.e. the starved cells in 0.5, 1.0, and 1.5 % formed food vacuoles after 1 h at a rate of 96, 77, and 52 %, after 3 h at a rate of 92, 63, and 46 %, and after 5 h at a rate of 97, 62, and 25 %, respectively, of the control value (100 %); moreover, after 24 h the rate was 89, 61, and 3 %, respectively. These values are lower than the rates of endocytosis, shown in Fig. 2, for cells exposed to 0.5, 1.0, and 1.5 % ethanol in the growth medium. After 24 h many dead cells were seen in 1.0 and 1.5 % ethanol in the IM medium which means that *Tetrahymena* is less tolerant to ethanol in the absence of nutrients. The highest sublethal concentration in the starvation medium is 0.5 % ethanol.

To investigate the high sensitivity of starved cells further, *Tetrahymena* was exposed to another nutrient, i.e. lager beer, after dilution to 1.75 % ethanol and adjustment of pH. The cells behaved much like cells in



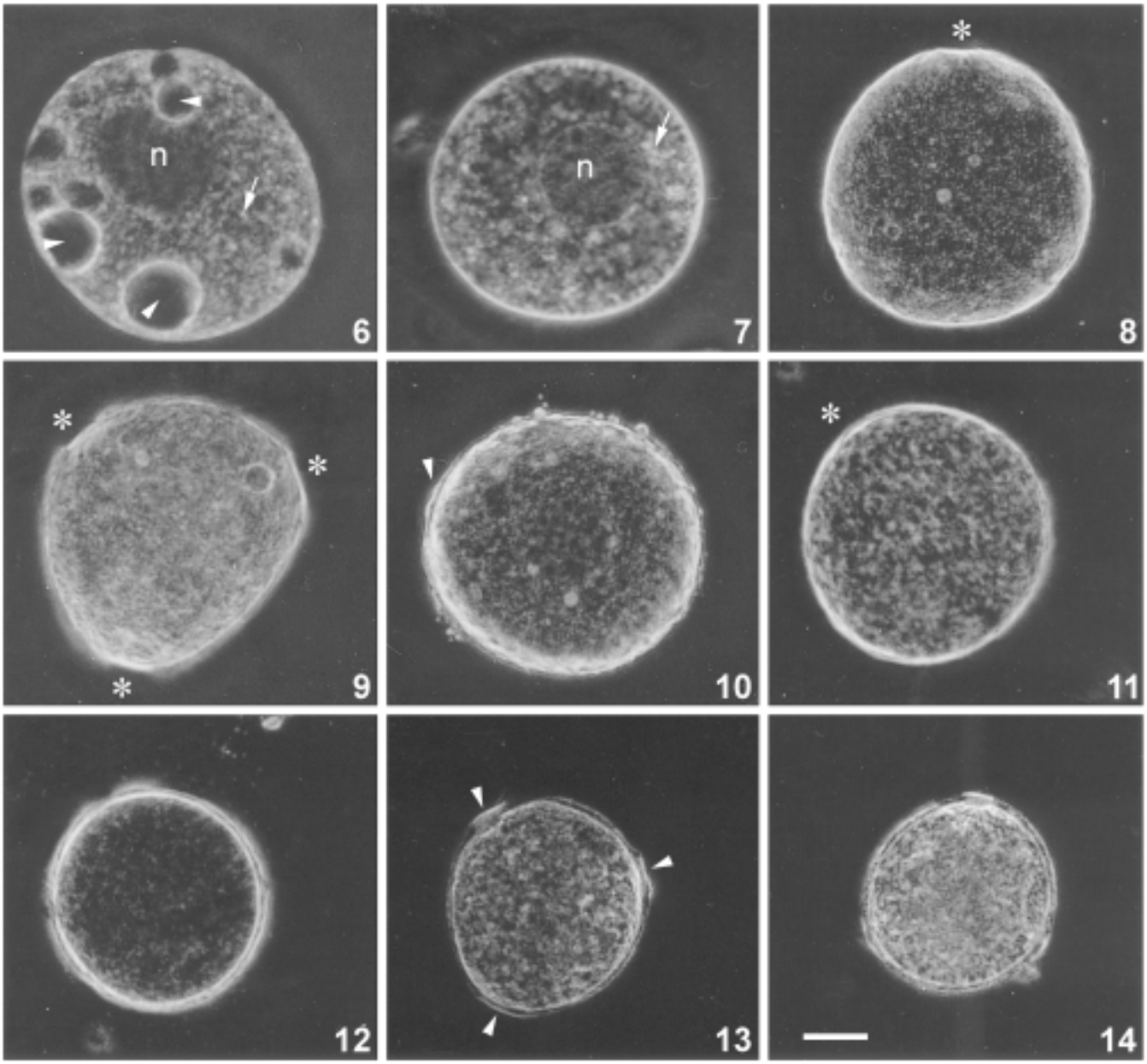
Figs 3-5. *In vivo* micrographs of compressed *Tetrahymena* exposed to 2 % ethanol in the growth medium. **3** - cell after 3.5 h exposure to ethanol; **4** - a cell from the "large cell" population after a 24 h exposure; **5** - a cell from the "small cell" population after a 24 h exposure. Nucleus (n); contractile vacuole (cv); food vacuoles (v); oral structure (oa); granules (arrows). Scale bar 10 µm.

the same concentration of ethanol in the PP medium (Fig. 1), i.e. a 5 h lag period, completion of less than one cell doubling after 24 h, and no cell death. The rates of vacuole formation after 1, 3, 6, and 24 h were 64, 65, 40, and 7 %, respectively, i.e. similar to the rates of cells in 1.75 % ethanol in the PP medium (Fig. 2). Hence, ethanol affects *Tetrahymena* identically in the two nutrient media, PP medium and beer, but starved cells only tolerate ethanol in lower concentrations.

Light microscopy

Light microscopical observation of ethanol-treated cells in the PP medium revealed normal cell behavior in the low concentrations. In the high ethanol concentrations, some cells lost their shape initially and became spherical with an enlarged contractile vacuole, concomitantly with the low 1 h values of phagocytosis (Fig. 2), but normal cell shape was restored by 3 h; this swelling of cells was also observed in the starvation medium. Small refractive granules appeared in all ethanol-treated cells, as typically seen in *Tetrahymena* during stress conditions (Nilsson 1976, 1989, 2003); the granules increased in number with time (Figs 3-5) and ethanol concentration. After 24 h, a distinct dose-dependent change occurred in the high ethanol concentrations (>1.5 %) as two distinct populations were seen, one of normal sized cells (uncompressed length/width = 1.7) and another of small, mostly spherical (uncompressed length/width = 1.1), cells (Figs 4, 5). This feature of two cell populations occurred also after 24 h in the ethanol-starvation medium and in beer. In PP medium, the number of small cells increased dose-dependently from a few % in 1.5 % ethanol and up to 90 % in 2.5 % ethanol after 24-48 h; hence, after 24 h in 1.75 % ethanol about 75 % of the cells were of normal size and shape, whereas the remaining 25 % were small, round cells lying at the bottom of the culture dish looking motionless, appearing dead.

On close observation, however, many of the small cells did move, not swimming in straight lines as the normal shaped cells but spinning around themselves. The cells were most often without visible oral structure or food vacuoles, only the macronucleus and contractile vacuole, or contractile vacuoles, were visible (Figs 5, 6). Transformation of pyriform cells to the small round precystic cells, was indicated by cells "dividing off" the pointed anterior end (round macronucleus remaining in the posterior end); the tiny triangular piece swam off leaving a round cell. Some cells did not move at all, they lacked cilia and did not have a contractile vacuole but they contained the macronucleus and numerous large granules (Fig. 7). Other cells were opaque, obscuring internal structures, and their smooth refractive outline showed indication of pore sites (Figs 8, 9). Precystic



Figs 6-14. *In vivo* micrographs of ethanol-induced encystment of *Tetrahymena*. Stages from the "small cell" population, after 24-48 h in 2 % ethanol in the growth medium, arranged in a possible sequential order. **6** - an early round precystic cell showing the macronucleus (n), several contractile vacuoles (arrow heads), and numerous granules (arrow); note, the absence of an oral structure; **7** - another spherical precystic stage with no sign of cilia; note, the macronucleus (n), the many large granules (arrow), and no contractile vacuole; **8** - a compact "cell" with a smooth, refractive outline and early indication of a pore site (star); **9** - probably a slightly later stage than that shown in the Fig. 8 with indication of pore sites (stars); **10** - a compact precystic stage with a rough, refractive outline due to extruded material for the cyst wall, note the indication of a double layered wall (arrow head); **11** - another compact stage within a thickened, refractive wall with indication of a pore site (star); **12** - an early cyst stage with a smooth, refractive double "shell"; **13** - a resting cyst showing 3 pore structures (arrow heads) in optical section and a layered cyst wall; **14** - another resting cyst with pore structures after 24 h in 1 % ethanol in the starvation medium. Scale bar 10 μm.

stages with an irregular outline due to extruded material (Fig. 10), probably for cyst wall formation as the refractive rim increased (Fig. 11) and thickened to form a less flexible (not compressible) early cyst with a layered smooth, refractive wall (Fig. 12); resting cysts had pores in the wall (Figs 13, 14). Although the sequential order of events is uncertain, the present findings seem to indicate that pore sites are created, or determined, prior to formation of the elaborate cyst wall; moreover, that the unusual appearance of several contractile vacuoles along the cell periphery (Fig. 6) may play a role in determining the site, or in creation, of the pores. The frequency of encystments was dose-dependent and highest in 2 % and 2.5 % ethanol but the yield of cysts was low, whereas the precystic stages were plentiful. No attempt was made to study or induce excystment.

As ethanol can induce encystment it means that the phenomenon must be a normal capacity of *T. pyriformis* and cysts should therefore occur also in old batch cultures in which the cells die out due to the increasing pH (> 8.5). Examination of the debris at the bottom of old batch (several days/weeks) cultures with a few swimming cells, revealed indeed numerous small round bodies, mostly ghosts but also moving cells, resembling the precystic stages induced by ethanol; resting cysts with pores were found but rarely.

DISCUSSION

Ethanol did not affect *Tetrahymena* much in low concentrations, the cells adjusted to up to 1 % (~175 mM) in the growth PP medium. In high concentrations, however, both phagocytosis and cell proliferation were affected dose- and time-dependently by ethanol. The present finding that ethanol affected cell proliferation, confirms the report by Nandini-Kishore *et al.* (1979) although the authors found growth of *Tetrahymena* in somewhat higher concentrations than seen in the present study. Chronic exposure of *Tetrahymena* to 1.6 % (w/v), or 0.35 M, ethanol caused profound changes in membrane fluidity (Nandini-Kishore *et al.* 1979, Goto *et al.* 1983), i.e. an altered membrane functioning, a feature which would affect the rate of endocytosis. Moreover, that ethanol affected vacuole formation in *T. pyriformis* with no, or little, inhibition in low concentrations and depression in high concentrations, resembles the increased activation of Kupffer cell phagocytosis in low, and depression in high, concentrations of ethanol (Eguchi *et al.* 1991). Another factor which may relate to the affected endocytosis in *Tetrahymena*, is that ethanol increases the $[Ca]_i$ in Kupffer cells which may disturb the phagocytic process (Hijioka *et al.* 1993). Incidentally, an increased $[Ca]_i$ in *Tetrahymena* would increase transport of excess calcium from the cytoplasm into the small refractive granules, i.e. also increase their number, to maintain homeostasis (see Nilsson 2003). That the endocytic capacity of starved cells was affected in a lower concentration of ethanol than in the nutrient media, could be ascribed to the fact that the starved cells were subjected to double stress: i.e. sudden transfer to starvation conditions followed by an exposure to ethanol.

The most surprising and unexpected effect of ethanol was the promotion of encystment in *T. pyriformis*, especially since cysts are not a recognized feature of this organism: “neither reproductive nor resting cysts are recorded” (Corliss1973). In the past, however, two reports on cysts have appeared. Watson (1946) described thin-walled, oval cysts formed after drying a *Tetrahymena* culture slowly on an agar plate; Corliss (1953) was unsuccessful in reproducing such cysts. Hurst (1957) reported in an abstract on the occurrence of “cysts” in *T. pyriformis* (variety 2): “the round, dark, sessile structures contained a micro- and a macronucleus but no nuclear reorganization was observed”, i.e. not enough information to identify the structure of the cysts (pores?).

Encystment is a common phenomenon in nature as a manner of survival. Free-living ciliates have seasonal blooms with interim periods of environmental stress (drought, frost, and snow) affecting their food supply so the ciliates must encyst to survive and often conjugation precedes encystment (e.g. Corliss and Esser 1974, Fenchel 1987). An arctic micronucleate *Tetrahymena* sp. isolated from a fresh-water pond in Greenland (Larsen 1992), is a typical ciliate living under extreme conditions. The ciliate was established in the laboratory in mixed cultures and cysts were commonly found in old cultures (unpublished observations), whether conjugation preceded encystment was not recorded. The cysts have pores in the wall and resemble the cysts (Figs 13, 14) induced by ethanol in *T. pyriformis*. The arctic *Tetrahymena* sp. probably belongs to the *T. pyriformis* complex (Corliss 1973, Nanney and McCoy 1976) which is not known to form cysts. Members of the *T. rostrata* complex are mostly parasitic species and form cysts readily. Descriptions of the cysts of *T. rostrata* (Corliss 1973, McArdle *et al.* 1980) do not mention pores in the cyst wall, hence they differ in structure from the *T. pyriformis* GL cysts described here.

Even though conjugation usually precedes encystment (e.g. Corliss and Esser 1974) it cannot be a prerequisite for formation of cysts since *T. pyriformis* GL can form cysts but is unable to conjugate. It was proven amicronucleate 65 years ago (Furgason 1940) and it has been maintained axenically in organic growth medium for even longer. Hence, it may be concluded from the present study that the genetic information of the micronucleus is not essential for encystment, or perhaps not entirely, as admittedly the yield of mature cysts was low whereas the yield of precystic stages was very high.

A last question is: why does ethanol promote encystment of *Tetrahymena pyriformis*? Without knowing the details, apart from the ethanol-induced changes in the membrane phospholipids (Nandini-Kishore *et al.* 1979, Goto *et al.* 1983), an answer may be that high concentrations of ethanol depress food vacuole formation in rapidly multiplying cells, i.e. the cells are abruptly brought to a state of starvation, a common trigger of encystment (Corliss and Esser 1974, Fenchel 1987).

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ACTA
PROTOZOOLOGICA

An Endocytobiont Harbouring *Naegleria* Strain Identified as *N. clarki* De Jonckheere, 1994

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Summary. A *Naegleria* strain harbouring two different populations of bacterial endocytobionts, together inhibiting the encystation of their host was isolated from a garden pond. Interestingly, the ability to form cysts was regained after removing one endocytobiont by antibiotic treatment. The trophozoite and the flagellate stage were investigated by light and electron microscopy and the temperature tolerance, under-agarose migration and tissue culture pathogenicity of the strain were assessed. Moreover, the entire gene of the SSU rRNA was sequenced and compared to published sequences of various *Naegleria* species and a cluster analysis was performed in order to reveal the phylogenetic position of this endocytobiont harbouring *Naegleria* strain. The amoeba was shown to grow well at 37°C, to migrate under-agarose and to lyse human HEp-2 cells, properties related to pathogenicity. With the help of SSU rDNA sequencing and subsequent cluster analysis the strain N_DMLG was identified as *N. clarki*. The *N. clarki* strains altogether formed a cluster with *N. gruberi* and the potentially pathogenic *N. italica, N. australiensis* and *N. tihangensis.*

Key words: amoebae, endocytobionts, *Naegleria clarki*, SSU rDNA.

INTRODUCTION

Naegleriae are free-living amoebae occurring predominantly in freshwater and soil. *N. fowleri* is pathogenic for man causing the almost always fatal primary amebic meningoencephalitis (Carter 1970). Besides their active pathogenicity free-living amoebae can also be of clinical relevance by acting as hosts and vehicles for pathogenic bacteria. Rowbotham (1980) was the first who confirmed the ability of *Legionella pneumophila* - the causative agent of legionnaires' disease - to grow within vacuoles of acanthamoebae. He also demonstrated the growth of this pathogen within *Naegleria* sp. While acanthamoebae and also hartmannellae frequently harbour intracellularly replicating bacteria (Barker and Brown 1994; Michel and Hauröder 1997; Michel *et al*. 1998, 2003; Horn *et al*. 2000, 2001; Greub and Raoult 2004) - some authors report that occasionally more than 20% of isolated *Acanthamoeba* strains are naturally infected with bacteria (Fritsche *et al*. 1993) - only very few naegleriae

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naturally harbouring bacteria have been found. Harf (1993) reported on the occurrence of intracellular Gram negative rods in *Naegleria* sp., Michel *et al*. (1998) found naturally occurring intracellular cocci within a *Naegleria* strain isolated from an aquarium and Thom *et al*. (1992) have shown experimentally within a period of 24 h, that *Vibrio cholerae* grow better in the presence of acanthamoebae and naegleriae. *Naegleria* spp. are also often found in *Legionella*-positive waters, but they usually do not carry bacteria inside their cells (Grimm *et al*. 2001).

A *Naegleria* strain (strain N_DMLG) isolated from a small private garden pond attracted our attention by its inability to form cysts. An initial microscopic study revealed that this strain harboured two different populations of bacterial endocytobionts - one (pc) replicating within the cytoplasm and the other (pn) growing within the nucleus of its host amoeba (Michel *et al*. 1999). The current study is a continuation of that investigation in order to characterise and identify this *Naegleria* strain serving as a host for such a rarely found double infection.

MATERIALS AND METHODS

Organisms and culture. The strain N_DMLG was isolated from a mixed population of various species of FLA from a garden pond (Michel *et al*. 1999) and was cultured by the non nutrient (NN)-agar plate culture method according to Page (1988). Briefly, mud samples were inoculated onto non-nutrient agar plates coated with a 24 h old culture of *Enterobacter cloacae* and incubated at RT. Initial cultures were diluted in order to eliminate co-contaminants by cutting a small piece of agar with a sterile scalpel and applying it centrally onto a fresh plate. For obtaining uniform genetic populations for taxonomic investigations, the isolate was cloned by transferring a single cyst to a fresh plate with a micromanipulator.

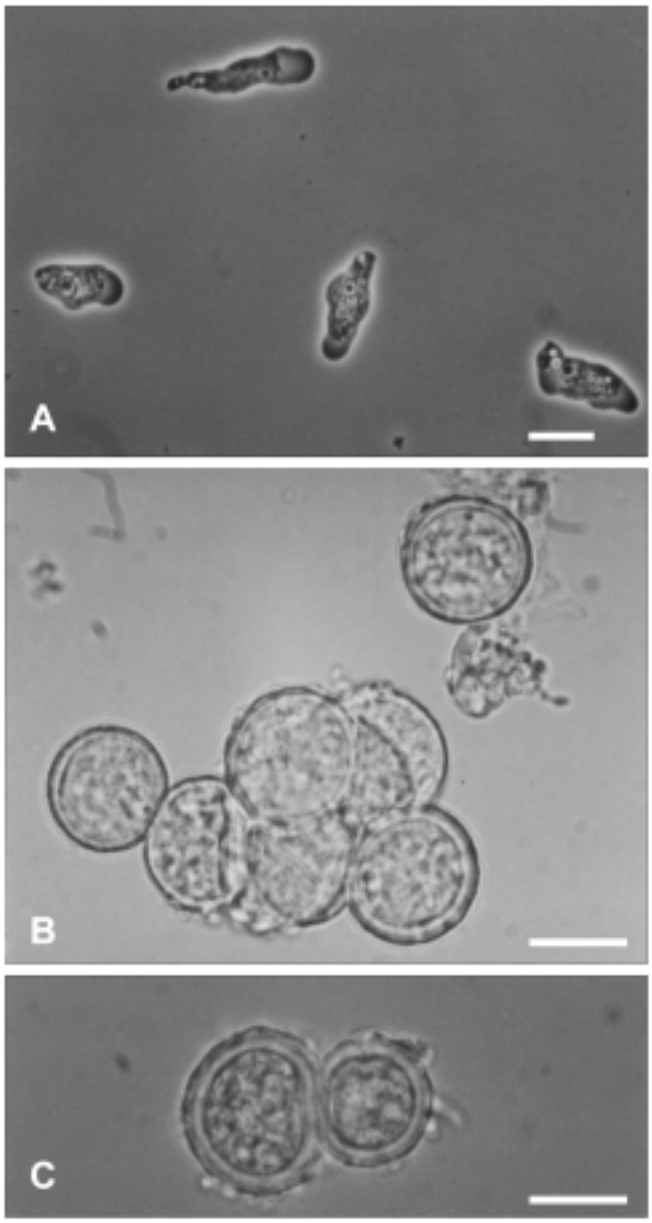
In order to confirm the ability of the strains to transform into the characteristic flagellate stage the trophozoites of a 48-72 h culture were transferred from the agar surface into amoeba-saline or distilled water and observed for one to two hours. The strain was maintained on NN-agar plates, subcultured by monthly serial transfers and later transferred to axenic SCGYE-medium (De Jonckheere 1977) under antibiotic treatment (200 IU penicillin and 200 µg/ ml streptomycin). Morphological investigations included measurements made with a calibrated ocular micrometer.

Electron microscopy. For electron microscopical investigations amoebae and flagellates were harvested from the plates and pelleted by centrifugation (500 g) for 10 min. The resulting pellets were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h, washed twice in the same buffer, postfixed for 1 h in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2), and embedded in Spurr resin. Finally, the sections were stained with uranyl acetate and Reynold's lead citrate and examined using a Leo EM 910 transmission electron microscope (Leo, Oberkochen).

Physiological investigations. Temperature tolerance was assessed by incubating fresh cultures at a range of temperatures (30°C, 34°C, 37°C, 40°C and 42°C) and observing them daily using phase contrast microscopy. The strain was considered tolerant to a certain temperature, if it was able to survive and to multiply at this temperature. Altogether, the growth of the amoebae was observed at each temperature for two weeks. The ability of the amoebae to migrate into the agar was recorded after 72 h. The ability of the amoebae to lyse human cells was revealed by coincubating the amoebae with a human cell monolayer (HEp-2 cells). The HEp-2 cells were cultured in a 1:1 mixture of PC-1TM (Bio Whittaker, Walkersville, Maryland) and CO_2 independent medium (Life Technologies Ltd., Paisley, Scotland) supplemented with L-glutamine (2 mM) in 75 cm^2 tissue culture flasks (Corning, Costar, Bodenheim, Germany) at 37°C under sterile conditions. 1 ml of a 10^5 cell/ ml axenized suspension was inoculated onto a monolayer of HEp-2 cells. Cocultures of amoebae and tissue cells were incubated at 30°C, 37°C and 42°C, respectively. Pathogenicity was defined as complete lysis of the monolayer within 48 h at 37°C. All experiments were carried out in triplicate and were repeated after 6 weeks.

Isolation of DNA. For molecular biological investigations ~10^6 amoebae from actively growing cultures were harvested and washed 3x in sterile 0.9% NaCl by centrifugation 500 ×g/ 7 min. Whole-cell DNA was isolated by a modified UNSET-procedure (Hugo *et al*. 1992). Briefly, the amoebae were suspended in 500 µl of UNSET-lysis buffer, overlaid with 500 µl phenol-chloroform-isoamylalcohol (PCI) and shaken gently for 5 h. DNA was extracted by multiple PCI-extraction, precipitated in alcohol, air dried and resuspended in 30 µl of sterile double-distilled water.

PCR and sequence analysis. The SSU rRNA gene (small subunit ribosomal RNA gene) was amplified using universal eukaryotic primers complementary to the conserved ends of the eukaryotic SSU rRNA genes (Gast *et al*. 1996) and a standard amplification program (30 cycles; 95°C 1 min, 51°C 2 min., 72°C 3 min.). The amplified gene (3285 bp long) was visualized by ethidium-bromide in an agarose-gel electrophoresis and then sequenced stepwise (by constructing the following internal primers, given in 5'-3' direction:
N1-CTGCAGCGATAATACTTG,
N2-GGAATCAGTGTTCGATTCC,
IN1-GTAGTGACAAGCTATAGTG,
IN2-CTGTAGGATGGTAACAAGC,
IN3-CAAGATTGATCGCGAATC,
IN4-CGAATGCTTGATTGGTGC,
IN5-CTGAGGAGGTCTCAATC,
IN6-GAATTGATGTTCGGTAGTC,
Nrev1-CAACGAGCTGATAGGTAAG,
Nrev2-GTTATCTACACCCAAATC,
Nrev3-CTGCAGTCCCATGCTAAATC,
INrev1-CTCGTTGCCGAGCGGTAC,
INrev2-CAATGGTCTGCCGTGTAG,
INrev3-GCAGTTTGACAACCATTC,
INrev4-CACAGCCTCCATTACCAC,
INrev5-CACTCTTAGGTATACTAC)
by direct sequencing from the PCR-product using the BigDye sequencing kit and a 310 ABI PRISM® automated sequencer (PE Applied Biosystems, Langen, Germany). Sequences were obtained from both strands.



Figs 1 A-C. *Naegleria clarki* strain N_DMLG. **A** - trophozoites in phase contrast, endocytobiotic bacteria visible; **B, C** - cysts, note the extremely thick cyst wall, encystation induced after antibiotic treatment in order to kill the endocytobiotic bacteria. Scale bars 10 µm (A); 15 µm (B, C).

Sequence data were processed with the GeneDoc (Nicholas *et al*. 1997) sequence editor and compared to the ones of strains published in GenBank using BLAST Search (Altschul *et al*. 1990). Multiple alignment was performed and sequence dissimilarities to published sequences of *Naegleria* spp. were assessed.

Phylogenetic analyses. Two cluster analyses were performed. One including various vahlkampfiid amoebae in order to proof the position of strain N_DMLG within the genus *Naegleria* using *Acrasis rosea* as outgroup and a second, more detailed cluster analysis in order to find out the exact position of strain N_DMLG within the genus *Naegleria* and in order to reveal the detailed branching order of the closely related species *N. clarki, N. gruberi, N. italica, N. australiensis* and *N. tihangensis*. In this second analysis *N. fowleri* and *N. lovaniensis* were used as an outgroup. Multiple sequence alignments were performed by subsequent pairwise alignment using the Clustal X application (Thompson *et al*. 1997). The alignments were controlled by eye and corrected manually.

Phylogenetic analyses were made using the PHYLIP (Felsenstein 1989) package. Primer sites, unique gaps, ambiguously aligned sites and introns were excluded from the analyses. The analyses were performed with different evolutionary models including maximum likelihood, neighbour joining and maximum parsimony. The confidence of the branching order was proven by the generation of 100 bootstrap replicates. Maximum likelihood was performed with invariable sites and a gamma distribution of among-site rate variation, neighbour joining with a Kimura two parameter corrections and maximum parsimony employing heuristic search with a random sequence addition option and counting all steps. Consensus trees were made from the resulting trees using Consense and drawn with the TREEVIEW software (Page 1996).

Sequence data. The sequence reported in this paper is available at GenBank under the following accession number: AF426157.

RESULTS

Morphology and physiology. The strain N_DMLG readily transformed to the flagellate stage when incubated in amoeba saline, but the amoebae were obviously unable to encyst. In order to prove that this was due to the intracellularly “parasitising” bacteria and also in order to be able to study the cyst morphology, it was aimed to induce encystment by eliminating the bacteria. Indeed, after several rounds of antibiotic treatment (200 IU penicillin and 200 µg/ ml streptomycin) and thus after elimination of at least the cytoplasmic population of the endocytobiotic bacteria, the amoebae did form cysts. Figure 1 shows trophozoites (Fig.1A) and cysts (Figs 1B, C). The cysts are round, 8-16 µm in diameter with clearly separated cyst walls and a slightly wrinkled ectocyst.

In order to investigate the endocytobiotic bacterial populations electron microscopy was performed. At the ultrastructural level we discovered not only coccoid organisms of a very prickly appearance (pc) within the cytoplasm but in addition cells with a more smooth outline within the nucleus - especially on the surface of the prominent nucleolus looming into the karyoplasm (pn) (Fig. 2). Both endocytobiotic bacterial populations were shown to be Gram negative and both could not be

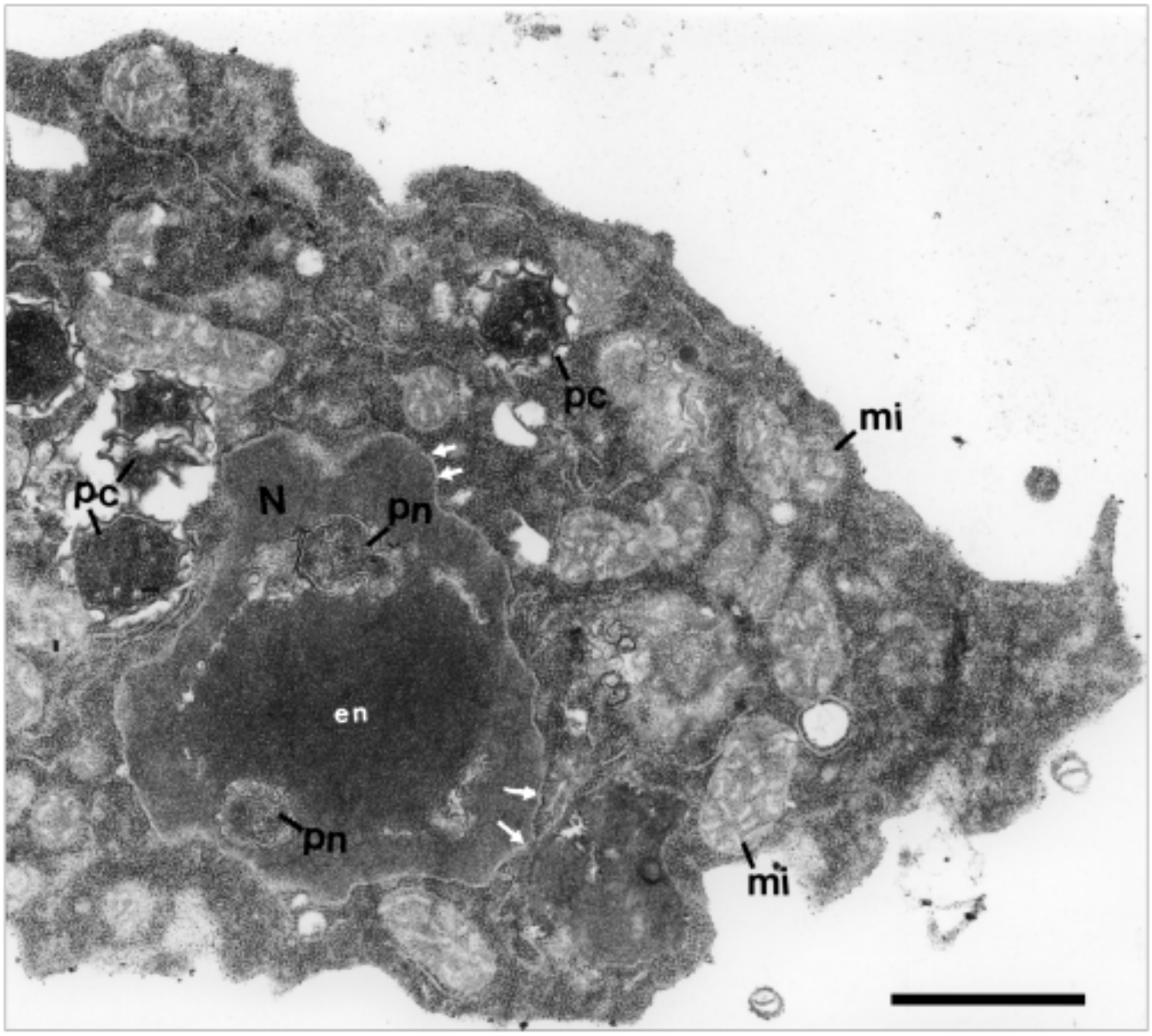
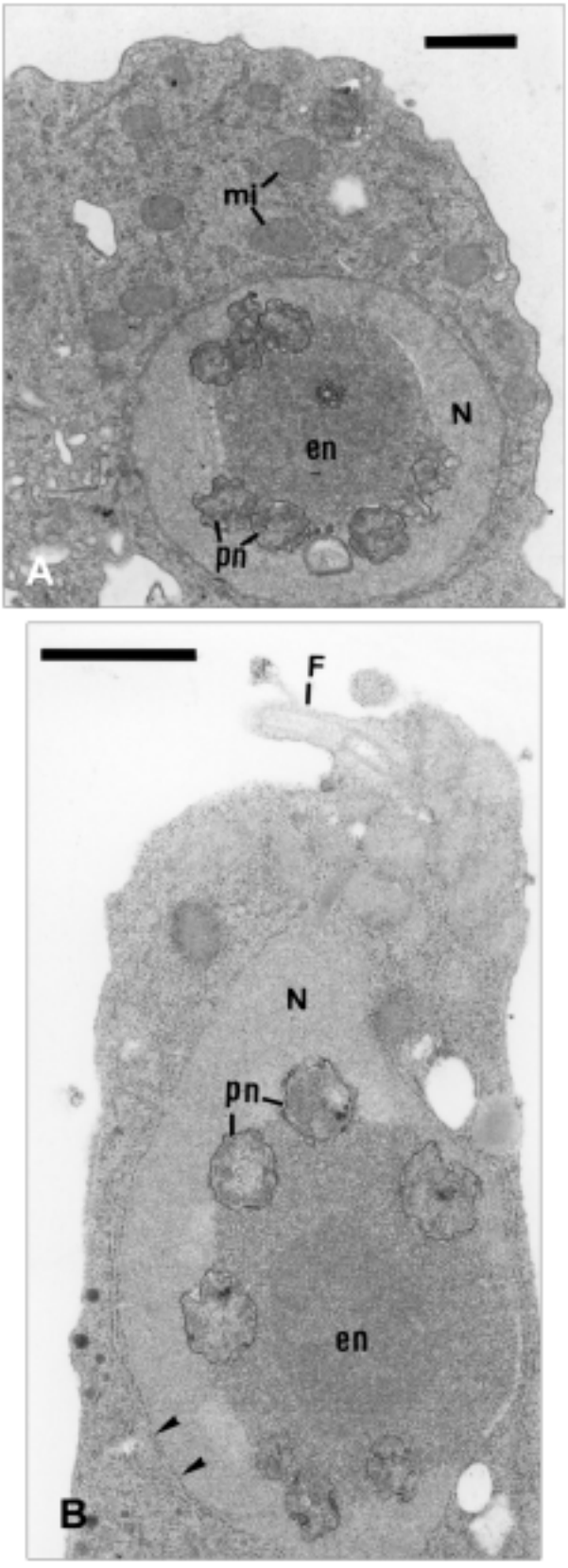


Fig. 2. *Naegleria clarki* strain N_DMLG trophozoite harbouring two different populations of endocytobiotic bacteria: one (pc) is multiplying within the cytoplasm, the other one (pn) is located within the nucleus (N) at the interphase between the prominent nucleolus (en) and the karyoplasm. mi - mitochondria. Scale bar 1 µm (30 000×).

grown on various synthetic media (Michel *et al*. 1999). However, the two bacterial populations could be grown separately as a "single infection" by variation of the culture conditions and application of antibiotics - both within substrains of the same host amoeba. Thus, it can be concluded, that indeed two different populations were present in the original isolate (Michel *et al*. in preparation). An example of a trophozoite harbouring solely the intranuclear population is shown in Fig. 3A. The original isolate with "double infection" as well as the experimentally derived amoebae with only a single infection were still able to transform into the flagellate stage thereby maintaining their respective endocytobionts such as the intranuclear population for instance shown in Fig. 3B.

Physiological investigations revealed the amoebae to be thermophilic (growing well at 37°C), to be able to



migrate under-agarose and to completely lyse a monolayer of human HEp-2 cells within 48 h, independently of the presence of the bacterial populations.

Molecular biology. It was shown that the SSU rDNA of the *Naegleria* strain N_DMLG is 3285 bp long. It has a group I intron of 1305 bp from base 631-1936 and a G + C content of 44.18 (47.53 without insert). Comparison to published sequences revealed that N_DMLG shows highest SSU rDNA sequence identity (99.95%) to strain 4564/IV of *N. clarki* (GenBank Accession No.: AF338419) isolated from the kidney of a *Perca fluviatilis* (Dykova *et al*. 2001). The sequence dissimilarity of N_DMLG is <0.36% to all *N. clarki* strains - to the most closely related one only 0.05% (the dissimilarities between all published *N. clarki* strains are up to 0.56%), while it is 0.66 to *N. gruberi*, the closest relative to *N. clarki* (from which it, however, differs by the intron N_DMLG and all *N. clarki* strains have, and which *N. gruberi* sensu stricto does not have), and >1% to all other naegleriae - to *N. fowleri*, *N. lovaniensis, N. jamiesoni* and *N. andersoni* even >5%.

Cluster analysis. In the first phylogenetic analysis (Fig. 4) it was shown that the strain N_DMLG grouped within the species *N. clarki* and the *N. clarki* strains altogether formed an imperfectly resolved cluster with *N. gruberi, N. italica, N. australiensis* and *N. tihangensis*. The whole genus *Naegleria* appeared as a monophyletic group with *Willaertia* being most closely related.

In the second analysis (Fig. 5) it was shown that the strain N_DMLG indeed groups with the 5 *N. clarki* strains isolated from organs of freshwater fish. Moreover, it was shown, that *N. gruberi* is the nearest relative and this grouping was supported by high bootstrap values. The subsequent closest relative was *N. italica* and these altogether formed a cluster with *N. australiensis* and *N. tihangensis*.

Figs 3A, B. *Naegleria clarki* strain N_DMLG. **A** - trophozoite harbouring only the **intranuclear** endocytobiotic population (pn) located at the interphase between nucleolus (en) and karyoplasm. N - nucleus, mi - mitochondria. Scale bar 1 µm (13 200×); **B** - flagellate stage harbouring the intranuclear population (pn) at the margin of the nucleolus (en). F - flagellum, N - nucleus. Arrowheads - nuclear membrane. Scale bar 1 µm (21 000×).

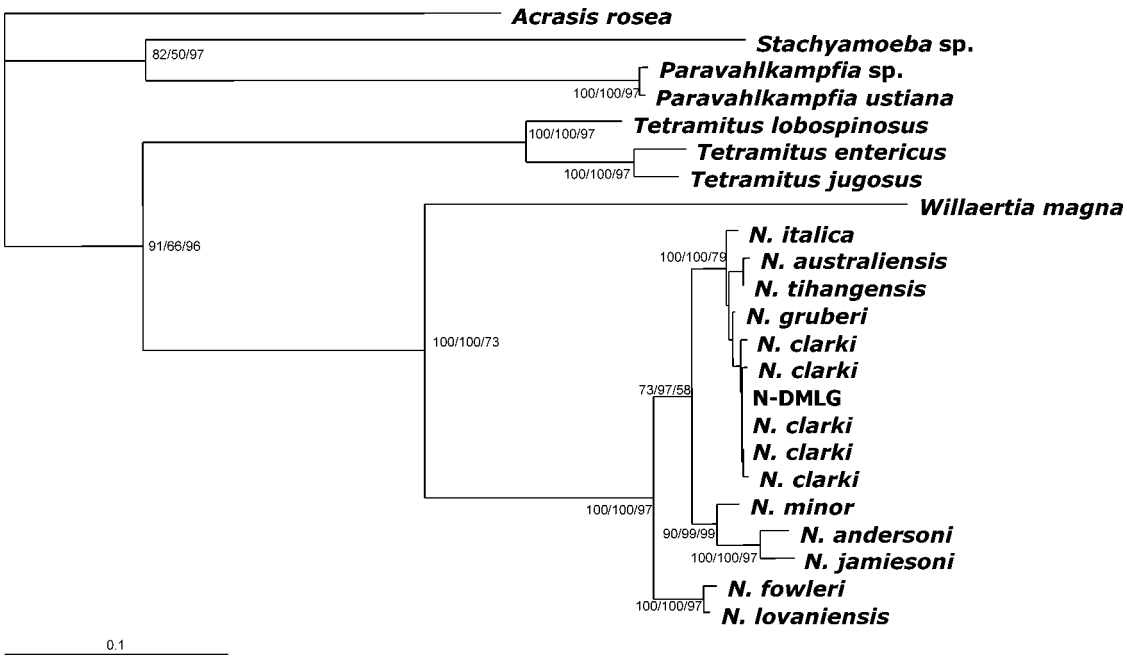


Fig. 4. 18S rDNA maximum likelihood analysis of the relationship of different vahlkampfiid amoebae using *Acrasis rosea* as outgroup. Bootstrap values are based on 100 replicates and are given at the nodes (ML/NJ/MP). The scale bar indicates the number of substitutions per site. Observe the clustering of *N. clarki* with *N. gruberi*, *N. italica*, *N. australiensis* and *N. tihangensis*. Strains and GenBank accession numbers: *Acrasis rosea* (strain T-235, AF011458), *N. andersoni* (strain PPMFB-6, U80057), *N. australiensis* (strain CB2B/I, AF338421), *N. clarki* (strain Pd72Z/I, AF338417; strain 4177/I, AF338418; strain 4564/IV, AF338419; strain 4709/I, AF338420; strain Pd56Z/I, AF338422), *N. fowleri* (strain MCM, U80059), *N. gruberi* (strain ATCC 30224, M18732), *N. italica* (strain AB-T-F3, U80060), *N. jamiesoni* (strain T56E, U80061), *N. lovaniensis* (strain C-0490, U80062), *N. minor* (strain WTO43, X93224), *N. tihangensis* (isolate TT, AY321362), *Paravahlkampfia* sp. (isolate li3_03, AJ550994), *P. ustiana* (strain CCAP 1588/6, AJ224890), *Stachyamoeba* sp. (strain ATCC 50324, AF011461), *Tetramitus entericus* (strain CCAP 1588/5, AJ224889), *T. jugosus* (strain ATCC 30703, M98050), *T. lobospinosus* (strain ATCC 30298, M98052), *Willaertia magna* (strain A1,2 PWCl1, AY266315)

DISCUSSION

A *Naegleria* strain isolated from a garden pond and harbouring two different populations of bacterial endocytobionts, one replicating within the cytoplasm, the other growing within the nucleus of the amoeba - together inhibiting the encystation of their host, was identified as *Naegleria clarki* De Jonckheere, 1994, by SSU rDNA sequencing.

Identification

Morphology alone is unfortunately not discriminative in the genus *Naegleria* (De Jonckheere 2002), and in this case the identification was especially intricate as these amoebae initially did not form cysts. All amoeboflagellates with plugged pores and with not dividing flagellate stages with two flagellae and without a cytostome have been assigned to the genus *Naegleria*. However, meanwhile it has become clear, that there are naegleriae that have no flagellates and there are also naegleriae that have dividing flagellates. Up to date more than 20 different species of *Naegleria* have been described (De Jonckheere 2002, 2004), whereby ribosomal DNA sequencing has contributed the main part of recent classification and identification of *Naegleria* isolates.

The SSU rDNA of strain N_DMLG was shown to be highly identical to 5 *N. clarki* strains isolated from organs of freshwater fish in the Czech Republic (Dykova *et al*. 2001) and as N_DMLG also clustered with these strains in phylogenetical analyses we identify our strain as *N. clarki*. The strains isolated from fish have not been described morphologically, so no light- and electron microscopical comparison to these strains was possible. The initially described *N. clarki* strains, strain RU30 and RU42, had been isolated from a spring in Rotorua (New Zealand). Also these two strains have a group I intron in their SSU rDNA (De Jonckheere 1993, 1994a), however, no entire SSU rDNA sequences are available at

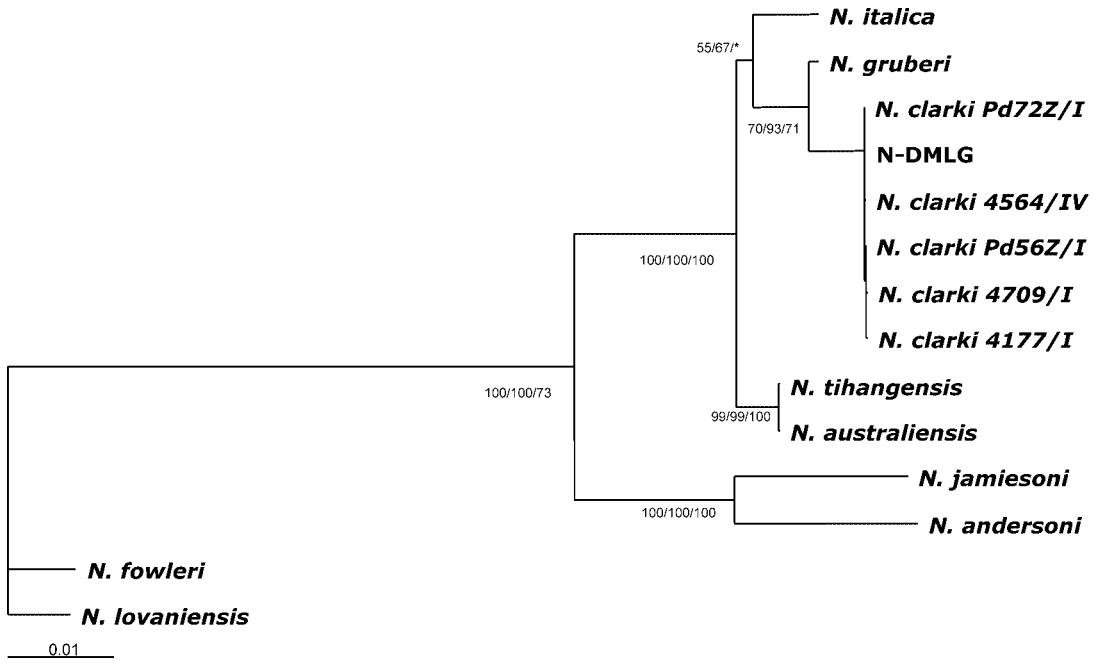


Fig. 5. 18S rDNA maximum likelihood cluster analysis of strain N_DMLG and different *Naegleria* spp. using *N. lovaniensis* and *N. fowleri* as outgroup. Strain N_DMLG clearly clusters with the *N. clarki* strains isolated from freshwater fish in the Czech Republic and *N. gruberi* is the closest relative of this group. Strains and GenBank accession numbers see legend of Figure 4.

GenBank, so no thorough molecular comparison was possible. Recently, De Jonckheere (2004) identified another *N. clarki* strain (strain BG6), but also for this strain no entire SSU rDNA sequence is available at GenBank.

The SSU rDNA of strain N_DMLG was shown to contain a 1305 bp group I intron and this intron was identical to the one Dykova *et al*. (2001) found in their 5 *N. clarki* strains and also to the group I intron of strain RU30 (De Jonckheere 1994a). Several species of *Naegleria* have group I introns in their SSU rDNA (De Jonckheere 1994a, De Jonckheere and Brown 1998). These group I introns of *Naegleria* are twintrons with two catalytic RNAs, which is rather unusual and only also found in *Didymium*, a myxogastrid mycetozoan (Einvik *et al*. 1998). De Jonckheere demonstrated that the *Naegleria* SSU rDNA group I intron was acquired in an ancestral state and lost in most *Naegleria* species (De Jonckheere and Brown 1994, De Jonckheere 2002).

Interesting is also the obviously broad temperature tolerance spectrum of the species *N. clarki*. The two New Zealandian *N. clarki* isolates came from a spring, the 5 Czech isolates from freshwater fish and our strain from a garden pond. The *N. clarki* strains isolated from fish all had a temperature limit below 37°C, while our isolate, although coming from a rather cold habitat exhibited temperature tolerance (growth at 37°C). Also the type strain of *N. clarki* RU30, as the majority of *N. clarki* strains, has an upper temperature limit of 37°C, however, the species *N. clarki* De Jonckheere, 1994 also includes a strain growing up to 40°C (De Jonckheere 2002). Griffin (1972) established temperature tolerance (at least 37°C) as a criterion for pathogenicity. All pathogenic *Naegleria* isolates are thermophilic, however, also several non-pathogenic species have been shown to be thermophilic, e.g. *N. lovaniensis*. Altogether, there are thermophilic isolates in 12 different *Naegleria* species (De Jonckheere 2002). Three species of *Naegleria* have been shown to kill mice after intranasal instillation, *N. fowleri*, *N. australiensis* and *N. italica*, although, until now, neither *N. australiensis* nor *N. italica* have been involved in human disease. The first two *N. clarki* strains, the ones from New Zealand, had initially been shown to be pathogenic for mice, but in later pathogenicity tests they did not kill mice after intranasal instillation (Willaert 1976) and the whole species was thus considered as

non-pathogenic. However, these strains had been isolated long before and only later assigned to this genus and it is known that at least *N. fowleri* strains tend to loose their pathogenicity after long term culture (Wong *et al*. 1977).

Cluster analysis

Strain N_DMLG clearly clustered with the *N. clarki* strains isolated from freshwater fish in the Czech Republic and the *N. clarki* strains altogether formed a cluster with *N. gruberi, N. italica, N. australiensis* and *N. tihangensis*. *N. tihangensis* was newly erected out of several *N. gruberi* strains (see below). *N. australiensis* was described in 1981 (De Jonckheere 1981). It grows up to 42°C and has no intron. The type strain had been isolated from water in Australia, but the species is meanwhile considered to occur all over the world. Recently, *N. australiensis* has also been isolated from the brain of fishes in the Czech Republic (Dykova *et al*. 2001). *N. italica* De Jonckheere, Pernin, Scaglia *et* Michel, 1984, was initially described as a subspecies of *N. australiensis*. Later it was found that the differences justify an own species name for these amoebae (Adams *et al*. 1989, De Jonckheere 1994b) and also in our analysis it rather grouped with *N. gruberi* and *N. clarki* than with *N. australiensis*, although this clustering was not supported by really high bootstrap values. *N. italica* has in contrast to *N. australiensis* a group I intron in its SSU rDNA (De Jonckheere 1993) and is also more virulent than *N. australiensis* (De Jonckheere 2002). *N. italica* has long been thought to occur only in Italy, however, recently two strains of *N. italica* have been isolated in Australia with an sequence identity of 100% in the 5.8S rRNA gene and the flanking ITS (Henderson *et al*. 2001, Robinson *et al*. 2004). Interestingly, the Australian isolate seems to be non-pathogenic to mice after intranasal instillation. While *N. lovaniensis* and *N. australiensis* are considered to be ubiquitously distributed (De Jonckheere 2004), it has not yet been clarified, if this is true for all *Naegleria* strains. A major problem is that from many strains only so few isolates have been investigated that no final decision can be drawn on this.

N. gruberi has long been a most heterogenous species, which was to a large extent also due to the fact, that before 1970, when the species *N. fowleri* was established (Carter 1970) for the pathogenic isolates, all *Naegleria* isolates were assigned to this in those days only described species. *N. gruberi* was first described in 1899 (Schardinger 1899) and is therefore the first described *Naegleria* species, although it was initially described as *Amoeba gruberi* and only later assigned to the newly established genus *Naegleria* Alexeieff, 1912. Today *N. gruberi* is considered to represent a complex of at least 10 species (Clark *et al*. 1989, De Jonckheere 2002), it has been divided into four clusters on the basis of rDNA sequence analyses (Clark *et al*. 1989) and these clusters were later given the species status (*N. gruberi* sensu stricto, *N. pringsheimi, N. pagei* and *N. tihangensis*, whereby *N. tihangensis* was then considered a subgroup of *N. australiensis* and is now considered the sister species to *N. australiensis*) (De Jonckheere 2002). Also the genus Vahlkampfia has been reevaluated and the species have now been divided between the four genera *Tetramitus, Vahlkampfia, Neovahlkampfia* and *Paravahlkampfia* (Brown and De Jonckheere 1999).

Endosymbionts in *Naegleria*

Our isolate was the first *N. clarki* shown to harbour bacteria and, moreover, the first *Naegleria* strain harbouring two different bacterial populations at the same time, one in the cytoplasm and one in the nucleus. At another occasion a similar coccoid Gram negative bacterium ("Knic") had been shown to replicate within the cytoplasm of a *Naegleria* sp. isolated from an aquarium (Michel *et al*. 1998). This host-amoeba was lost unfortunately whereas the endoparasites could be transferred and maintained on other *Naegleria* strains (Michel *et al*. 2000). They have a more smooth outline compared to "Pc" and look like little golf balls seen with SEM. The taxonomic position of both the endocytobionts of the present paper as well as of "Knic" could not yet be identified unequivocally. Endoparasites in the nucleus are altogether very rare. Sabaneeva *et al*. (2002) reported on intranuclear bacteria in *Paramecium*.

In summary, it was shown that *N. clarki* can harbour living bacteria, and thus can act as vehicle for bacteria. Moreover, the results of our study corroborate the close relationship between *N. clarki* and the potentially pathogenic *N. italica, N. australiensis* and *N. tihangensis* and demonstrate that also *N. clarki* shows physiological properties related to pathogenicity.

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The Life Cycle and Pathogenicity of *Eimeria fulva* Farr, 1953 in Domestic Goslings

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Summary. The life cycle and pathogenicity of *Eimeria fulva* were studied. Eighteen, 10-day-old, artificially-reared coccidia-free goslings (*Anser cygnoides* var. *domestica*) were inoculated orally with 7.0×10^5 -1.5×10^6 sporulated oocysts of *Eimeria fulva* and killed at intervals from 24 to 288 h post-inoculation (PI). Parts of the visceral organs including intestines, kidney, liver, gallbladder, and spleen from inoculated goslings were fixed, sectioned, and observed microscopically. The results revealed that at least 2 generations of meronts occurred in the life cycle of *E. fulva*. The first generation completed maturation at about 72 h PI, and the second at about 144 h PI. Each meront contained about 15 merozoites. Development of gamonts began at about 144 h PI. The prepatent period was 7.5 days and patency continued for 2.5 days. Sporulation of oocysts occurred in 60-84 h at 25°C. Developmental stages were present in the epithelial cells of the villi and crypts, and in the lamina propria of the whole intestine and cloaca, resulting in local desquamation and necrosis of the epithelium (including crypt epithelium), edema, hemorrhages, and infiltration of inflammatory cells. Histological lesions were pronounced in the jejunum and the ileum. The infected goslings mainly showed diarrhoea, slight bloody feces, but no death. The results suggest that *E. fulva* may be mildly pathogenic for goslings.

Key words: coccidium, *Eimeria fulva*, gosling, life cycle, pathogenicity.

INTRODUCTION

Coccidiosis is an important protozoan disease of domestic animals. The disease is manifested by severe symptoms such as diarrhea, dehydration, and deaths in young animals (Levine 1985). Although coccidia are among the most common parasites of geese worldwide, coccidiosis in geese is not generally known as a severe problem (Soulsby 1982, Gajadhar *et al*. 1983). Nevertheless, there are numerous case reports where extensive morbidity and mortality have occurred in both domestic and wild flocks in some areas, especially in Europe, where domestic geese are of great economic importance (Gajadhar *et al*. 1983). In China, goose coccidiosis has been paid little attention for a long time because of its little economic significance, and relevant reports are few. With changes in agricultural practices in recent years, however, geese are now raised on a larger scale. As a result, coccidiosis has occurred in many flocks in many areas, resulting in considerable losses (Dai *et al*. 2004).

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Since the first coccidium *E. truncatum* (then named *Coccidium truncatum*) was observed by Railliet and Lucet in 1890 in the domestic goose (*Anser anser domesticus*) in France, 17 species of the family Eimeriidae, belonging to 3 genera have been described and named from a variety of goose hosts in many areas of the world, primarily identified on the basis of oocyst morphology though the validity of some remains in doubt (Railliet and Lucet 1890, Gajadhar *et al*. 1983). Most species, except *E. truncata*, which is known to occur in kidney, are found in the intestines of geese (Gajadhar *et al*. 1982, 1983). Experimental studies, particularly those with single species infections, have rarely been reported, and the life cycles and pathogenicity of many species are yet to be resolved.

Eimeria fulva Farr, 1953 was first reported from the eastern Canada goose (*Branta canadensis canadensis*) and experimentally transmitted to the domestic goose (Farr 1953). Later, it was found in the lesser snow goose (*A. caerulescens caerulescens*), Aleutian Canada geese (*B. canadensis leucopareia*), and the domestic geese (*A. a. domesticus* in Europe and *A. cygnoides* var. *domestica* in China) (Hanson *et al*. 1957, Greiner *et al*. 1981, Arslan *et al*. 2002, Xie *et al*. 1988, Tao *et al*. 2003, Dai *et al*. 2004). The domestic goose was believed to be the experimental host of *E. fulva*, however, it was found widely in domestic geese with relatively high prevalence (Arslan *et al*. 2002, Tao *et al*. 2003, Dai *et al*. 2004). It appears that the host specificity of *E. fulva* (as well as *E. hermani*) is not so strict as researchers expect (Hanson *et al*. 1957, Gajadhar *et al*. 1983), and that the domestic goose may be the natural host of *E. fulva* (Dai *et al*. 2004). Gajadhar *et al*. (1983) reviewed the life cycle and pathogenicity of *E. fulva*; however, detailed information on them is inadequate until the present study.

MATERIALS AND METHODS

Oocysts. The pure cultures of *E. fulva* were produced in our previous experiment (Dai *et al*. 2004). Briefly, 160 sporulated oocysts of *E. fulva* were picked out under the microscope from a sample obtained in an experimental infection in which this species predominated. Eighty oocysts each were inoculated into 2, coccidia-free, 10-day-old goslings. The feces from both birds were collected during the period of oocyst release (9-10 days post-inoculation [PI]). Oocysts in feces were harvested by screening, sedimentation, flotation in saturated salt solution, and washings. They were then placed in 2% potassium dichromate and incubated at 25°C for 4 days to permit oocysts to sporulate. Sporulated oocysts were examined repeatedly under light microscopy to ensure its purity. Two other coccidia-free goslings were each inoculated with 5000 sporulated oocysts obtained from the previously inoculated birds to produce adequate numbers of oocysts for the present study. Sporulated oocysts were stored at 4°C for about 2 months prior to use.

Experimental birds. Domestic geese (*A. cygnoides* var. *domestica*) were incubator hatched and obtained when 1-day-old. All birds were reared individually in wire cages in isolation rooms and provided with a non-medicated ration and water *ad libutum*. Fecal samples from those birds were collected daily and examined for the presence of oocysts by fecal flotation technique before inoculations were performed. Only coccidia-free birds were used in this study. At 10 days of age, 18 goslings were orally inoculated with 7.0×10^5-1.5×10^6 sporulated oocysts of *E. fulva* and killed with an overdose of pentobarbitone at 24 to 288 h PI (Table 1). Considering the parasites in tissues of infected birds might be few and difficult to find at the early stage of infection, those goslings killed early were inoculated with the higher doses (1.2×10^6-1.5×10^6 oocysts). During the experiments, the clinical signs and gross lesions of infected goslings were observed and recorded in detail.

Collection and preparation of tissues. Immediately after the goslings were killed, intestine, kidney, liver, gallbladder, and spleen were collected and fixed in 10% phosphate buffered formalin (pH7.2). For the intestine, beginning at the pylorus, 3 samples from the duodenum, 4-5 at 15 cm intervals from the jejunum, 2 from the ileum, 3 from the caecum, 2 from the rectum, and 1 from the cloaca were taken. Tissues were embedded in paraffin wax and later sectioned at 4 ìm, then stained with hematoxylin and eosin (H. E). Digital photographs were taken by Cell Image System (CIS-1000, Shanghai Tanon Science & Technology Co., Ltd., Shanghai, China).

Examination and sporulation of oocysts. To determine the prepatent and patent periods, feces from some infected goslings were collected twice daily and examined for the presence of oocysts by flotation using saturated salt solution. When oocysts were present, they were harvested by preparing a slurry of feces and water that was passed through a 400 µm nylon sieve and washed with water twice after centrifugation. Subsequently, oocysts were placed in 2% potassium dichromate in covered Petri dishes at 25°C, and observed 4 times daily until most had completed sporulation.

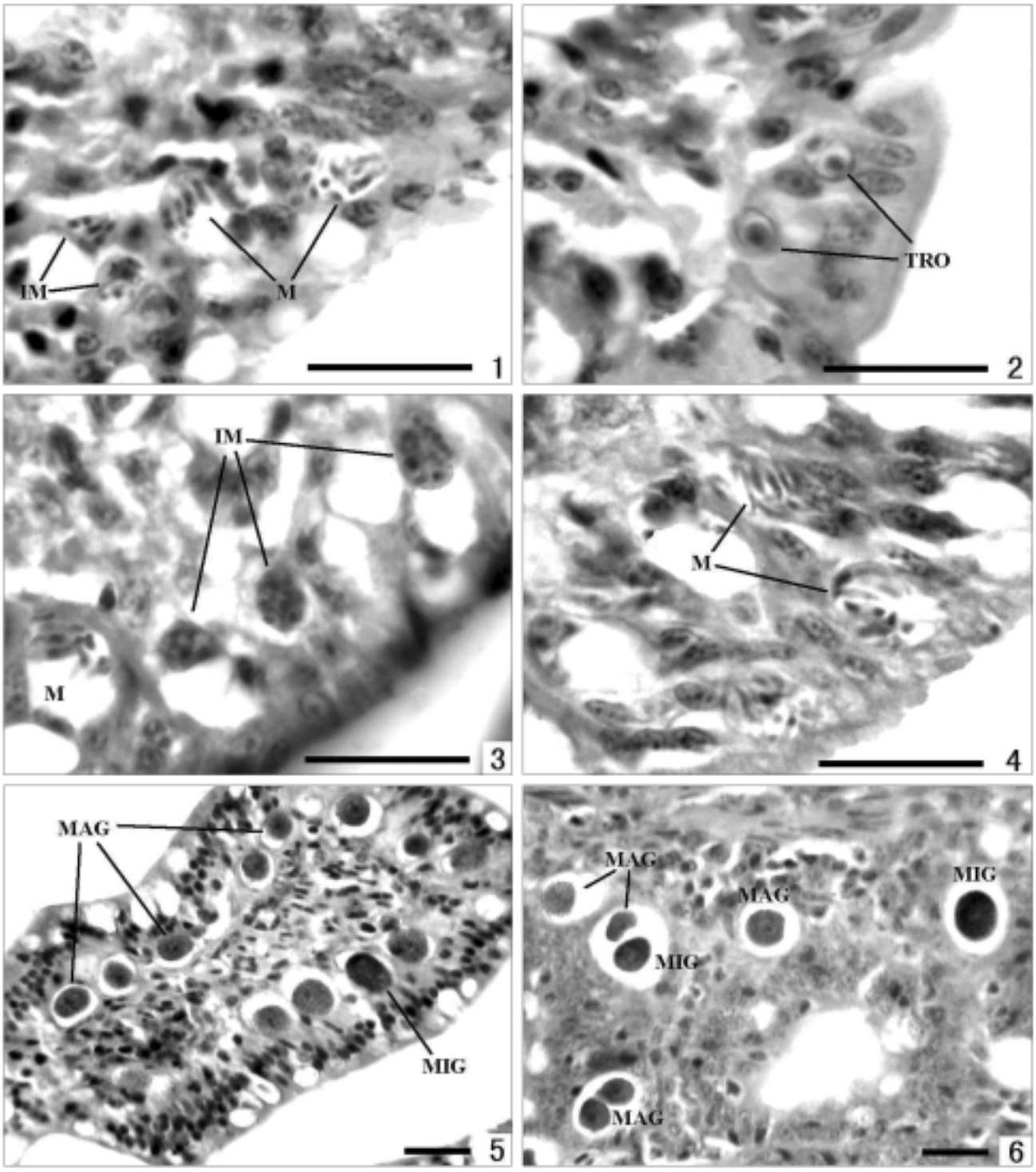
RESULTS

Clinical signs

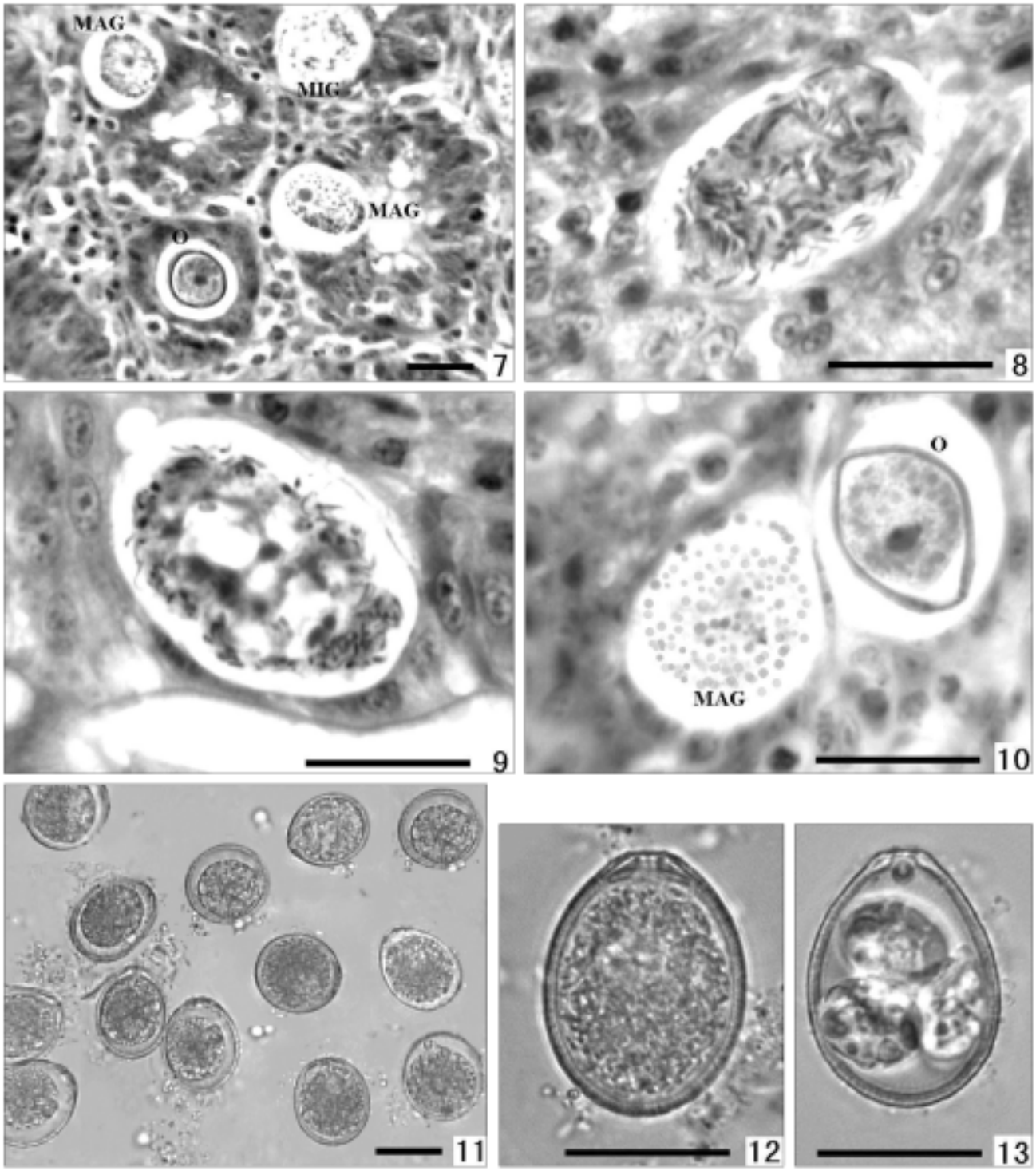
All unkilled birds began to show diarrhea at day 4 PI, and the diarrhea continued through day 9 PI. During this period, the feces generally contained a small amount of necrotic debris, mucus, and blood, and the infected birds remained normal in appetite and vigor. Microscopically, a large number of lipid-like drops were found in the feces. Beginning at day 10 PI, the feces gradually returned to normal.

Life cycle

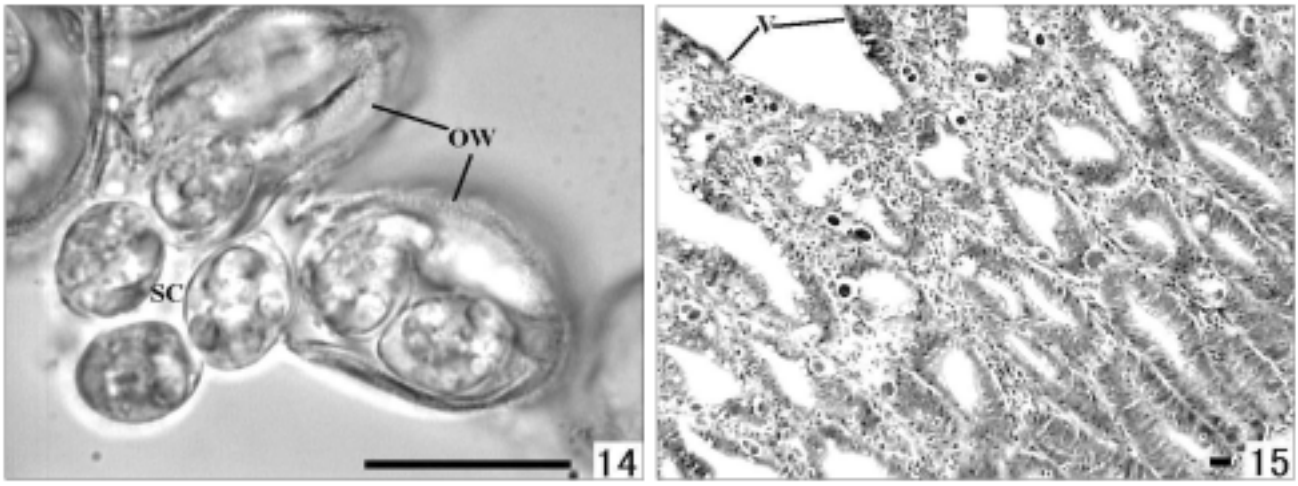
The various stages in the life cycle of *E. fulva* found in infected goslings were showed in Table 1 and fully detailed as follows.



Figs 1-6. *Eimeria fulva*. **1** - immature and mature meronts in the epithelial cells of a villus of the jejunum at 72 h post-inoculation (PI); **2** - trophozoites in the epithelial cells of a villus of the ileum at 72 h PI; **3** - immature and mature meronts in the epithelial cells of a villus of the jejunum at 96 h PI; **4** - mature meronts in the epithelial cells of a villus and in the lamina propria of the ileum at 120 h PI; **5** - young macrogamonts and microgamonts in the epithelial cells of a villus of the ileum at 168 h PI; **6** - young macrogamonts and microgamonts in the crypts and the lamina propria of the jejunum at 192 h PI. IM - immature meront, M - mature meront, MAG - macrogamont, MIG - microgamont, TRO - trophozoite. Scale bars 20 µm.



Figs 7-13. *Eimeria fulva.* **7** - mature macrogamonts, microgamont, and oocyst in the crypts and the lamina propria of the rectum at 216 h post-inoculation (PI); **8** - mature microgamont at 216 h PI with numerous fully developed microgametes; **9** - mature microgamont at 216 h PI with microgametes migrated to its periphery; **10** - mature macrogamont and oocyst in the lamina propria of the caecum at 216 PI; **11** - fresh oocysts isolated from the feces at 204 h PI; **12** - an amplified unsporulated oocyst; **13** - sporulated oocyst. MAG - macrogamont, MIG - microgamont. O - oocyst. Scale bars 20 µm.



Figs 14-15. 14 - *Eimeria fulva* crushed sporulated oocysts. Pitted oocyst walls and sporocytes are showed; **15** - gamonts at various stages in the epithelial cells of the villi and crypts, and in the lamina propria of the ileum at 168 h post-inoculation. Note that no endogenous stages occur in the crypts in the deeper region of mucosa. OW - oocyst wall, SC - sporocyte, V - villus. Scale bars 20 µm.

Meronts and merozoites. Meronts mainly occurred in epithelial cells of the villi and lamina propria of the jejunum, ileum, basis ceci, and rectum, occasionally in the lower duodenum. At 24 h PI, some sporozoites were found in the epithelial cells of villi of the jejunum and ileum. Thereafter, the meronts of different developmental stages were observed at 48-144 h PI (Table 1). Trophozoites, and meronts containing 2-4 nuclei, were found in the epithelial cells of villi and lamina propria at 48 h PI, apparently arising from the sporozoites. Immature and mature meronts, as well as trophozoites, were present at 72 h PI (Figs 1, 2), and both immature and mature meronts at 96 and 120 h (Figs 3, 4). At 144 h PI, only few mature meronts were present; most were trophozoites.

Immature meronts contained 2 to 20 or more nuclei, depending on their developmental degree (Figs 1, 3). Mature meronts were approximately 10 µm in diameter and contained variable numbers of fully developed merozoites (Figs 1, 3, 4). In histological sections, no obvious differences were found among meronts of various generations. Mature meronts contained 8-28 merozoites ($\overline{X}$ =14.92 ± 5.41 SD, n=50).

Fully developed merozoites were slightly curved, with one end rounded and the other end bluntly pointed (Fig. 4). They were approximately 5.0-6.0 µm long and 1.0-1.5 µm wide. Their spherical nuclei were at the wide end of the merozoites. There appeared to be a small amount of undifferentiated residuum left after formation of merozoites in some meronts. Many merozoites were noted in the process of rounding up and becoming trophozoites; apparently, these parasites had just penetrated epithelial cells of villi and crypts (Fig. 2). Trophozoites were located at the base of cytoplasm of host cells and below the nuclei. They contained central nuclei and were surrounded by a clear area.

Gamonts and gametes. Gamonts occurred in the epithelial cells of villi and crypts and in the lamina propria of whole intestines, and cloaca. Development of gamonts apparently began at about 144 PI, but macrogamont and microgamont were undistinguishable morphologically at that time, since both of them developed from a trophozoite, and were in their very early stages. At 168 h PI, young macrogamonts and microgamonts were clearly visible, and gamonts of various stages were present until 240 h PI (Table 1). The macrogamonts were more numerous than the microgamonts.

During the development of macrogamonts, the sizes increased gradually and the color of the cytoplasm paled. Meanwhile, eosinophilic, plastic granules (wall-forming bodies) in their cytoplasm increased in numbers as development progressed. In young macrogamonts, these granules were evenly scattered throughout the cytoplasm (Figs 5, 6); in mature macrogamonts that may have been fertilized those larger, deeply stained granules were in close contact with each other at the periphery of the parasites; others remained dispersed in cytoplasm (Fig. 7). Each of macrogamonts contained a nucleus with a sharply delineated nucleolus in its center.

Table 1. Developmental stages of *E. fulva* found in experimental goslings.

Hours post-inoculation	Gosling No.	Oocysts inoculated ($\times 10^5$)	Developmental stages found
24	B_1 0374	15.0	Some sporozoites in the epithelial cells of villi of the jejunum and ileum.
48	B_1 0376 B_1 0325	12.0 15.0	Trophozoites and meronts containing 2-4 nuclei in the epithelial cells of villi of the jejunum and ileum.
72	B_1 0377	8.0	Meronts at various stages and trophozoites present. Mature meronts and trophozoites predominated. Meronts in the same sites as those at 48 h post-inoculation (PI). Trophozoites in the epithelial cells of villi and lamina propria from middle duodenum to rectum (including basis ceci).
96	B_1 0378 B_1 0324	8.0 7.0	All endogenous stages as at 72 h PI. They were in the same sites as trophozoites at 72 h PI.
120	B_1 0346 B_1 0330	7.0	All endogenous stages as at 96 h PI.
144	B_1 0365	10.0	Few mature meronts in the same sites as the endogenous stages at 96 h PI, and large numbers of trophozoites in the whole intestine and cloaca.
168	B_1 0366	7.0	Trophozoites and young gamonts in the same sites as the trophozoites at 144 h PI.
192	B_1 0364 B_1 0329	10.0 8.0	Large numbers of gamonts at various stages and oocysts in the same sites as the endogenous stages at 168 h PI. A few oocysts shed in the feces.
216	B_1 0379	7.0	All endogenous stages as at 192 h PI. Great numbers of oocysts shed in the feces.
228	B_1 0371 B_1 0324	7.0 8.0	Few mature gamonts and oocysts present. Small numbers of oocysts shed in the feces.
240	B_1. 394	7.0	Very few endogenous stages as at 228 h PI. Few oocysts shed in the feces.
264	B_1. 391	7.0	No endogenous stages present, and no oocysts were detected in the feces.
288	B_1 0328	8.0	Same as at 264 h PI.

Different developmental stages of microgamonts were seen. They contained nuclei stained intensively and uniformly dark blue with hematoxylin. The number of nuclei varied with the developmental degree of the microgamonts. Very young microgamonts were small and contained only a few nuclei. Young microgamonts were in the shape of the host cell, and contained many nuclei (Figs 5, 6). Mature microgamonts contained large numbers of microgametes (Figs 7-9); in some, the microgametes migrated to the periphery of the parasite, with some apparent residual material remaining in the center (Fig. 9). The fully developed microgametes were slender and crescentic or comma-shaped; they measured 4.5-5.5 μm long and about 0.5 μm wide (Figs 8, 9).

Oocysts. Oocysts were found in the same sites as gamonts. The process of fertilization was not observed

in the present study. Young oocysts were found in sections as early as 192 h PI. They possessed a well developed wall and finely granular cytoplasm evenly distributed over the whole oocyst; each contained a nucleus with a central nucleolus (Figs 7, 10). The oocysts differed from the mature macrogamonts in that the eosinophilic, plastic granules at the periphery of the latter had coalesced to form the oocyst wall.

Shedding of oocysts in the feces began at 192 h PI, and continued until 240 h PI (Table 1). The number of oocysts in the feces reached its peak at 204 and 216 h PI, and sharply decreased over the next 24 h. All fresh oocysts (Figs 11, 12) had a bumpy surface and a prominent micropyle; they were filled with the granular cytoplasm (sporont). In 2.5% potassium dichromate solution, oocysts underwent sporogony and were fully sporulated at 25°C after 60-84 h. Sporulated oocysts (Figs 13, 14) were characteristic of *E. fulva*. They were broadly ovoid and slightly flattened at the narrower pole. One hundred oocysts measured 29.79 ± 0.99 (28.0-31.5) × 24.21 ± 1.15 (20.2-25.2) μm with a shape index (length: width ratio) of 1.23 ± 0.08 (1.15-1.38). The bilayered wall was 2.0-2.5 μm thick. The outer layer was brownish yellow, pitted, and transversely striated; the inner layer was smooth and colorless, and expanded around the micropyle. The micropyle was 2.0-3.0 μm wide, and lacked a cap. There was a large refractile polar granule near the micropyle. No oocyst residuum was present. One hundred and twenty sporocysts (Fig. 14) measured 14.22 ± 0.73 (12.5-15.0) × 10.08 ± 0.25 (10.0-11.0) μm with a shape index of 1.41 ± 0.09 (1.25-1.5). Each sporocyst had a prominent Stieda body, and contained a large, coarsely granular residuum and two folded sporozoites.

Pathological changes

Gross autopsy findings. At necropsy, no obvious gross lesions were observed in organs of the goslings at 24 and 48 h. At 72 to 216 h PI, infected birds had watery intestinal contents containing parasites of different stages, and a small amount of blood, and necrotic debris; the intestinal walls were slightly thickened, with some small hemorrhagic points in the mucosa.

Histopathological changes. Examination of histological sections revealed that the meronts mainly developed in epithelial cells of the upper two-thirds of villi, occasionally in those of the bases of villi. Before 72 h PI, meronts were found only in the jejunum and ileum (Table 1), and they were relatively few in number and small in size, no other obvious lesions were present except for the degeneration and necrosis of the infected cells. With further development of the parasite, its numbers increased and the affected region progressively extended upwards and downwards. Until gamogony, the developmental stages were present in the whole intestine (including caecum) and cloaca, but the majority were noted in the jejunum and ileum (Table 1). Gamonts occurred principally in the epithelial cells of villi and in the lamina propria, as well as in the upper regions of crypts (Figs 5-7, 15), except in the duodenum where they were present within epithelial cells only from the middle to tips of the villi. The histological lesions began to manifest progressively at 72 h PI, and were pronounced in the jejunum and ileum during the gamogony and oocyst shedding. Desquamation and necrosis of epithelium of intestine and crypts, infiltration of inflammatory cells, and slight hemorrhage and mucosal edema were usually associated with aggregates of endogenous stages, whereas the tissue structures of intestine generally remained normal. Beginning at 264 h PI, no endogenous stages were present, and only local chronic enteritis was present in some affected areas.

No endogenous stages or histological lesions were found in other tissues.

DISCUSSION

In all coccidia described from geese, four species, *E. fulva*, *E. magnalabia*, *E. striata* and *E. stigmosa*, have sculptured oocyst walls which are pitted and appear striated. *E. fulva* markedly differs from other 3 species in its much larger oocysts and longer prepatent period (Farr 1953, Gajadhar *et al*. 1983, Dai *et al*. 2004). In our previous study (Dai *et al*. 2004), four, 12-day-old coccidia-free goslings were inoculated with oocysts of *E. anseris*, *E. fulva*, *E. hermani*, *E. nocens*, *E. stigmosa* and *Tyzzeria parvula*, and their feces were collected and examined for the presence of oocysts every 12 h after inoculation. When oocysts were present, they were isolated and then identified. At 204 and 216 h PI, the oocyst shedding of *E. fulva* reached its peak, and that of *E. stigmosa* (its prepatent and patent period were 108 h and 60 h, respectively) had ceased. During this period, *E. fulva* oocysts predominated and no *E. stigmosa* oocysts, which might confuse with *E. fulva* oocysts, were present in the fecal samples. It was easy to pick out *E. fulva* oocysts from these samples under light microscope; misidentification was unlikely. Furthermore, the purity of oocysts was repeatedly checked in the

process of oocyst preparation. So we ensure the inoculum used in this study was of a single species.

Like other *Eimeria* spp., *E. fulva* underwent 3 developmental stages, merogony, gamogony, and sporogony, in its life cycle. The observations in the present study showed that first generation meronts matured at about 72 h PI, and the meronts at various stages were present up to 144 h. Gamonts began to develop at about 144 h PI. Accordingly, it was estimated that at least 2 generations of meronts developed in the life cycle of *E. fulva*. There appeared to be no obvious differences among meronts of various generations. Gajadhar *et al.* (1983) described that both meronts and gamonts of *E. fulva* were present in the epithelial cells of tips and sides of villi and in the lamina propria, as well as in the necks and fundi of the intestinal glands at 169 h PI, and that endogenous stages of *E. fulva* require 9 days to complete the life cycle. In the present study, only few meronts were found at 144 h PI, and by 168 h PI no meronts were present. At 192 h PI, oocysts could be detected in the feces, so the prepatent period was about 7.5 days.

Several goose coccidia, *E. hermani*, *E. kotlani*, *E. nocens*, *E. stigmosa* and *Tyzzeria parvula* have been reported to develop intranuclearly (Antukhayev 1976, Ponizovskii and Shibalova 1978, Skeene and Fernando 1978, Shibalova and Morozova 1979, Gajadhar *et al*. 1986). In this study, both meronts and gamonts of *E. fulva* were found to develop beneath the nuclei of host cells. The endogenous development of *E. fulva* appeared to be relatively brief. The prepapent period was about 7.5 days, and all oocysts were released within about 60 h. The short patent period could be explained by the closely synchronous development of endogenous stages.

Severe illness and death of 1- to 2-week-old goslings have resulted from mixed infections with multi-species of coccidia (Randall and Norton 1973, Arslan *et al*. 2002, Dai *et al*. 2004). Severe infections of *E. fulva* in both domestic and Canada geese resulted in a thickening and congestion of the intestinal wall and an accumulation of greenish mucus in the intestinal lumen (Farr 1953, Gajadhar *et al*. 1983). Arslan *et al*. (2002) described the macroscopic and microscopic lesions in intestines and kidneys of goslings that died of severe diarrhea. These birds might be infected with multi-species of coccidia including the renal coccidium *E. truncata*. They had edematous and hyperemic intestinal mucosae, and the intestinal content was watery and brownish red. Hyperemic vessels, epithelial desquamates, and endogenous stages (meronts, gamonts) of coccidia were seen in lamina propria and epithelial cells of the intestinal villi. The kidneys revealed tubular nephrosis and tubular necrosis, and endogenous developing forms of *E. truncata* were also seen in the epithelium of the renal tubules. The results of this study showed that *E. fulva* could invade the whole intestine and cloaca, and cause intestinal lesions similar to those mentioned above. Marked histological changes were present during the gamogony and oocyst shedding, and most pronounced in the jejunum and ileum. However, *E. fulva* appeared to be mildly pathogenic for goslings based on the obtained data. The inoculating doses of 7.0×10^5-1.0×10^6 oocysts could cause diarrhea, slightly bloody feces, but no death. Histological examination showed that of *E. fulva* mainly developed in the superficial regions of intestinal mucosa, and caused less severe lesions than those in the deeper regions.

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Free-living Heterotrophic Flagellates from Freshwater Sites in Tasmania (Australia), a Field Survey

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Summary. In order to contribute to an understanding of the geographic distribution of free-living freshwater flagellates, we have investigated the diversity of heterotrophic flagellates occurring in a number of freshwater sites in Tasmania (Australia). This community of organisms has not previously been studied in Tasmania, and the isolation of the freshwater habitats from like habitats elsewhere in the world increases the probability of the emergence of an endemic community. Forty-four species are described with uninterpreted records based on light-microscopy of living cells in natural communities. The records include an account of one new species: *Ploeotia tasmanica* sp. n. Of the 44 species (excluding unidentified taxa), 23 species are new to Australian freshwater sites but the majority of the species encountered here have also been found at other locations worldwide including Australian marine sites. The new records for Australian freshwater sites are: *Adriamonas peritocrescens, Ancyromonas sigmoides, Astasia* cfr. *gomphonema, Bodo saltans, Ciliophrys infusionum, Colpodella vorax, Goniomonas truncata, Gyromonas ambulans, Heteromita globosa, Heteronema exaratum, H. globuliferum, Microcometes paludosa, Neobodo designis, N. saliens, Parabodo caudatus, Petalomonas poosilla, Ploeotia tasmanica* sp. n., *Protaspis obliqua, P. simplex, Reclinomonas americana, Rhynchomonas nasuta, Trepomonas agilis, Urceolus cyclostomus.* The relative lack of novelty provides little support for the existence of endemic biota among this group of organisms, as has been shown to also be the case with marine species. This suggests that contiguity of habitats has not been a major determinant of the distribution of heterotrophic flagellates.

Key words: Australia, biogeography, cosmopolitanism, endemism, heterotrophic flagellates, *Ploeotia tasmanica* sp. n., Protista, Tasmania.

INTRODUCTION

The significance of free-living heterotrophic flagellates as consumer of small organisms and prey for ciliates and copepods in aquatic ecosystems, and at least in some benthic ecosystems has now been broadly accepted (e.g. Azam *et al*. 1983, Arndt *et al*. 2000, Lee and Patterson 2002). However, this acceptance has emerged in the absence of a broad understanding of the natural history of the organisms concerned. In attempts to redress this, we are seeking to develop a genus-level overview of these organisms (Patterson and Larsen, 1991) and an understanding of their geographic distribution in marine sites (Larsen and Patterson 1990; Vørs 1992a, b, c, 1993a, b; Vørs *et al.* 1995; Patterson *et al*. 1993; Ekebom *et al*. 1996; Patterson and Simpson 1996; Tong 1997a, b, c; Tong *et al*. 1997, 1998; Lee and Patterson 1998, 2000; Bernard *et al*. 2000; Al-Qassab *et al*. 2002; Lee 2002; Lee *et al*. 2003). To date, we have not been able to detect any major biogeographic trends (Lee and Patterson 1998, Patterson and Lee 2000, Lee

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et al. 2003) for flagellates in marine sites. This may be attributable, at least in part, to the contiguous nature of marine environments. The aim of the present paper is to extend the biogeographic study of free-living heterotrophic flagellates to freshwater environments. Since these are temporally and spatially isolated habitats, their inhabitants may be more prone to diversifying influences and thus biogeographic trends are expected. The hypothesis that we address is that the geographical location of discontinuous environments does not influence the species that occur in communities of heterotrophic flagellates.

The existing literature on the identity and taxonomy of heterotrophic flagellates from freshwater sites in Australia is impoverished, and appears to be restricted to Schewiakoff (1893), Playfair (1914, 1921), Gillies (1915), Stickland (1898, 1924), Stickland and Stickland (1895), Skvortzov and Noda (1971, 1972), Croome and Tyler (1985, 1986, 1988a, b), Croome *et al*. (1987), Dürrschmidt and Croome (1985), Foissner *et al*. (1988), Robinson *et al*. (1989) and Schroeckh *et al*. (2003). There is as yet no synthesis of this work, although the literature on the heterotrophic members of lineages traditionally treated as "algae" and occurring in Australia is summarised in Day *et al*. (1995). The generic identities of free-living heterotrophic flagellates are covered by Patterson and Larsen (1991), and the following items are useful guides to many species descriptions: Stein (1878), Kent (1880-1882), Klebs (1893), Lemmermann (1910, 1913, 1914), Skuja (1939, 1948, 1956, 1964), Huber-Pestalozzi (1955), Hänel (1979). There are not many detailed studies documenting the species diversity of free-living benthic heterotrophic flagellates in a single area of freshwater sites, but many from marine sites. We present here a detailed study of heterotrophic flagellates from Tasmanian freshwater sites as a future reference source for diversity studies.

We adopt a standard methodology in order to make comparisons with our previous studies (Lee and Patterson 1998) possible. These studies emphasize a study of living cells from natural communities on site. This ensures that we recover information on a large number of active species. It precludes detailed studies that might be based on cultures or preserved materials, but such studies can only be conducted on a small subset of the taxa. Studies limited to subsets of taxa present cannot be compared in order to address biogeographic questions (Lee and Patterson 1998, Patterson and Lee 2000).

MATERIALS AND METHODS

Sampling sites. Samples of vegetation, water and sediment from 7 sites were collected in April 1996. The sampling sites are (1) Bothwell site - a slow moving stream with floating duckweed and *Azolla*, located north of Bothwell; (2) Hollowtree site - a small pond with dense growth of charalean algae, located north of Hollowtree; (3) Meadowbank site - dead and decaying reeds and other plant material from the shores of the Derwent River; (4) Carlisle beach site- a small pond with cladophoralean algae near ocean, South of Dunalley; (5) Carlton River site - a small stream with rich diatomaceous slime, west of Copping; (6) Hobart University site - a small lily pond located on Hobart University campus; (7) Ladies Tarn site - a mountain lake of Hartz mountains.

Sample collection and process. The samples were processed as described by Larsen and Patterson (1990) in that surface sediment was collected, larger macrofauna and plant material were usually removed by sieving, the residue placed in layers 1 cm deep in trays, allowed to settle for several hours, excess water drained off, and the material was covered with a sheet of lens tissue upon which 22×22 mm No.1 coverslips were placed. Flagellates tended to concentrate under the coverslips after 12-72 h. The coverslips were then removed and the associated flagellates were observed using a Zeiss (Axioplan) and Leica (DMR) microscopes equipped with photographic facilities. The flagellates were also recorded on U-MATIC video tapes and records were also made with video prints. Specimens were also drawn. The samples were maintained at room temperature (~20°C) for 5 days. This method is not designed to obtain all species that are present or to reflect the relative abundance of species. The technique is designed for consistency between different sampling sites. Original samples were maintained for variable periods after collection, and these were used as crude cultures that allowed us to collect additional information on any species that survived these conditions.

RESULTS

The organisms are reported in two categories: (1) those taxa identified to species and / or with adequate documentation, and (2) organisms which could not be identified to species level, but which were none-the-less distinctive. Lists of species encountered during this study are presented in Table 1.

Category 1. Organisms identified to species

Adriamonas peritocrescens Verhagen, Zölffel, Brugerolle *et* Patterson, 1994 (Figs 1a; 2a, b)

Observations: Pseudodendromonad. Cells about 5.5 µm long, laterally compressed and somewhat triangular in profile. Two flagella with narrowed tips, and both almost twice the length of the cell emerge from one

Table 1. List of species encountered during this study.

Species / Sites	New to Australia	Bothwell	Hollowtree	Meadowbank	Carlisle	Carlton	University	Ladies Tarn
Adriamonas peritocrescens	x					x		
Amastigomonas debruynei		x						
Ancyromonas sigmoides	x	x				x		
Anisonema acinus						x		
Anthophysa vegetans						x		
Astasia cfr. *gomphonema*	x						x	
Bicosoeca sp.		x						
Bodo saltans	x	x				x		
Cercomonas sp.				x				
Ciliophrys infusionum	x		x					
Codosiga botrytis					x			
Colpodella vorax	x			x				
Cryptomonas paramecium		x				x		
Entosiphon sulcatum		x		x	x	x		
Goniomonas truncata	x	x		x	x	x		x
Gyromonas ambulans	x					x		
Heteromita globosa	x		x	x		x		x
Heteronema exaratum	x					x		
Heteronema globuliferum	x					x		
Hexamita sp.						x		
Jenningsia fusiforme		x	x	x		x		
Mastigamoeba sp. 1						x		
Mastigamoeba sp. 2						x		
Menoidium obtusum							x	
Microcometes paludosa	x				x	x		
Neobodo designis	x	x				x		
Neobodo saliens	x					x		
Notosolenus apocamptus		x	x			x		
Notosolenus mediocanellatus						x		
Notosolenus steini				x			x	
Parabodo caudatus	x	x				x		
Peranema dolichonema		x	x	x		x		
Peranema inflexum						x		
Peranema trichophorum		x	x	x		x		
Petalomonas abscissa						x		
Petalomonas minuta						x		
Petalomonas poosilla	x					x		
Petalomonas sp. 1						x		
Petalomonas sp. 2						x		
Petalomonas sp. 3				x				
Ploeotia obliqua				x		x		
Ploeotia plana			x			x		
Ploeotia sp.						x		
Ploeotia tasmanica sp. n.	x					x		
Protaspis obliqua	x					x		
Protaspis simplex	x					x		
Reclinomonas americana	x					x		
Rhipidodendron splendidum						x		
Rhynchobodo sp.					x			
Rhynchomonas nasuta	x		x	x		x		
Sphenomonas angusta								x
Sphenomonas teres						x		
Trepomonas agilis	x					x		
Urceolus cyclostomus	x					x		

anterior ventral pole of the cell, the ventral face of the cell is slightly grooved. There is a curving cytopharynx leading into the cell from the posterior end of the groove. Cell movement not observed. Three cells seen from Carlton River.

Remarks: This species was observed originally by Verhagen *et al*. (1994) from soils in Northern Europe and has not been reported subsequently in Australia. Like Verhagen *et al*. (1994) we take the position that *Adriamonas* is a pseudodendromonad, and in the absence of cultures, cells of this species may not be distinguished from single cells from the colonial *Pseudodendromona*s. The genus *Cyathobodo* can be distinguished from *Adriamonas* because the latter is stalked.

Amastigomonas debruynei De Saedeleer, 1931 (Fig. 1b)

Observations: Gliding ovoid apusomonads, cells about 5 µm long. Two flagella are thin and insert anteriorly. The anterior flagellum is surrounded by a motile sleeve, and may protrude slightly from its end; the sleeve is the same width for the whole of its length, or dilates slightly at the distal end. The posterior flagellum is equal to cell length or slightly longer. Strands of cytoplasm may trail from the posterior of the cell. Observed from Bothwell.

Remarks: Members of *Amastigomonas* are distinguished from other gliding flagellates by possessing a non-bulbous sleeve and thin flagella. Species within the genus are distinguished largely on size and the relative lengths of the sleeve and flagellum (Molina and Nerad 1991). The cells seen in this study correspond well to recent descriptions of the species from marine sites including tropical (Larsen and Patterson 1990, Lee *et al*. 2003), northern polar (Vørs 1993a), northern temperate (Vørs 1992a, b; Tong 1997b) and subtropical Australia (Ekebom *et al*. 1996, Patterson and Simpson 1996, Tong 1997a, Lee and Patterson 2000). Although most recently reported from marine sites, the species was initially reported from freshwater (De Saedeleer 1931) in N. Europe. We confirm the overall similarity of the species from freshwater and marine locations.

Ancyromonas sigmoides Kent, 1880 (Figs 1c, 2c)

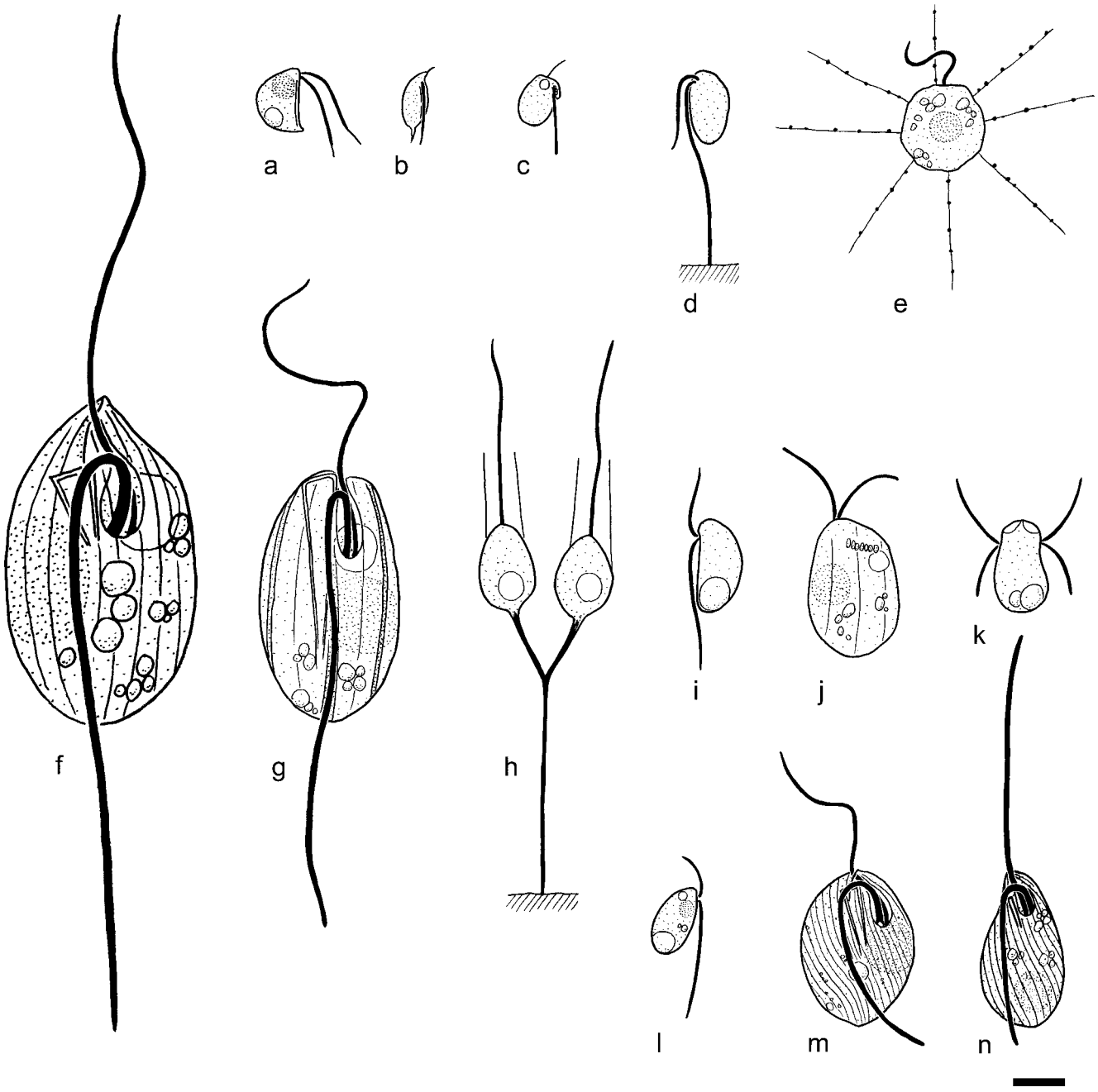
Observations: The genus is of uncertain affinities. Dorso-ventrally flattened cells with a reniform profile, 3.5-6 µm long, with two flagella. The anterior flagellum emerges from a depression in the anterior margin of the cell, is fine and shorter than the cell body. The posterior flagellum inserts in a fold that sets off a snout-like anterio-lateral prominence which may have refractile granules. The posterior flagellum is 2-2.5 times the length of the cell, and is sometimes acronematic. A contractile vacuole is located near the insertion of the flagellar. Cells glide with the posterior flagellum trailing and the anterior flagellum beating stiffly from side to side. Observed from Carlton River and Bothwell.

Remarks: Observations in accordance with contemporary identity of this species (e.g. Larsen and Patterson 1990, Lee and Patterson 2000, Al-Qassab *et al*. 2002) but with contractile vacuole. There are two other species in the genus: *Ancyromonas melba* Patterson *et* Simpson, 1996 which differs from *A. sigmoides* in possessing an anterior flagellum of the same width as the posterior flagellum, and *A. sinistra* Al-Qassab, Lee, Murray, Simpson *et* Patterson, 2002 which is dorso-ventrally flattened with a rugose flange on the left anterior-lateral margin of the cell. Patterson and Simpson (1996) incorrectly included *A. contorta* in this genus [see Patterson and Zölffel (1991) where it is correctly placed in *Phyllomonas*]. *Ancyromonas sigmoides* has been reported as having a widespread distribution in marine habitats (Lee and Patterson 1998, 2000; Al-Qassab *et al*. 2002; Lee 2002), however so far has only been reported from European freshwater locations (e.g. Hänel 1979).

Anisonema acinus Dujardin, 1841 (Figs 1f; 2e, f)

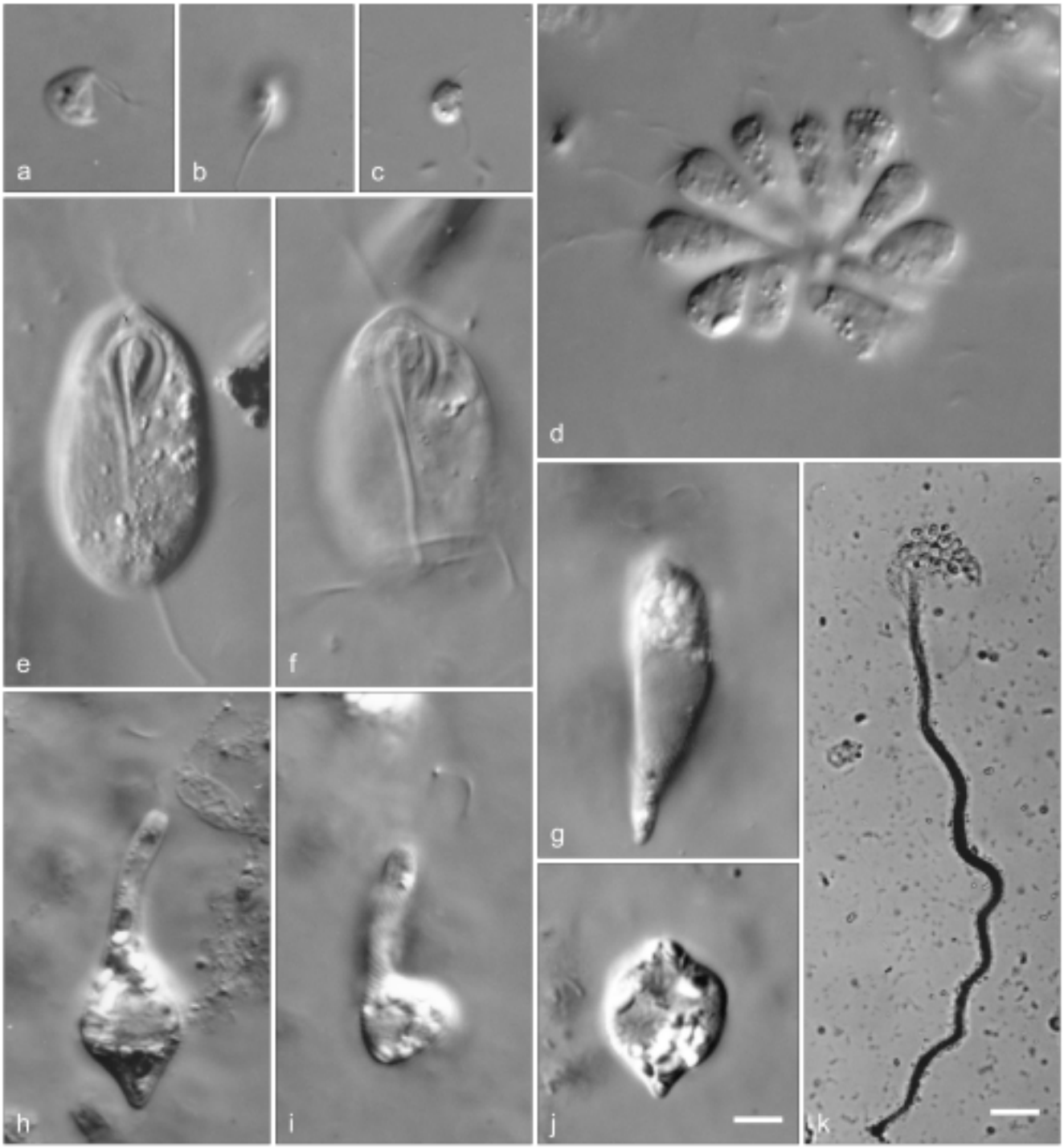
Observations: Gliding euglenid with profile similar to a barley-grain, 25-47 µm long, mostly about 30 µm, dorso-ventrally flattened. With a wedge-shaped ingestion organelle which may be difficult to observe. With two flagella, the anterior flagellum about 1.5 times as long as the cell, and beating with an undulating and sweeping motion in front of the cell. The posterior flagellum is up to twice the length of the cell, very thickened at its base and follows an almost semi-circular loop as it leaves the flagellar pocket, emerging under a ventral fold of the cell surface. The posterior flagellum can cause occasional reversals of movement. With the flagellar pocket to left of cell and with an associated contractile vacuole. Nucleus with maculate texture and to right of the cell near mid-line. About 8-10 very delicate grooves on each side of body, grooves sometimes not seen. Sometimes contain algal materials, although no ingestion organelle was observed. Common and widespread found in Carlton River.

Remarks: The genus *Anisonema* contains slightly metabolic gliding euglenids with the capacity for reversal movements. The confirmation of an ingestion apparatus



Figs 1 a-n. a - *Adriamonas peritocrescens*; **b** - *Amastigomonas debruynei*; **c** - *Ancyromonas sigmoides*; **d** - *Bodo saltans*; **e** - *Ciliophrys infusionum*; **f** - *Anisonema acinus*; **g** - *Entosiphon sulcatum*; **h** - *Codosiga botrytis*; **i** - *Colpodella vorax*; **j** - *Goniomonas truncata*; **k** - *Gyromonas ambulans*; **l** - *Heteromita globosa*; **m** - *Heteronema exaratum*; **n** - *Heteronema globuliferum.* Scale bar 5 µm for all figures.

in this genus will require a review of the composition of this and adjacent genera and is outside the scope of this paper. The cells observed here correspond in size with *A. acinus* as summarised by Lee and Patterson (2000). Marine isolates lack contractile vacuoles (Larsen and Patterson 1990, Lee and Patterson 2000, Al-Qassab *et al.* 2002). It has been found in several freshwater sites in mainland Australia (Schroeckh *et al*. 2003).



Figs 2 a-k. a, b - *Adriamonas peritocrescens*, **a** - general appearance of cell, **b** - ventral view; **c** - *Ancyromonas sigmoides*; **d, k** - *Anthophysa vegetans*; **e, f** - *Anisonema acinus*; **g-j** - *Astasia* cfr. *gomphonema*, **h** - general appearance of cell, **j** - cell in maximum contraction. All micrographs are DIC images. Scale bars 5 µm (a-j); 25 µm (k).

***Anthophysa vegetans* (Müller, 1773) Stein, 1878 (Figs 2d, k)**

(Basionym: *Volvox vegetans*)

Observations: This heterotrophic stramenopile occurs in spherical colonies of cells, 8-10 µm long, borne on the end of irregular branching brown stalk which tends to be paler near unattached end of the stalk. Cells with one long and short flagella emerging apically. The cells are usually in clusters of 20-30 cells, and are borne at the end of branched stalk. From Carlton River.

Remarks: The genus is most usually interpreted as being monospecific (Starmach 1985, Preisig *et al.* 1991, but see Hänel 1979 for differing point of view), and the organisms observed here correspond well with previous accounts of *A. vegetans*. This species has been widely reported from the Northern Hemisphere, and has previously been reported in Australia by several workers (Schewiakoff 1893; Stickland 1898, 1924; Gillies 1915; Playfair 1921).

***Astasia* cfr. *gomphonema* Pringsheim, 1942 (Figs 2g-j)**

Observations: Swimming heterotrophic euglenid with a metabolic body, with waves of constrictions passing along some or all of its length. Cells 25-40 µm long (mostly 25-30 µm) when fully extended, as short as 13 µm when contracted. Body circular in cross-section, posterior end not pointed sometimes with papilla, anterior end rounded or with slightly protruding apex from which the flagellum emerges. The single flagellum inserts into an anterior flagellar pocket with associated contractile vacuole, is about the length of the fully extended cell, and beats with loops passing from base to tip. Contracted cells may not have a visible flagellum. Nucleus located near middle of cell. Cytoplasm typically with numerous reserve granules with a slightly elongate angular shape. Cell surface with several grooves. Found in University pond.

Remarks: We identified the cells observed here as *Astasia gomphonema* with uncertainty because the identification of species in this genus is not straightforward since the degree of intraspecific variation was not recorded clearly, and there is considerable overlap of identity of a number of species. The following nominal species overlap in form, reserve granules and surface grooves with the organisms observed from Tasmania: *A. agilis* Christen, 1959 (17-20 µm), *A. applanate* Pringsheim, 1942 (39-47 µm), *A. clava* Pringsheim, 1942 (20-25 µm), *A. comma* Pringhseim, 1942 (28-32 µm), *A. cylindrica* Pringsheim, 1942 (16-20 µm), *A. inflata* var. *minor* (20 µm), *A. kathamerios* Skuja, 1948 (18-22 µm), and *A. thiophila* (30-40 µm) (all reviewed in Huber-Pestalozzi 1955). *Astasia communis* and *A. detrita* (in Skvortzov 1957) are similar to *A. gomphonema* in size and shape, but the surface of these is said to be smooth. The number and size of reserve granules may vary within a species, and may prove not to be a sound criterion upon which to establish morphospecies identities. *Astasia variabilis* Skvortzov (in Huber-Pestalozzi 1955) is said to have punctate pellicular ridges. Clearly, further work is required to confirm the number of small species of *Astasia* and to establish inter-specific boundaries.

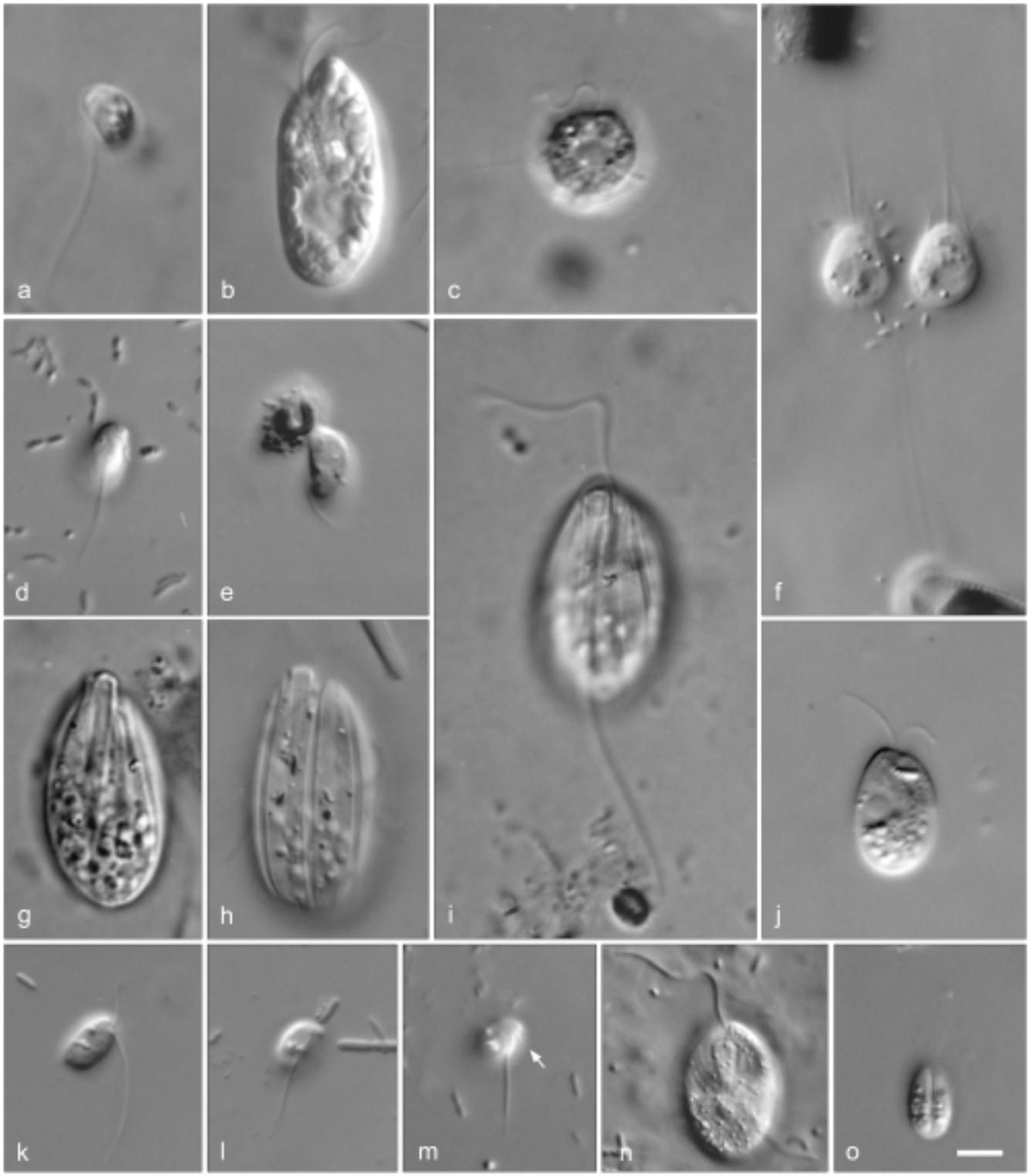
***Bodo saltans* Ehrenberg, 1832 (Figs 1d, 3a)**

Observations: Obovate kinetoplastid flagellates with body measuring 5-10 µm long, mostly 7-8 µm long. Two flagella insert in a sub-apical depression. The anterior flagellum is recurved, sometimes lying adjacent to the body and in a slight curving groove; sometimes the flagellum is lifted in an arc extending slightly away from the body; this flagellum generates a current of water, and the species consumes suspended bacteria. The posterior flagellum also curves back over the body to attach to the substrate by its tip, this flagellum is 3-3.5 times the body length, and bends quickly such that the cell has characteristic kicking movements. A contractile vacuole is located near site of flagellar insertion. The species may also release from the substrate and swim rapidly while rotating. Observed widely from Bothwell and Carlton River.

Remarks: Despite a poor initial description (Ehrenberg 1832), the characteristic kicking motion of this species has assured it a clear identity - but sometimes under the name *Pleuromonas jaculans* (Zhukov, 1971). This species is common in freshwater habitats, has been detected in brackish water (Vørs 1992a) and hypersaline environments (Post *et al.* 1983, Patterson and Simpson 1996, Al-Qassab *et al.* 2002), but rarely in normal marine environments (Vørs 1993b, Lee 2001).

***Ciliophrys infusionum* Cienkowski, 1876 (Figs 1e, 3c)**

Observations: Pedinellid stramenopile, cell about 9 µm wide in heliozoan form. The cell is more or less spherical, with delicate pseudopodia bearing extrusomes arising from the cell surface and extending radially from



Figs 3 a-o. a - *Bodo saltans* showing general appearance; **b** - *Cryptomonas paramecium* showing general appearance; **c** - *Ciliophrys infusionum*, note flagellum; **d, e** - *Colpodella vorax*, **d** - note short flagellum, **e** - grazing on a prey; **f** - *Codosiga botrytis* showing general appearance; **g-i** - *Entosiphon sulcatum*, **g** - showing ingestion organelle, **h** - showing surface grooves, **i** - general appearance of cell; **j** - *Goniomonas truncata* showing general appearance; **k-m** - *Heteromita globosa*, **k** - general appearance of cell, m - note short flagellum (arrow); **n** - *Heteronema exaratum* showing general appearance; **o** - *Petalomonas minuta* showing general appearance. All micrographs are DIC images. Scale bar 5 µm for all figures.

the body. With single flagellum held in a figure-of-eight and a central nucleus. With contractile vacuoles. Swimming not observed. One cell observed from Hollowtree.

Remarks: This species was initially reported from freshwater habitats but - accepting the synonymy with *C. marina* - it has more frequently been reported from marine habitats (e.g. Lee and Patterson 1998, 2000; Al-Qassab *et al.* 2002). Schewiakoff (1893) described *Ciliophrys australis* from Australia distinguished by the immotility of the non-heliozoan form. *Ciliophrys azurina* has been reported from tropical Australia (Lee *et al.* 2003, Mikrjukov and Patterson 2001) and is distinguished from *C. infusionum* (above) because *C. azurina* is considerably larger, has a longer flagellum which is held in a double 'figure of 8', and has the arms which taper from base to tip.

***Codosiga botrytis* (Ehrenberg, 1838) Kent, 1880 (Figs 1h, 3f)**

(Basionym: *Epistylis botrytis*)

Observations: Colonial choanoflagellate, cells about 10 µm long, with single apical flagellum measuring up to 30 µm long, with collar of delicate pseudopodia located around the flagellum. Cells attached to each other and to a broad hollow stalk by dichotomous branches. Rarely observed from Carlisle beach.

Remarks: This colonial choanoflagellate has been widely reported. It would appear that organisms with two different identities have been given the same name. The organisms described by Ehrenberg (1838), like the species reported here, has a broad stalk, whereas the organisms reported by Vørs (1992a) are said to have a delicate stalk. As pointed out by Vørs (1992a), there are taxonomic problems with this species and further work is desirable. Previously reported from Australia by Stickland (1924).

***Colpodella vorax* (Kent, 1880) Simpson *et* Patterson, 1996 (Figs 1i; 3d, e)**

(Basionym: *Dinomonas vorax*)

Observations: Apicomplexan flagellate, bean-shaped cells measuring 10-21 µm long, but mostly 12-14 µm long. With two flagella inserting on the body surface slightly sub-apically, with one flagellum extending forwards and the other trailing. The portion of the body anterior to the flagellar insertion protrudes slightly to form a small rostrum. Body without surface marks or grooves, but long cells may be slightly twisted. Body often contains one large posterior inclusion. All non-feeding and unencysted cells were observed swimming rapidly with a spiral motion (swimming cells may be very difficult to distinguish from some bodonids, especially swimming *Bodo saltans*). A predatory flagellate, attacking other protists (e.g. colourless, heterotrophic stramenopiles and *Bodo caudatus*), to which they attach by the rostrum, using this to withdraw cytoplasm from the prey. Prey cells may be released while alive. Well-fed cells form thin-walled cysts in which the cells divide into four. Commonly found at Meadowbank on one occasion.

Remarks: This genus has been reviewed by Simpson and Patterson (1996), with further species added by Patterson and Simpson (1996) and Mylnikov (2000). The four-way division in a cyst, and the mode of attack of prey are distinctive for *Colpodella* (Simpson and Patterson 1996). *Colpodella vorax* may be distinguished from most species in the genus because it lacks a long rostrum and surface grooving or gutters, and is not flattened. *Colpodella gonderi* is smaller, has longer flagella, and appears to feed exclusively on colpodid ciliates (Foissner and Foissner 1984). *Colpodella pontica* Mylnikov, 2000 is distinguished from *Colpodella vorax* at the LM level only be the lack of a contractile vacuole (it is a marine isolate). There are ultrastructural differences between the type species of the genus *C. pontica* and organisms identified as *Colpodella vorax* from freshwater (e.g. Brugerolle 2002). *Colpodella vorax* has previously reported only from the northern hemisphere, often under the names *Alphamonas coprocola*, *Bodo celer*, *Dinomonas vorax* and *Spiromonas angusta* (Simpson and Patterson 1996).

***Cryptomonas paramecium* (Ehrenberg, 1838) Hoef-Emden *et* Melkonian, 2003 (Fig. 3b)**

(Basionym: *Chilomonas paramecium*)

Observations: Elongate ovate cryptomonad cell measuring 14-28 µm long. With two flagella equal in length and slightly shorter than cell length inserting sub-apically near the opening of a pocket that extends to almost half the length of the cell and is lined by refractile ejectisomes. Viewed laterally, dorsal anterior portion of cell is more prominent, and is the site of the contractile vacuole. Several refractile crystals may also be present in the cell. Body frequently with many reserve granules. Usually observed resting with flagella against the substrate or actively swimming. Cell occasionally jumps backwards. Commonly observed from Bothwell and Carlton River.

Remarks: This species has usually been referred to as *Chilomonas paramecium*, with *Chilomonas* being

one of two heterotrophic genera of cryptomonads (the other being *Goniomonas*) (Hill 1991). *Chilomonas* has been reported widely in freshwater and marine locations, but the species usually reported from marine habitats (*Chilomonas marina*) has been transferred to the genus *Leucocryptos*, a kathablepharid (Vørs 1992c). Recently, *Chilomonas* has been shown to be an aplastidic lineage within *Cryptomonas* (Hoef-Emden and Melkonian 2003). Hill (1991) is of the view that many of the nominal species assigned to *Chilomonas* from freshwater sites are synonymous with *C. paramecium* - and we concur. We attribute the jumping motion to the explosive discharge of extrusomes. The organisms observed here are in good agreement with observations of *Cryptomonas paramecium* from the Northern hemisphere (see Hill 1991, Kugrens and Lee 1991). Also reported from Australia by Schewiakoff (1893), Gillies (1915), Playfair (1921) and Bernard *et al.* (2000), but not from Tasmania.

***Entosiphon sulcatum* (Dujardin, 1841) Stein, 1878 (Figs 1g, 3g-i)**

(Basionym: *Anisonema sulcata*)

Observations: Ovate gliding euglenid, 20-26 µm long. Body with six deep grooves with or without an additional six lesser grooves that alternate with the large grooves. Slightly flattened dorso-ventrally. With two flagella; the anterior flagellum about the length of the cell and beating with a rapid sweeping motion, the posterior flagellum about 1.5 times the length of the cell and trailing against the substrate. The conspicuous feeding organelle extends from near the front of the cell to the posterior, and is capable of protruding slightly and withdrawing. The flagellar pocket lies in the anterior part of the cell, and a contractile vacuole empties into it. Mostly consuming bacteria and detritus. Found widely and commonly at Bothwell, Carlisle beach, Meadowbank, and Carlton River.

Remarks: The genus *Entosiphon* is widespread and *E. sulcatum* has been subject to detailed study (Mignot 1966, Solomon *et al.* 1987, Triemer and Fritz 1987 inter alia). The species of *Entosiphon* described to date, with the exception of Goldyn's observations (Goldyn 1989) were reviewed and synonymised by Larsen and Patterson (1990) and Schroeckh *et al.* (2003). Larsen and Patterson (1991) suggested that *Marsupiogaster* of Schewiakoff (1893) is a synonym of *Entosiphon.* Given the inadequacy of the descriptions of Skvortzov's species, Schroeckh *et al.* (2003) suggested that all of the freshwater species may be referred to *Entosiphon sulcatum* or *Ploeotia obliqua.* By the criteria used in Schroeckh *et al.* (2003), the organisms described above can be identified as *E. sulcatum*. This species has 6 well-developed grooves and 6 lesser grooves, and a protrusible siphon. Reported previously from freshwater and marine habitats world-wide (Schroeckh *et al.* 2003).

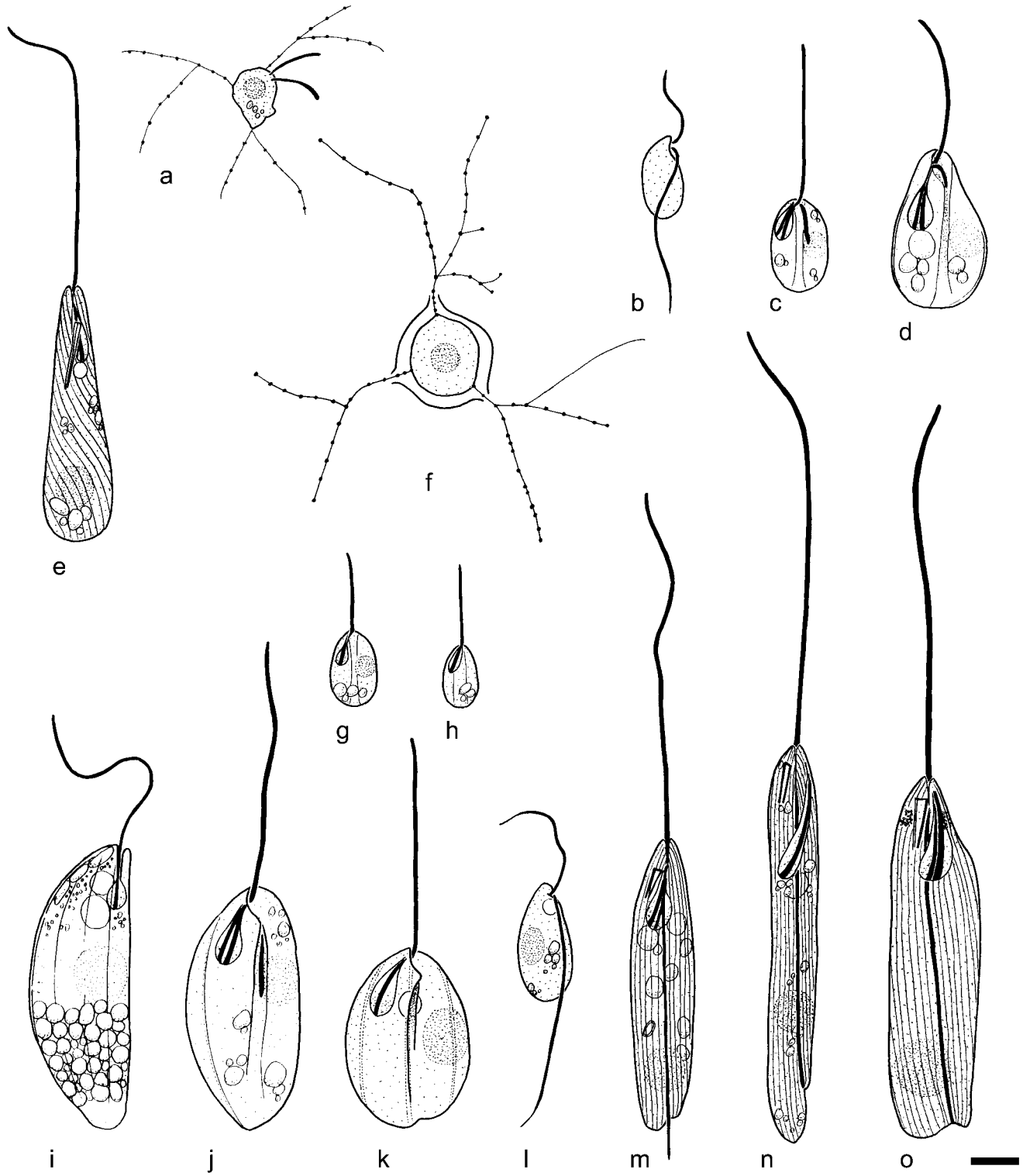
In respect of the genus *Entosiphon*, we have chosen to apply the operational criteria suggested by Larsen and Patterson (1990, 1991), and admit to this genus only those species which have a protrusible siphon. Species with a non-protrusible siphon are assigned to the genus *Ploeotia*.

***Goniomonas truncata* (Fresenius, 1858) Stein, 1878 (Figs 1j, 3j)**

(Basionym: *Monas truncata*)

Observations: Cryptomonad. Cell oval in profile but with a flattened anterior margin, cell body 8-14 µm long and very compressed. Cells often have 4 faint longitudinal striations following a curving path across both faces of the cell - some cells may have 3 or 5 striations, and in other cells (especially smaller ones) it was not possible to observe striations. Striations may appear granular. A single lateral row of extrusomes (ejectisomes) runs across the cell just behind the anterior face. Two equal length flagella are roughly two-thirds cell length and insert in a small pocket near the dorsal margin of the anterior face. Nucleus is located near the middle of the body behind the flagellar pocket. Contractile vacuole anterior. Some cells with food vacuoles. Cell swims either with one face against the substrate, or through the fluid with rotation. Observed from Bothwell, Meadowbank, Ladies Tarn, Carlisle beach and the Carlton River.

Remarks: *Goniomonas truncata* (previously also referred to as *Cyathomonas truncata* - see Larsen and Patterson 1990) has not previously had a clear identity. It was originally described from freshwater, and descriptions from freshwater refer to organisms ranging in size from 3 to 25 µm (Vørs 1992a) and having no-to-many ridges (Vørs 1992a, Skuja 1939). The uncertainty of the identity of the species *G. truncata* and the poor quality of the original account are now being resolved along the lines indicated by Vørs (1992a), that is to use the name *G. truncata* for a cell that is generally larger than *G. amphinema* and *G. pacifica*, the only other species in the genus and typically with more than 3 ridges per side, but not with many finely separated ridges. The presence of food vacuoles confirms the case presented by Kugrens and Lee (1991) that this species is



Figs 4 a-o. a, f - *Microcometes paludosa*; **b** - *Neobodo designis*; **c** - *Notosolenus apocamptus*; **d** - *Notosolenus mediocanellatus*; **e** - *Jenningsia fusiforme*; **g** - *Petalomonas minuta*; **h** - *Petalomonas poosilla*; **i** - *Menoidium obtusum*; **j** - *Notosolenus steini*; **k** - *Petalomonas abscissa*; **l** - *Parabodo caudatus*; **m** - *Peranema dolichonema*; **n** - *Peranema inflexum*; **o** - *Peranema trichophorum*. Scale bar 5 μm for all figures.

phagotrophic. This species has been reported widely in the Northern Hemisphere but not previously from Australia.

***Gyromonas ambulans* Seligo, 1886 (Fig. 1k)**

Observations: Diplomonad, cells measuring 6 and 7 µm long and with two pairs of opposed flagella arising from the anterior margin of the cell. The anterior flagellum of each pair is about the cell length and is directed laterally. The body may be sac-like and contain food vacuoles. The cells were observed clambering clumsily around detritus using flagella. Paired nuclei located at the anterior part of the cell. Two cells observed from Carlton River.

Remarks: Differs slightly from original description (Seligo 1886) in that the posterior flagella are slightly shorter and no twisting of the body was observed. Nonetheless the accounts are similar in respect of size, profile, and mode of locomotion. *Gyromonas* is the only diplomonad genus with four emergent flagella arranged as two pairs. The two species in the genus, *G. ambulans* and *G. salinus* (Ruinen 1938) are currently distinguishable because the latter is slightly larger the former and has slightly longer posterior flagella than anterior flagella, although future work may demonstrate that these distinctions are spurious. *Gyromonas ambulans* has previously been reported from the Northern hemisphere (e.g. Hänel 1979) and *G. salinus* from Australia (Ruinen 1938).

***Heteromita globosa* (Stein, 1878) Kent, 1880 (Figs 1l, 3k-m)**

(Basionym: *Bodo globosa*)

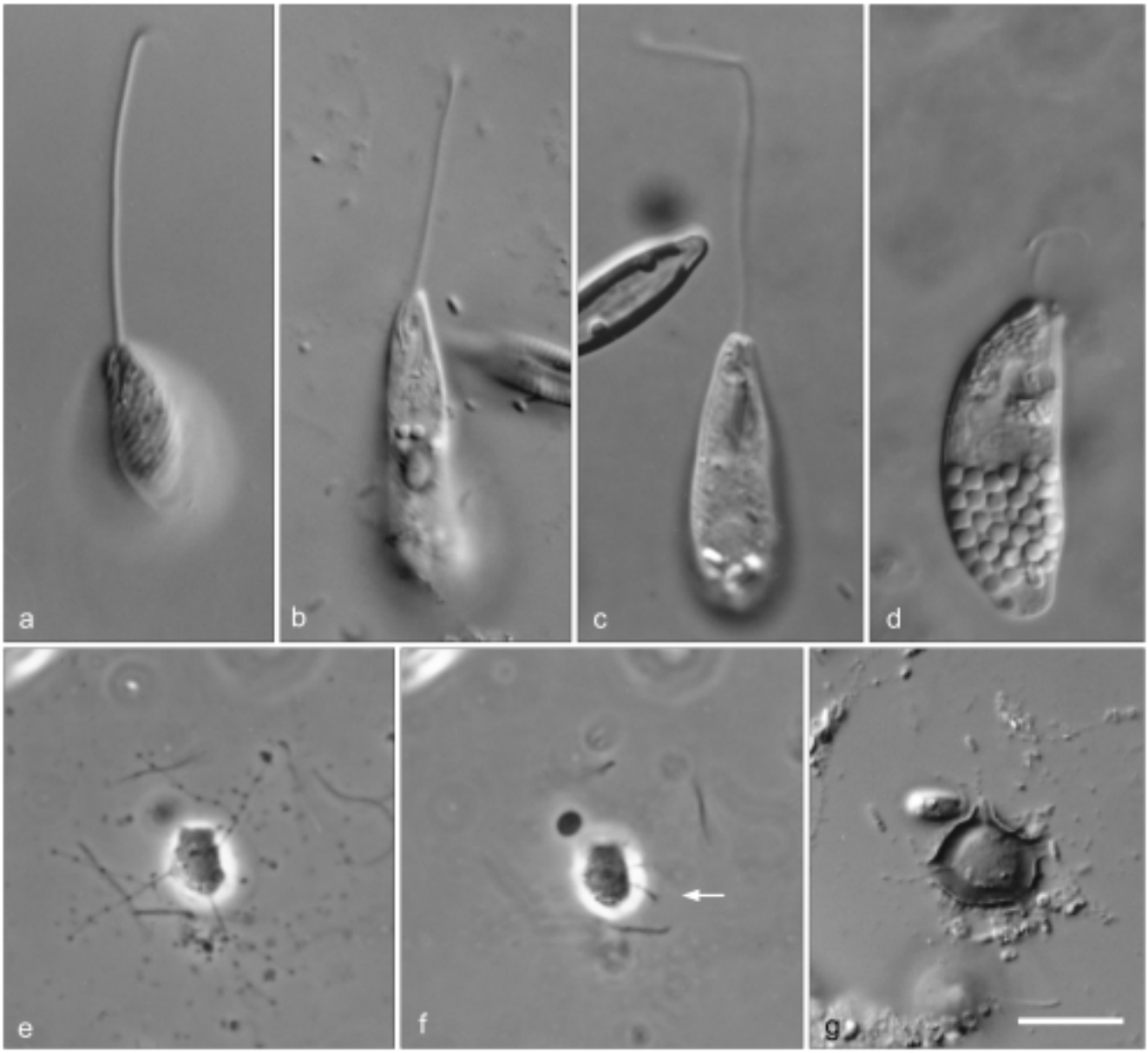
Observations: Gliding cercomonad, rounded to ovoid cell, with a slightly protruberant anterior end, 5.5-11 µm long (mostly 6-7 µm). With two flagella inserting at about 60° to each other in a sub-apical depression. The anterior flagellum varies in length from a short stub to almost the same length as the cell. The posterior flagellum is 2-2.5 times the length of the cell, and acronematic and trails against the substrate. In cells with a long anterior flagellum, the flagellum may beat with a slow paddling motion. Cells move with their anterior ends close to the substrate, posterior ends raised, and with a fairly consistent wobbling motion which is distinctive. The nucleus is located near centre of cell and has a round nucleolus. The contractile vacuole is located from slightly anterior to mid-line to the posterior of the cell. Particles are engulfed at the anterior end of the cell. Commonly observed from Hollowtree, Carlton River, Ladies Tarn and Meadowbank.

Remarks: Despite being reported as extremely abundant and widespread - especially in soils, the members of this genus are very poorly identified, and more work is required. The most detailed records are that of MacDonald *et al.* (1977). MacDonald *et al.* (1977) and Robertson (1928) indicate flagella of variable lengths but make no specific comment on this - and we are of the view that the variation in flagellar lengths does not indicate the presence of more than one species. Recorded from Europe, America and Antarctica (Robertson 1928, Smith 1973, MacDonald *et al.* 1977). This species is quite similar to *Metopion fluens* but is most easily distinguishable by the length of the shorter flagellum and the flexibility of the body in *H. globosa*.

***Heteronema exaratum* Larsen *et* Patterson, 1990 (Figs 1m, 3n)**

Observations: Fast skidding/swimming euglenid measuring 16-25 µm long. Cells ovate and plastic. Two flagella insert into a large anterior flagellar pocket and emerge somewhat laterally from a fold in the cell surface. Both flagella are about the same length as the cell. The anterior flagellum is directed anterio-laterally, while the posterior flagellum is most commonly coiled below the swimming cell. Ingestion organelle comprised of two rods with distinct caps opens near the flagellar pocket. The contractile vacuole opens into the flagellar pocket, and the nucleus is located near the centre of the cell posterior to the flagellar pocket in the left side of the cell. Pellicular grooves spaced about 1 µm apart or slightly more, often with refractile granules located adjacent to them, and usually similar on both surfaces, but sometimes faint and sometimes well marked, especially dorsally. Observed on several occasions from Carlton River.

Remarks: This species was described by Larsen and Patterson (1990) from marine sites. The current observations differ in that a contractile vacuole was observed but otherwise the organism resembles the marine isolates in size, and presence of small globules alongside the pellicular grooves. Unlike the original observations, we were unable to record a consistent pattern to the variation in the grooving (originally said to be light ventrally and well marked dorsally). *Heteronema exaratum* has previously been reported from marine sites world-wide (Lee and Patterson 1998, 2000; Lee 2002; Lee *et al.* 2003), but not freshwater sites.



Figs 5 a-g. a - *Heteronema globuliferum* showing general appearance; **b, c -** *Jenningsia fusiforme*, **c -** general appearance of cell; **d -** *Menoidium obtusum* showing general appearance; **e-g -** *Microcometes paludosa*, **f -** note flagella (arrow). All micrographs are DIC images with the exception of (e, f) which are phase contrast images. Scale bars: 8 µm for Fig. a and 5 µm for Figs b-g.

***Heteronema globuliferum* (Ehrenberg, 1838) Stein, 1878 (Figs 1n, 5a)**

(Basionym: *Trachelius globulifer*)

Observations: Gliding euglenid, cell highly plastic, pear-shaped, 16-25 µm long. With two flagella arising in a flagellar pocket and emerging from a small collar at the anterior of the cell. Anterior flagellum thick and 1.5-2.5 times as long as the cell, posterior flagellum about the same length as the cell. Moves typically by gliding with anterior flagellum most active at its tip, and with the posterior flagellum trailing on the substrate. Contractile vacuole located near flagellar pocket. Ingestion organelle formed of a single broad rod is often difficult to see; often with refractile granules between it and cell surface. Pellicular grooves spaced about 1 µm apart. Observed on several occasions from Carlton River.

Remarks: This species corresponds in body form to *H. abruptum, H. nebulosum, H. sacculus* and *H. globuliferum* (Huber-Pestalozzi 1955). Larsen and

Patterson (1990) regard *H. sacculus* of Skuja (1948) (incorrectly cited as *H. sacculosus* Skuja 1939) as a synonym of *H. globuliferum*, and Lee and Patterson (2000) regard *H. abruptum* as a synonym of *H. globuliferum*. Klebs (1893) also comments on the similarity of *H. nebulosum* and *H. globuliferum*. The organism observed here is smaller than the size (40-60 µm) reported for *H. globuliferum* and *H. nebulosum* (Huber-Pestalozzi 1955) but Larsen and Patterson (1990) point out that there is little basis for the estimate of size for *H. globuliferum*. With the exception of the presence of a contractile vacuole in the organisms we observed here, our observations are in good agreement with the descriptions of marine *H. globuliferum* from Fiji, Brazil and subtropical Australia (Larsen and Patterson 1990, Lee and Patterson 2000).

Jenningsia fusiforme (Larsen, 1987) Lee, Blackmore *et* Patterson, 1999 (Figs 4e, 5b-c)

(Basionym: *Peranema fusiforme*)

Observations: Gliding metabolic euglenid, with elongate body measuring 20-35 µm long. Body narrowed anteriorly, and broadened posteriorly to a greater or lesser extent; posterior end rounded. Body breadth up to one-third body length. Flagellum very thick, about as long or slightly longer than the cell, emerging apically, but arising in a sub-apical flagellar pocket with an associated contractile vacuole. Flagellum beats with a flailing motion or is extended in front of the cell while gliding. Flagellar pocket opens with a short slit running along ventral surface. Two rods measuring about one quarter the length of the cell open near the posterior end of the slit, and appear thick when viewed from the side. Pellicular grooves are very delicate and spaced 0.75-1 µm apart. The cell consumes algae and other detritus. The nucleus is located in posterior part of body. No extrusomes or reserve grains observed, but some cells with globular refractile inclusions. Observed from Bothwell, Hollowtree, Meadowbank and Carlton River.

Remarks: This species was described by Larsen (1987) as *Peramena fusiforme*, based on observations from the Wadden Sea. It was transferred to *Jenningsia* (Lee *et al*. 1999), because it has only one emergent flagellum. This species is distinguished from other *Jenningsia* and *Peranema* spp. because of its fusiforme shape and smaller size. Larsen and Patterson (1990) state that *P. fusiforme* has thin rods, but we note that the rods appear thick when viewed laterally. This species has been observed from marine (e.g. Larsen and Patterson 1990; Lee and Patterson 1998, 2000; Al-Qassab *et al.* 2002) and Australian freshwater sites (Schroeckh *et al.* 2003).

Menoidium obtusum Pringsheim, 1942 (Figs 4i, 5d)

Observations: Rigid euglenid, 25-34 µm long, compressed, lens-shaped with dorsal margin convex and ventral margin flat or very slightly concave, anterior end indented at site of emergence of flagellar pocket, posterior end rounded. A single emergent flagellum arises from a flagellar pocket located to the ventral side of the cell. Cell surface with a few widely spaced ridges per face - but these are often hard to see. Cytoplasm with variously-sized polygonal or rounded grains, some appearing to be less dense centrally. Smaller granules often near site of flagellar insertion. Common in the University pond.

Remarks: We identify our species with *M. obtusum* because of the rather broad anterior margin - but we note that there is considerable similarity of many species in this genus. Previously reported from freshwater sites in Europe, Brazil and recently Australia (Pringsheim 1942, Schroeckh *et al*. 2003).

Microcometes paludosa Cienkowski, 1876 (Figs 4a, f; 5e-g)

Observations: This genus is of uncertain affinities. Organisms with cell body 5-7 µm in diameter living in a lorica measuring 10-13 µm in diameter, the lorica organic, becoming brown with age, presumably due to accumulated metal salts. Fine, occasionally branching pseudopodia with small granules emerge from 4-8 collared apertures. With two short relatively inactive flagella that are hard to see except in cells that have settled recently and are without lorica or with a transparent lorica. With contractile vacuole, nucleus with round nucleolus. Extremely common, from Carlisle beach and Carlton River.

Remarks: This genus was first described by Cienkowski (1876) as an amoeba of uncertain affinities. It has been reported infrequently from Northern Europe and America (e.g. Leidy 1879, Penard 1902). It is not surprising that this organism was mistaken for an amoeba, given the difficulty of observing the flagella in all but recently settled cells. The genus has similarities with naked amoebo-flagellates having fine pseudopodia such as *Massisteria* (Larsen and Patterson 1990, Patterson and Fenchel 1990) and *Gymnophrys* (Mikrjukov and Mylnikov 1995) - but ultrastructural or molecular studies

will be required to establish if *Microcometes* is closely related to either of these. This species has not previously been reported from Australia.

***Neobodo designis* (Skuja, 1948) Moreira, Lopez-Garcia *et* Vickerman, 2004 (Fig. 4b)**

(Basionym: *Bodo designis*)

Observations: Kinetoplastid flagellate, slightly elliptical cell body measuring 7-11 µm long, with a sub-apical flagellar pocket that sets off a small rostral region. Two flagella insert into the pocket, one is acronematic, almost three times the length of the cell and trails behind the cell as it skids, the other is directed anterior-laterally. Cells rotate rapidly during swimming, but stop to feed, usually with the anterior flagellum wrapped around the front of the cell. A contractile vacuole is located near the site of flagellar insertion. Observed on a number of occasions from Bothwell and Carlton River.

Remarks: This species was described from freshwater by Skuja (1948) and he included reference to the distinctive peculiar rotating motion by which this species is most quickly distinguished from other *Bodo*-like kinetoplastids. It has been reported under the name of *Bodo designis* from freshwater around the world (Skuja 1948, Eyden 1977, Heyden *et al*. 2004) and been more widely reported - without a contractile vacuole - from marine sites around the world (Lee and Patterson 1998, 2000; Al-Qassab *et al*. 2002; Lee 2002; Lee *et al*. 2003). Excepting for the contractile vacuole, the marine and freshwater forms appear indistinguishable by light microscopy.

***Notosolenus apocamptus* Stokes, 1884 (Fig. 4c)**

Observations: Cell length 10-12 µm. A euglenid, cell ovate to rhomboidal, anterior end narrowed, dorso-ventrally flattened, with a broad longitudinal dorsal groove running along the entire length of the cell. Cell convex both sides of the dorsal groove, ventral side fairly flat. Two flagella of unequal length emerge from an ovate flagellar pocket. Anterior flagellum up to two times cell length and posterior flagellum up to three-quarters the length of the cell but usually about one quarter. Contractile vacuole empties into the flagellar pocket. Cells move by smooth gliding with anterior flagellum against substrate and ventral face of cell angled relative to substrate. Common, observed from Bothwell, Meadowbank and Carlton River.

Remarks: This species has been observed in marine and freshwater sites world-wide (Huber-Pestalozzi 1955; Larsen and Patterson 1990; Ekebom *et al*. 1996; Patterson and Simpson 1996; Lee and Patterson 1998, 2000; Al-Qassab *et al*. 2002; Schroeckh *et al*. 2003). It is similar to *Petalomonas minuta*, but is distinguished because *N. apocamptus* has two flagella.

***Notosolenus mediocanellatus* (Stein, 1878) Schroeckh, Lee *et* Patterson, 2003 (Figs 4d; 6a, b)**

(Basionym: *Petalomonas mediocanellatus*)

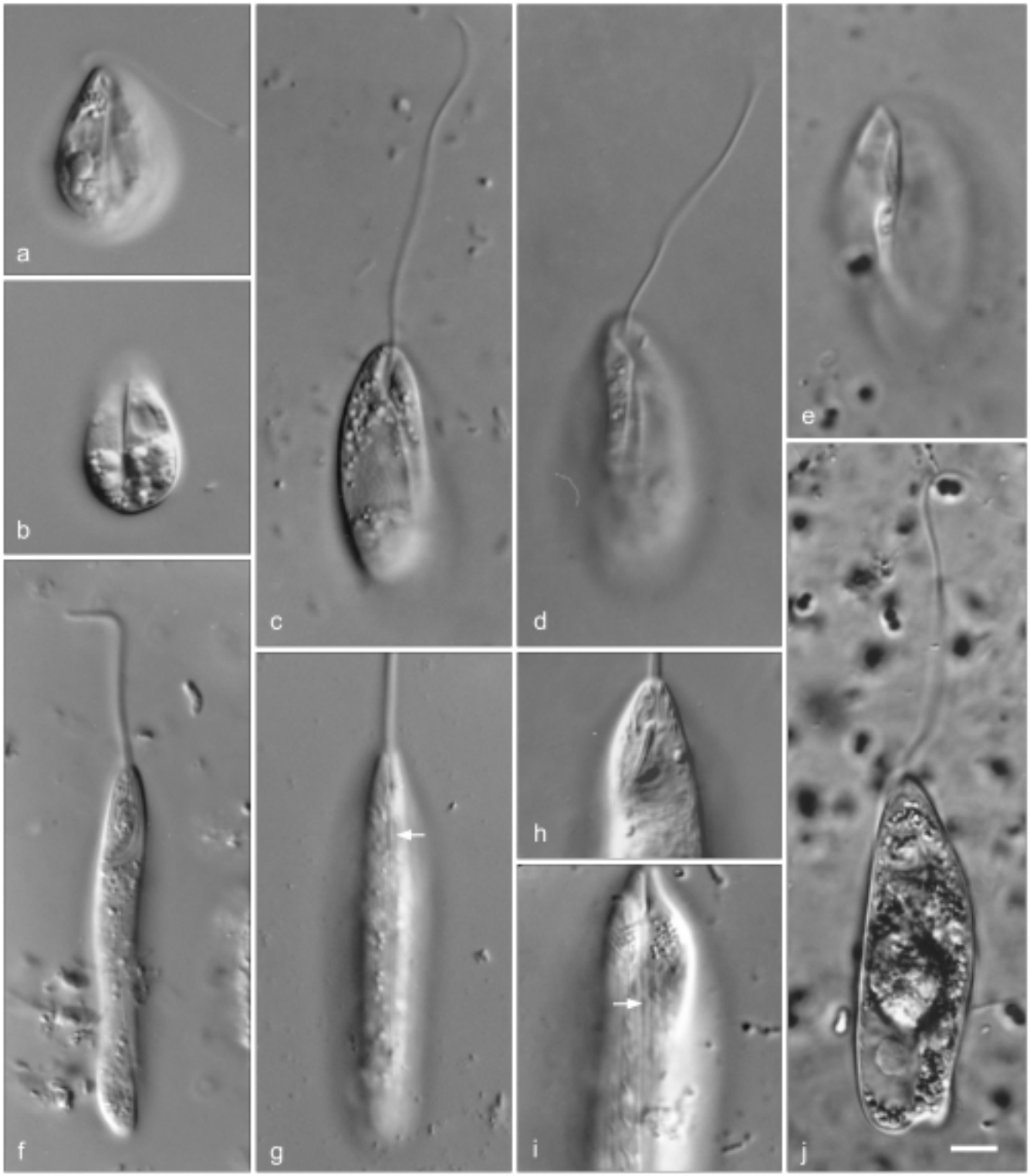
Observations: Rigid gliding euglenid, about 20 µm long, flattened with a highly ovate dorso-ventral profile. One pronounced median dorsal groove and less developed median ventral groove that lead to a wide, sub-apical, ventral opening to the flagellar pocket. The anterior flagellum, about the same length as the cell, directed anteriorly, and the short posterior flagellum may be difficult to observe. Flagellar pocket and associated contractile vacuole to the right side of the cell. Nucleus is on the left. Numerous small granules, possibly extrusomes, in the anterior portion of the cell. One cell observed from Carlton River.

Remarks: Corresponds well with the original description by Stein (1878), except for the presence of the ventral groove in addition to the dorsal groove (Huber-Pestalozzi 1955). The ventral groove has been noted by other workers (e.g. Skuja 1939, Schroeckh *et al.* 2003). The literature indicates a range of sizes for this species from 7 to 39 µm (Huber-Pestalozzi 1955, Schroeckh *et al.* 2003). Previously reported widely in freshwater (e.g. North America, Europe, and Australia (Skuja 1939, Huber-Pestalozzi 1955, Schroeckh *et al.* 2003).

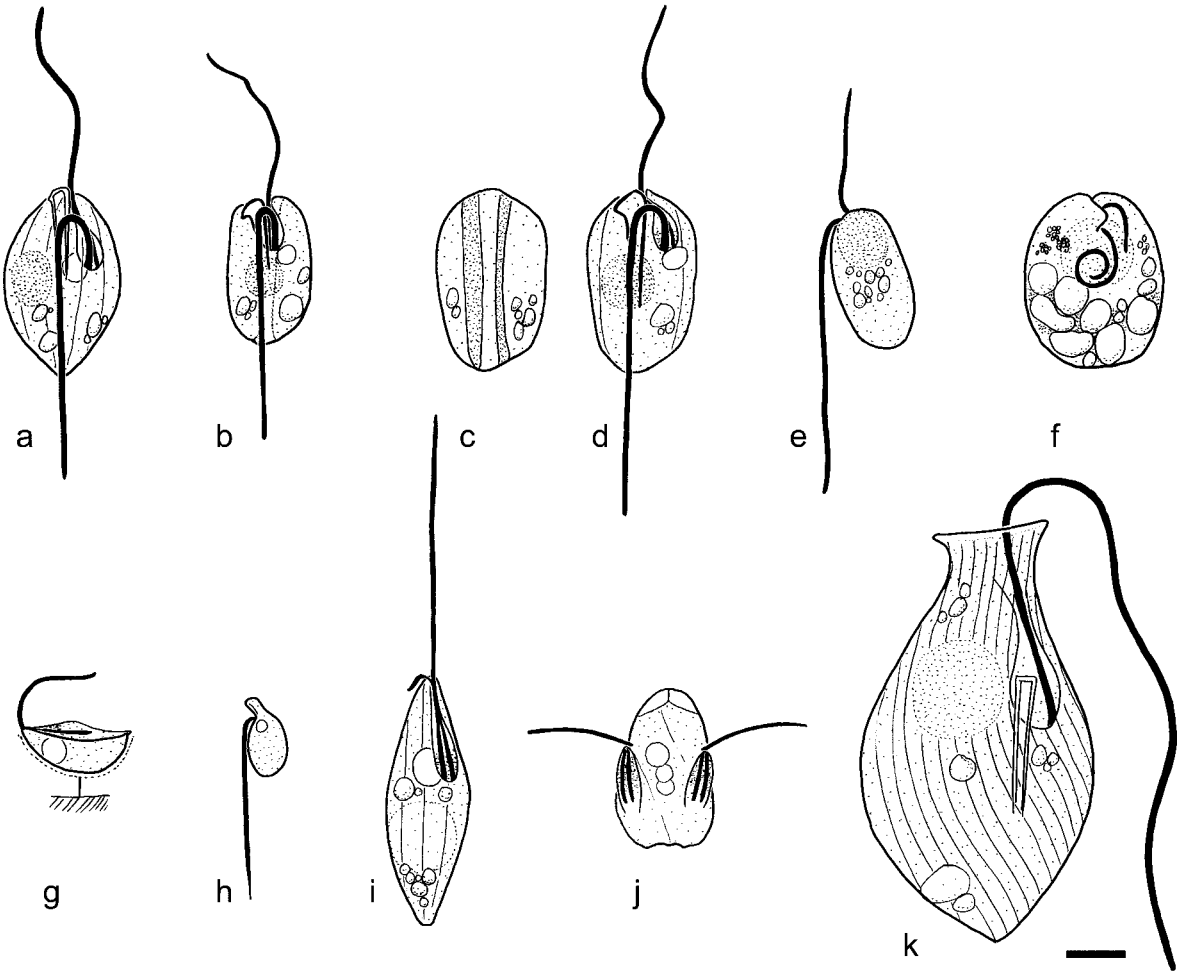
***Notosolenus steini* (Klebs, 1893) Schroeckh, Lee *et* Patterson, 2003 (Figs 4j, 6c-e)**

(Basionym: *Petalomonas steinii*)

Observations: Flattened gliding euglenid with oval or ovate body 23-36 µm long, rounded and slightly narrowed anteriorly, slightly pointed posteriorly. Cell rigid with right side concave and left side convex. Two flagella insert in a flagellar pocket which is located to the left of the cell. The opening of the pocket continues posteriorly as a groove, the left margin is strengthened to form a ridge and has a cusp near the point of emergence of the anterior flagellum. The fold extends towards the dorsal of the cell and converges on the right margin of the cell. The right margin of the groove is raised slightly. The posterior flagellum lies between the fold and ridge, and is about one half the length of the cell; it can be very difficult to see. The anterior flagellum is about as long as the cell and projects anteriorly. The flagellar pocket is narrow, and runs from the ventral part of the cell to



Figs 6 a-j. a, b - *Notosolenus mediocanellatus*, **a** - ventral view, **b** - dorsal view showing a groove; **c-e** - *Notosolenus steini*, **e** - dorsal ridge; **f, g** - *Peranema inflexum*, **f** - general appearance of cell, **g** - ventral view showing posterior flagellum in groove (arrow); **h-j** - *Peranema trichophorum*, **h** - ingestion organelle, **i** - note ventral striations and posterior flagellum in groove (arrow), **j** - general appearance of cell. All micrographs are DIC images. Scale bar 5 μm for all figures.



Figs 7 a-k. a - *Ploeotia obliqua*; **b** - *Ploeotia plana*; **c, d** - *Ploeotia tasmanica* sp. n.; **e** - *Protaspis simplex*; **f** - *Protaspis obliqua*; **g** - *Reclinomonas americana*; **h** - *Rhynchomonas nasuta*; **i** - *Sphenomonas teres*; **j** - *Trepomonas agilis*; **k** - *Urceolus cyclostomus*. Scale bar 5 µm for all figures.

terminate near the dorsal surface. There is an associated contractile vacuole. The right hand part of the dorsal surface is usually lifted up as a broad ridge that is triangular in profile. Occasionally this ridge does not project but curves over to set off a gutter running near the right margin of the cell. The nucleus is situated near the mid line of the cell towards the left margin, is maculate in appearance but with a nucleolus. The anterior region of the cell may have numerous small refractile droplets. Common in University pond, also observed from Meadowbank.

Remarks: This species has been transferred to *Notosolenus* by Schroeckh *et al*. (2003), because it has two flagella. As the posterior flagellum can be extremely difficult to see, we think that it has been overlooked by earlier authors. We note that the flagellar pocket is on the left, whereas Larsen and Patterson (1990) noted that in this genus it is characteristically located to the right. This species has been described from freshwater habitats in the Northern Hemisphere (Klebs 1893) and in Australia (Schroeckh *et al.* 2003).

Parabodo caudatus (Dujardin, 1841) Moreira, Lopez-Garcia *et* Vickerman, 2004 (Fig. 4l)

(Basionym: *Amphimonas caudata*)

Observations: Skidding or gliding kinetoplastid flagellate measuring 6-21 µm long (most were under 15 µm). Cell usually ovate but with considerable variation among individuals, large individuals often very flattened. Two flagella insert at the 'dorsal' end of a sub-apical groove

than runs across one face of the cell. Anterior flagellum extends forward and is recurved to point towards the rear; it beats with a paddling motion. Posterior flagellum acronematic, 2-2.5 times the length of the cell, attached to body anteriorly, trailing behind cell as it glides. Contractile vacuole empties near location of flagellar insertion, tear-drop kinetoplast also visible in larger cells adjacent to where flagella insert. Bacterial food includes rods more than 10 µm long. Observed from Bothwell and Carlton River.

Remarks: This species was originally described by Dujardin (1841) from freshwater habitats in N. Europe. It has been reported under the name of *Bodo caudatus* from a normal marine habitat in tropical Australia (Lee *et al*. 2003), and also reported from hypersaline environments in Australia (Ruinen 1938, Post *et al*. 1983, Al-Qassab *et al*. 2002). The flattened form, the range of sizes and the orientation of the anterior flagellum may be regarded as characteristic.

***Peranema dolichonema* Larsen *et* Patterson, 1990 (Figs 4m, 8a-c)**

Observations: Gliding metabolic euglenid, measuring 20-45 µm, mostly about 30 µm long. Body narrowed anteriorly, broadening posteriorly, to a width 1/3-1/2 of the cell length. The posterior end rounded or dimpled. Anterior flagellum thick, about 1.5 times as long as the cell, arising in a sub-apical flagellar pocket with an associated contractile vacuole, and beating with flailing motion or extends in front of the cell while gliding. The flagellar pocket opens with a short slit running along ventral surface. The posterior flagellum emerges from the slit and extends along the body and may or may not project from the posterior end - the body in this region may be depressed around the flagellum. We observed individuals with fine grooves spaced about 0.5 µm apart and individuals with grooves about 1 µm apart. Two fine ingestion rods measure about one quarter the length of the cell and open near the posterior end of the slit of the flagellar pocket. The rods are narrow and taper posteriorly, their anterior ends are thickened and joined by an arc of cytoskeletal material. Cells eat diatoms, other algae and detritus. The nucleus is located near the centre of the body and has a lobed nucleolus. Extrusomes short and refractile, typically in small clusters and oriented at right-angles to cell surface; most often seen in the 'neck' region (area of ingestion rods). Observed from of Bothwell, Hollowtree, Meadowbank and Carlton River.

Remarks: This species was first identified by Larsen and Patterson (1990) and distinguished by the projection of the posterior flagellum beyond the posterior end of the cell when gliding. The species observed here agrees well in size and overall appearance. No reference is made to a contractile vacuole in the original description, nor was reference made to extrusomes, although they are visible in the illustrations (Fig. 8a). Although individual extrusomes of *P. dolichonema* appear similar to those in *P. trichophorum*, those of *P. dolichonema* are typically found in clusters and oriented perpendicular to the cell - rather than along the direction of the grooves. Individuals with fine-spaced grooves and/or a non-projecting posterior flagellum have some similarity with *P. inflexum*, but we distinguish these two species by the differing aspects and lengths of the body, the different profile of the posterior end and the absence of extrusomes from *P. inflexum*. Reported from marine and freshwater sites in Australia (Larsen and Patterson 1990, Schroeckh *et al*. 2003).

***Peranema inflexum* Skuja, 1939 (Figs 4n, 6f, g)**

Observations: *Peranema* is a euglenid with an elongate body measuring 40-50 µm long and with a characteristically narrow (cylindrical) aspect, body breadth only rarely as wide as one quarter of body length. Anterior flagellum broad, and about as long as the cell, projecting forwards in gliding cells. Posterior flagellum delicate and extending to the posterior end of the body in a groove. Flagellar pocket located about one quarter down length of cell, contractile vacuole empties into this pocket. Ingestion rods appear very delicate. The nucleus is located near the middle of the body with one or more rounded nucleoli. No extrusomes observed, no starch grains observed, but some cells with globular refractile inclusions. Only seen from Carlton River.

Remarks: *Peranema inflexum* can be distinguished from other species of *Peranema* by its narrow cylindrical aspect, the delicate nature of the ingestion organelle, and the lack of extrusomes. *Peranema hyalina* as well as *P. acuta, P. ocellata* and *P. planctonica* described by Skvortzov (1957) are regarded as junior synonyms of *P. inflexum* (Schroeckh *et al*. 2003). Reported from freshwater sites world-wide (see Schroeckh *et al*. 2003).

***Peranema trichophorum* (Ehrenberg, 1832) Stein, 1878 (4o, 6h-j)**

(Basionym: *Trachelius trichophorus*)

Observations: Gliding metabolic euglenid, with elongate body measuring 45-65 µm, mostly 55-60 µm long. Body narrowed anteriorly, broadened posteriorly (width up to 1/3 of cell length) where it may be rounded,

Figs 8 a-k. a-c - *Peranema dolichonema*, **a** - showing posterior flagellum behind the cell body, **b** - posterior flagellum in groove (arrow), **c** - ingestion organelle; **d,e** - *Petalomonas abscissa*, **d** - ventral view, **e** - dorsal view showing dorsal groove; **f, g** - *Ploeotia obliqua*, **f** - ventral view, **g** - note ingestion organelle; **h-k** - *Ploeotia plana*, **h** - general appearance of cell, **i-k** - dorsal groove, **j** - ventral view. All micrographs are DIC images. Scale bar 5 µm for all figures.

dimpled. Anterior flagellum very thick, about as long or slightly longer than the cell, emerging apically, but arising in a sub-apical flagellar pocket with an associated contractile vacuole. Flagellum beats with flailing motion or is extended in front of the cell while gliding. Flagellar pocket opens with a short slit running along ventral surface, and posterior flagellum emerges from this slit and extends almost the full length of the cell lying in a ventral groove. Body with fine grooves spaced about 1 µm apart. With two ingestion rods measuring to about

1/4 the length of the cell and opening near the posterior end of the slit of the flagellar pocket. The rods appear hollow, are thickened at their most anterior margin and are linked by an anterior arc of refractile material. Cells eat diatoms, other algae and detritus. The nucleus is located in the posterior part of the body and has a lobed nucleolus. The cells may have refractile rod-shaped structures presumed to be extrusomes oriented more-or-less along the long axis of the body in more-or-less complete rows. They appear to be attached to inner surfaces of pellicular folds. Sometimes there is a densely packed collar of extrusomes underlying the cell surface in the region of the flagellar pocket. Some cells were observed with few extrusomes, but they were otherwise similar to isolates with many extrusomes. With variable quantities of refractile (paramylon?) granules. Observed at a variety of locations inclusive of Bothwell, Hollowtree, Meadowbank and Carlton River.

Remarks: This species has been reported from marine and freshwater sites world-wide with lengths from 22 to 81 µm (Huber-Pestalozzi 1955; Larsen and Patterson 1990; Lee and Patterson 1998, 2000; Schroeckh *et al*. 2003). See Schroeckh *et al*. (2003) for more detailed discussion.

***Petalomonas abscissa* (Dujardin, 1841) Stein, 1878 (Figs 4k; 8d, e)**

(Basionym: *Cyclidium abcissum*)

Observations: Rigid gliding euglenid, ovate with slightly pointed posterior end, cell 15-20 µm long. The lateral margins of the cell are thin flanges. With two dorsal ridges; left one well-developed, right one often more weakly developed. Ventrally one narrow groove forming a double ridge which bends like a dog-leg near the front. The margins of the ventral groove arise as extensions of a strengthened collar around the opening of the flagellar pocket. One emergent flagellum, 1.5-2 times cell length, directed anteriorly. Flagellar pocket to the right ventral side with associated contractile vacuole. Observed occasionally from Carlton River

Remarks: We have identified this organism with the taxon referred to as *P. abscissa* by Larsen (1987), Larsen and Patterson (1990), Ekebom *et al.* (1996), Patterson and Simpson (1996) and Lee and Patterson (2000) - despite some differences in the profile of the posterior end (not concave) and two dorsal ridges, and that the cells observed from freshwater have contractile vacuoles - unlike the marine cells. We are swayed by the following similarities: size, narrow marginal flange, double ridge with a dog-leg bend ventrally, by other records of contractile vacuoles in this species, and by the range of form previously accredited to this species (Huber-Pestalozzi 1955). Previously reported widely from marine and freshwater habitats, Northern and Southern Hemispheres (Lee and Patterson 1998, 2000; Lee 2002; Lee *et al*. 2003; Schroeckh *et al.* 2003).

***Petalomonas minuta* Hollande, 1942 (Figs 3o, 4g)**

Observations: Gliding, flattened euglenid, cell ovate, 6-12 µm long. With a median groove down the length of the dorsal surface. This groove varies in depth and visibility. Single flagellum about same length as body and pointing anteriorly. Flagellar pocket with associated contractile vacuole to right. The nucleus is located in the left side of the cell. Food vacuoles contained algal remains. Observed from Carlton River.

Remarks: This species has been described from marine habitats world-wide as well as from freshwater sites in Europe and Australia (Lee and Patterson 1998, 2000; Al-Qassab *et al.* 2002; Lee 2002; Lee *et al.* 2003; Schroeckh *et al*., 2003). This species is very similar in size to *P. minor,* which can be distinguished by a prominent dorsal ridge, and to *P. poosilla,* which has no grooves. *Petalomonas poosilla* is somewhat smaller to *P. minuta* and has but can be distinguished from *P. minuta* by delicate ridges.

***Petalomonas poosilla* Larsen *et* Patterson, 1990 (Fig. 4h)**

Observations: Gliding euglenid, cell ovate, about 6-7 µm long, anteriorly truncate. Extremely delicate ridges sometimes observed arising from the anterior pole of the cell, more easily seen ventrally. Single flagellum about the length of the cell and pointing anteriorly Flagellar pocket with associated contractile vacuole to right. The nucleus is located in the left side of the cell. Food vacuoles may contain algal remains. Observed from Carlton River.

Remarks: This species has been described from freshwater and marine habitats world-wide (Skuja 1948; Huber-Pestalozzi 1955; Lee and Patterson 1998, 2000; Al-Qassab *et al.* 2002; Lee 2002; Lee *et al.* 2003). The observations of delicate dorsal ridges confirm those of Patterson and Simpson (1996) and Lee *et al*. (2003). The species name "*poosilla*" was introduced for nomenclatural reasons under the International Code of Zoological Nomenclature (ICZN) only, *P. pusilla* being the correct name under the provisions of the International Code of Botanical Nomenclature (ICBN).

***Ploeotia obliqua* Schroeckh, Lee *et* Patterson, 2003 (Figs 7a; 8f, g)**

Observations: Small gliding euglenid, 11-16 µm long, ovoid with slight prominences to right anteriorly and centrally posteriorly, giving a profile similar to an elongate lemon. The anterior prominence is caused by the ingestion organelle and this extends to the posterior of the body. No pumping movements of the ingestion organelle were observed. Body slightly flattened dorsoventrally, one fold arising ventrally just posterior to the ingestion organelle, extending about half the length of the cell and from under which arises the posterior flagellum. Each face of the body with about four very delicate ridges. With two flagella, the anterior one with a quick undulating beat, and posterior one trailing under the cell. Flagellar pocket in anterior half of the cell to the left hand side, a contractile vacuole feeds into this. Common, being observed from Carlton River and Meadowbank on numerous occasions.

Remarks: Unlike most members of the genus *Ploeotia, P. obliqua* is rounded in cross-section, and among species with this feature it is smaller than *Ploeotia laminae, P. scrobiculata* and *P. robusta* and differs from these species also by surface texture / sculpting. It differs from *P. azurina, P. costata* and *P. oblonga* by number and nature of ridges, from *P. plumosa* by the absence of curved extrusomes, and differing from all of these and from *P. pseudanisonema* by the pointed posterior margin. Our observations agree well in size and appearance with those of *P. obliqua* from freshwater on mainland Australia by Schroeckh *et al.* (2003).

***Ploeotia plana* (Christen 1959) Schroeckh, Lee *et* Patterson, 2003 (Figs 7b, 8h-k)**

(Basionym: *Entosiphon planum*)

Observations: Small rigid gliding euglenid measuring 11-14 µm long. Body more or less ovate, with slight prominence in the right anterior margin where the ingestion organelle protrudes slightly. The sides of cell may be slightly convex, flattened, or - especially to the left - concave. The posterior end may be rounded or slightly dimpled or with a slight prominence. Cell flattened, with a deep and broad groove with undulating margins on the dorsal side, and four fine ridges ventrally and two fine ridges dorsally. With two flagella, the posterior one trailing and slightly longer than the cell, the anterior one beating with a quick sweeping motion and about the length of the cell. Motion generally somewhat jerky. Flagellar pocket with contractile vacuole to left-hand side of cell. Nucleus to the left, near the mid-line of the cell. The ingestion organelle slightly more substantial anteriorly, not seen to move. Observed from Hollowtree and Carlton River on several occasions**.**

Remarks: *Ploeotia* contains rigid gliding euglenids with the anterior flagellum free during gliding, and with a visible but non-motile ingestion organelle (in contrast to *Entosiphon* which has a motile ingestion organelle). Within *Ploeotia*, the combination of pronounced flattening and a broad dorsal groove is distinctive to *P. planum*. *Ploeotia plana* has previously been recorded only from freshwater in Europe and mainland Australia (Christen 1959, Schroeckh *et al*. 2003).

***Ploeotia tasmanica* sp. n. (Figs 7c, d; 9a-c)**

Diagnosis: *Ploeotia* measuring 13-15 µm long with two deep dorsal grooves.

Observations: Rigid gliding euglenid with elongate/oval cell body, 13-15 µm long, slightly flattened, posteriorly rounded. Dorsal face with two deep longitudinal grooves, one slight ventral median groove posterior and two faint grooves may lie to either side of the ventral groove. Feeding organelle opens apically to the right of mid-line, not observed to pump. Nucleus near the middle of the body and slightly right of centre. Two flagella emerge from flagella pocket, which is to the left of the centre-line. Anterior flagellum about the same length as the cell, beating in front of the gliding cell. Posterior flagellum about twice the length of the cell and adhering to the substrate during gliding, somewhat thicker than the anterior one. Contractile vacuole empties into the flagellar pocket. Observed occasionally from Carlton River.

Remarks: This species may be distinguished from all other species of *Ploeotia* by its two deep dorsal grooves. We do not believe that this organism has been recorded previously, under any name. It is distinguished from *Ploeotia laminae, P. oblonga, P. robusta* and *P. scrobiculata* by its smaller size and by having two deep dorsal grooves. This species is similar to *P. plana* and *P. obliqua*, but *P. plana* has one dorsal groove and *P. obliqua* is rounded in cross-section. It is distinguished from *P. azurina, P. costata, P. plumosa* and *P. pseudanisonema* by the two dorsal grooves. *Ploeotia plumosa* has curved extrusomes.

***Protaspis obliqua* Larsen *et* Patterson, 1990 (Figs 7f, 9g)**

Observations: Flattened cells with a rounded dorsoventral profile 15-17 µm long, with two flagella inserting sub-apically, the insertions being separated by a small

protrusion from the cell surface. There is a broad shallow groove in the middle of the ventral face of the cell. The anterior flagellum is 0.5-0.7 the cell length, and the posterior flagellum is slightly longer than the cell and is coiled in the rest cell (Figs 7 f; 9 g). The nucleus is large and rounded, with a rounded nucleolus. It is situated in the anterior of the cell, somewhat to the left of centre. The posterior of the cell often contained large highly refractile inclusions. Cells not observed moving. Several cells observed from Carlton River.

Remarks: The genus *Protaspis* is of uncertain affinities. *Protaspis obliqua* was distinguished from other species in the genus by the protruberance between the insertion points of the two flagella (Larsen and Patterson 1990, Lee and Patterson 2000). The organisms observed from freshwater comply well with the previous descriptions from marine sites in tropical Australia, Fiji, England and Korea (Larsen and Patterson 1990, Lee and Patterson 2000, Lee 2002).

***Protaspis simplex* Vørs, 1992 (Figs 7e, 9f)**

Observations: Gliding biflagellated cell measuring 7-14 µm long. The cell has a rounded to elongate ovoid profile, is flattened, and the two flagella insert subapically on the ventral side. The anterior flagellum is almost the length of the cell, and beats stiffly, flexing at its base and with the beat envelope directed to one side; the posterior flagellum is about twice the length of the cell and trails under the cell. Nucleus with nucleolus is located near the front of the cell and has cap in at least some cells. Some cells with fine surface granules. Contractile vacuole located posteriorly, among food vacuoles which often contain remains of algae. Six cells observed from Carlton River

Remarks: The genus *Protaspis* is of uncertain affinities. The cells seen in this study are similar to those observed by Vørs (1992a) from marine sites and like her we note a considerable range of size. *Protaspis simplex* has previously been reported from marine sites in Canada and northern Europe (Tong 1997b; Vørs 1992a, b), and Australia (Tong *et al*. 1998). This species exhibits considerable size range and further work is required.

***Reclinomonas americana* Flavin *et* Nerad, 1993 (Figs 7g; 9d, e)**

Observations: Biflagellated jakobid flagellate about 8 µm long with dorsal face adpressed to a dish-shaped lorica which is attached to the substrate by means of a short perpendicular stalk. The ventral face of the cell has a groove. The flagella insert at the anterior end of the cell, at the head of the groove and are about the length of the body or slightly shorter. One flagellum lies in the groove, while the other is directed ventro-posteriorly, lies further out from the cell and is more active. One cell observed from Carlton River.

Remarks: Our observations broadly in concordance with description by Flavin and Nerad (1993), but the presence of an epipodium was not confirmed. *Histiona* and *Reclinomonas* are colourless biflagellate cells that 'recline' in a hyaline lorica, thus distinguishing them from the more 'upright' loricate stramenopiles (Flavin and Nerad 1993). *Reclinomonas* is distinguished from *Histiona* by both flagella being easily visible, and in lacking a ventro-posterior projection (Flavin and Nerad 1993, O'Kelly 1993). This species is distinguished from the other nominal species in this genus (*R. campanula*) by the angle between the stalk and lorica (Flavin and Nerad 1993). Previously reported from Northern America and New Zealand (O'Kelly 1993).

***Rhipidodendron splendidum* Stein, 1878 (Fig. 10n)**

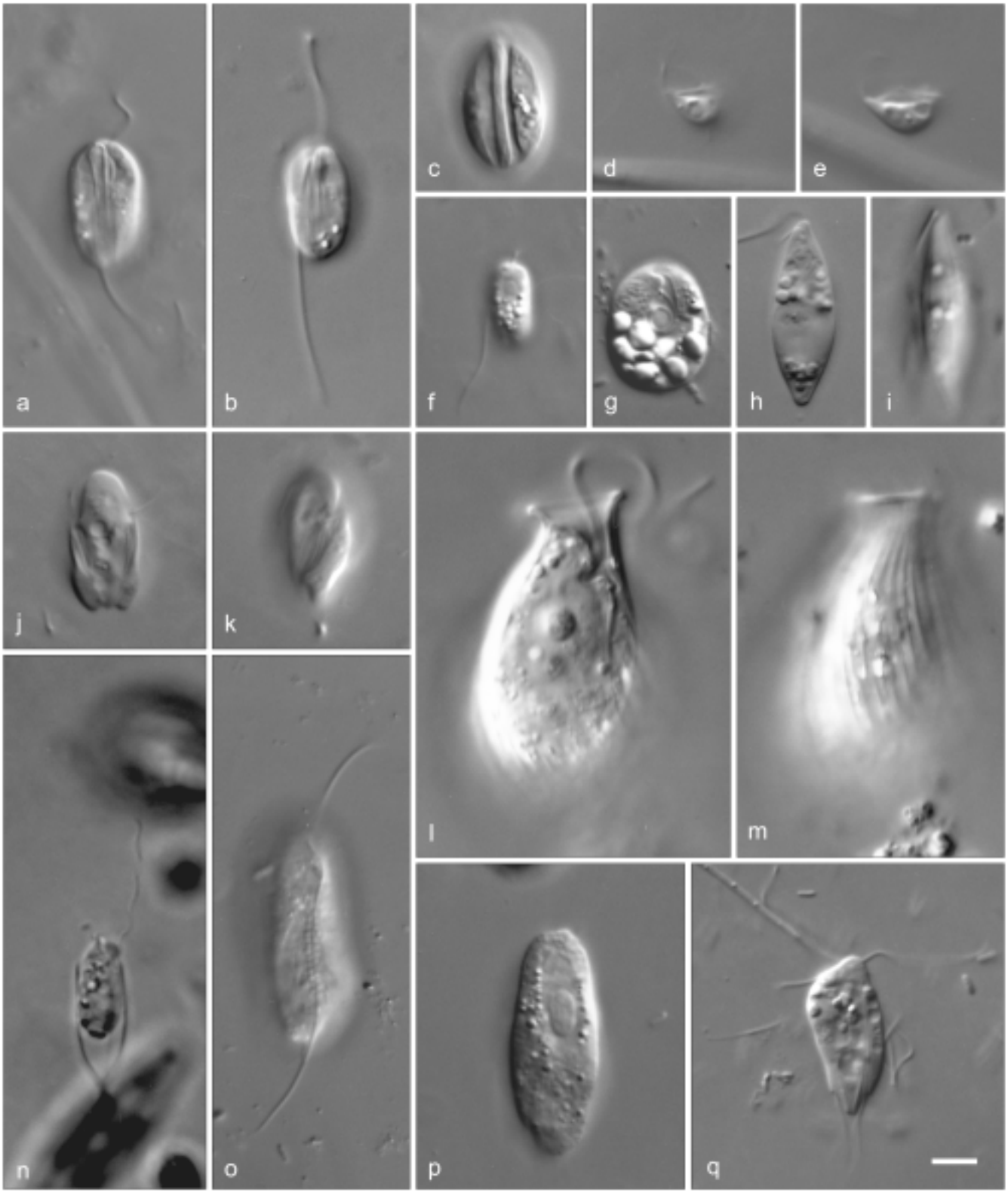
Observations: Cells measure about 5 µm and live in colonies up to 200 µm long. Cells embedded in globular organic material that forms tubes, the tubes dividing distally. Inner walls of the tubes appear smooth. The cells are spherical or elongate, and are located mostly at the open end of the tubes, but may occasionally be located non-apically. Two equally long flagella (about 20 µm) extend from the cell. Details of the cells not observed. Also frequently seen were 'encrustations' on the substrate of globular material enclosing 2-8 cells which may be of the same species. Observed from Carlton River

Remarks: This taxon is of uncertain affinities. This species corresponds broadly with *Rhipidodendron splendidum* as described by Kent (1880-1882). A more familiar second species is the larger *Rhipidodendron huxleyi*. Ultrastructural studies were conducted by Hibberd (1976). This species has previously been reported in Australia by Stickland (Stickland and Stickland 1895, Stickland 1924) and by Playfair (1921). We assume that the encrusting forms involving 2 or 8 cells are early colonies of the larger form and are not a species from the related genus *Spongomonas*.

***Rhynchomonas nasuta* (Stokes, 1888) Klebs, 1893 (Fig. 7h)**

(Basionym: *Heteromita nasuta*)

Observations: Gliding oval kinetoplastids, 4-7 µm in length, with a mobile bulbous snout. The posterior flagel-



Figs 9 a-q. a-c - *Ploeotia tasmanica* sp. n., **a** - general appearance of cell, and note ingestion organelle and fine ridges, **c** - dorsal view showing two grooves; **d, e** - *Reclinomonas americana*, **d** - note a stalk, **f** - *Protaspis simplex*; **g** - *Protaspis obliqua*, note a protublence; **h, i** - *Sphenomonas teres*; **j, k** - *Trepomonas agilis*; **l, m** - *Urceolus cyclostomus*, **m** - showing striations; **n** - *Bicosoeca* sp.; **o, p** - *Cercomonas* sp., **o** - ventral view showing rows of extrusomes, **p** - nucleus; **q** - *Hexamita* sp. All micrographs are DIC images with the exception of (n) which is a phase contrast image. Scale bar 5 µm for all figures.

lum inserts at the base of the snout in a small depression, is acronematic and about 2.5 times cell length. Observed from Hollowtree, Meadowbank and Carlton River, in a few samples, in large numbers.

Remarks: The cells observed are in good agreement with cells seen in previous accounts (e.g. Swale 1973, Larsen and Patterson 1990, Lee and Patterson 2000). Distinguished from other gliding flagellates by the mobile bulbous snout, and because only a single, thickened flagellum is easily visible by light microscopy (Larsen and Patterson 1990). *Rhynchomonas nasuta* has previously been reported from marine, soil and freshwater habitats in a wide variety of locations worldwide (e.g. Lee and Patterson 1998, 2000; Al-Qassab *et al.* 2002; Lee 2002; Lee *et al*. 2003).

***Sphenomonas teres* (Stein, 1878) Klebs, 1893 (Figs 7i; 9h, i)**

(Basionym: *Atractonema teres*)

Observations: Rigid gliding euglenid, slim elongate elliptical cell, 14-24 µm long. Two emergent flagella, the anterior about 1.5 times the length of the cell, the posterior about 0.1-0.2 times the length of the cell. Flagella insert into the flagellar pocket which is located dorsally, and has an associated contractile vacuole. Body surface with a number of longitudinal grooves (up to 10, but usually 6-8). Occasional cells with deep broad groove. Cells typically have one very large hyaline inclusion near the posterior end of the cell. A small number of cells are much broader - but this may be as a result of the inclusions. Seen frequently from Carlton River.

Remarks: There are 15 nominal species (Playfair 1921, Huber-Pestalozzi 1955, Skuja 1956, Christen 1959, Schroeckh *et al*. 2003). Our observations have demonstrated that there is a range of size of almost a factor of two and that there may be a variable number of surface grooves. We have observed a number of individuals with broad grooves with well-defined edges. Having observed a range of forms at other sites (Schroeckh *et al*. 2003), we regard such individuals as belonging to the same species as those without such deep grooves but are otherwise similar. The most similar species in the genus is *S. laevis* (of Skuja 1948) which can be distinguished by its smooth body and more pointed posterior. There is some similarity in profile with *S. quadrangularis*, but this species has four very strong ridges.

***Trepomonas agilis* Klebs, 1893 (Figs 7j; 9j, k)**

Observations: Diplomonad cells measuring 13 and 17 µm long, with twisted lateral grooves. Two sets of four flagella emerging from opposing anterio-lateral margins, one flagellum of each set extends laterally from a slightly pointed structure and about the same length as the cell, the other flagella are shorter than the cell and are directed posteriorly into grooves. Two elongate nuclei are located anteriorly. Two cells observed from Carlton River.

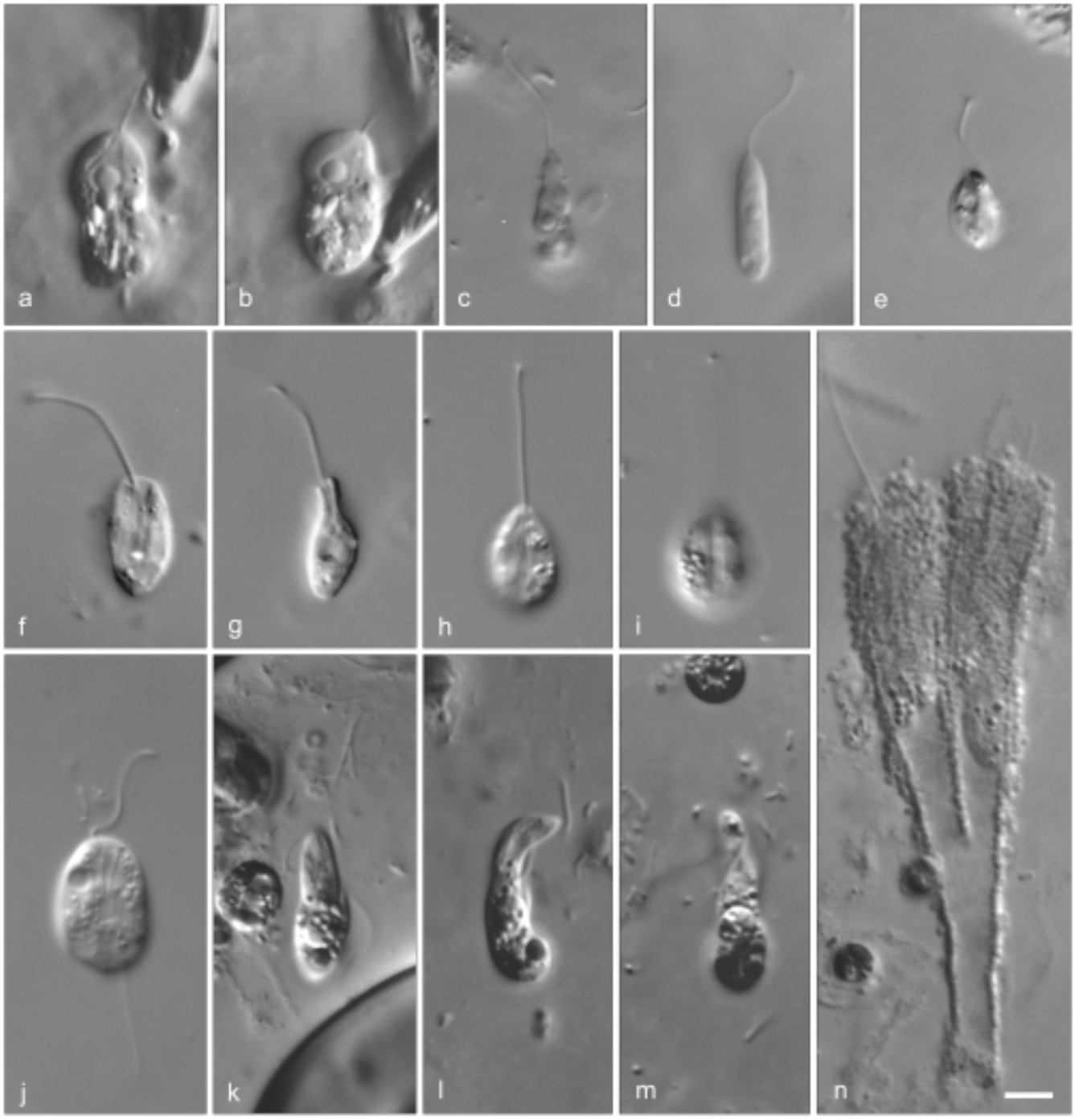
Remarks: The free-living diplomonad flagellates are reviewed by Mylnikov (1985), and *Trepomonas* studied in detail by Eyden and Vickerman (1975) and reviewed by Bernard et al. (2000). The organism observed here complies well with the observation of *T. agilis* by Bernard *et al.* (2000). The cosmopolitan species has been reported from marine and freshwater sites worldwide (Dujardin 1841, Gibbons 1874, Klebs 1893, Schewiakoff 1893, Playfair 1921, Eyden and Vickerman 1975, Bernard *et al*. 2000, Lee 2001).

***Urceolus cyclostomus* (Stein, 1878) Mereschkowsky, 1881 (Figs 7k; 9l, m)**

(Basionym: *Phialonema cyclostomum*)

Observations: Gliding metabolic euglenid, 40 µm long, sac shaped body, anterior end flared, slightly pointed posteriorly. Flagellum about 1.5 times the length of the cell, inserting into a flagellar pocket. With an ingestion organelle comprised of two rods. Body surface with well developed ridges. Nucleus is located near the centre of the cell. Observed from Carlton River.

Remarks: The members of this genus have been reviewed by Huber-Pestalozzi (1955), Larsen (1987) and by Larsen and Patterson (1990). There can be considerable ambiguity in species identity because of the poor nature of original descriptions and because detail is often obscured by adhering material. *Urceolus macromastix* has an irregular collar, and *U. cristatus* is considerably smaller and has a ridge on one side of the body. *Urceolus pascheri*, *U. ovatus* and *U. gobii* are also considerably smaller species. *Urceolus sabulosus* is similar in size but distinguished by adhering particles, a criterion which may not prove to be robust. *Urceolus cyclostomus* has previously been reported from freshwater and marine in Australia (Playfair 1921, Lee 2001).



Figs 10 a-n. a, b - *Mastigamoeba* sp. 1., showing nucleus; **c, d** - *Mastigamoeba* sp. 2, **c** - note pseudopodia; **e** - *Petalomonas* sp. 1.; **f, g** - *Petalomonas* sp. 2; **h, i** - *Petalomonas* sp. 3, **h** - ventral view showing a groove, **i** - dorsal view showing a groove; **j** - *Ploeotia* sp.; **k-m** - *Rhynchobodo* sp., **l** - flexible body, **m** - showing groove; **n** - *Rhipidodendron splendidum*. All micrographs are DIC images with the exception of (q) which is a phase contrast image. Scale bar 5 µm for all figures.

Other species were encountered during the study, but not recorded adequately: *Neobodo saliens, Sphenomonas angusta*.

Category 2: Taxa unassignable to species

Finally, we have also observed organisms from the several other genera but which we are unable to assign to species.

***Bicosoeca* sp.** (Fig. 9n): Stramenopile. Cells are oval and 10 µm long in cylindrical lorica measuring about 15 µm long, lorica slightly narrowed at its opening. Two flagellar insert sub-apically. The front flagellum is about 25 µm long and the posterior flagellum is slightly longer than the cell. Observed from Bothwell.

***Cercomonas* sp.** (Figs 9o, p): Cercomonad. Cells are fusiformis and 20-25 µm long with rows of granules running alongside the adherent portion of the posterior flagellum. The anterior flagellum is slightly shorter than the cell and the posterior flagellum is about 1.5 times the length of the cell. Nucleus is in the anterior part of the cell. Observed from Meadowbank.

***Hexamita* sp.** (Fig. 9q): Diplomonad. Cell measuring 16 µm long. Several contractile vacuoles are located below the midline of the cell. Two nuclei are located anteriorly. Observed from Carlton River.

***Mastigamoeba* spp.**: Pelobiont. Several cells observed 11-15 µm long (Figs 10a, b), with single long anterior flagellum, posterior end of some individuals capable of producing pseudopodia, body may be compact or elongate with nucleus anterior or near midline of cell, may represent several species. Observed from Carlton River. The organisms are similar to *M. punctachora* Bernard, Simpson *et* Patterson, 2000. Another organism (Figs 10c, d) observed from Carlton River is about 14 µm long and similar to *M. simplex* Kent, 1880.

***Petalomonas* spp.**: Euglenid. The first species (Fig. 10 e) is about 8 µm long with a pointed posterior end and thin lateral margins and probably a single broad median ridge. Observed from Carlton River. Two cells were observed of the second species (Figs 10f, g), from Carlton River. They measured 10 and 14 µm long. Anterior end flattened, flagellar pocket with wide opening, with faint surface ridges and punctae. A third species (Figs 10h, i) is about 12 µm long with two grooves; the ventral one is like that of *Notosolenus scutulum*. The single flagellum is about 1.5 times the length of the cell. Observed from Meadowbank. The organism was also found at Botany Bay (Lee 2001).

***Ploeotia* sp.** (Fig. 10j): Euglenid. One cell observed, about 14 µm long and flattened with ingestion organelle. There are three ventral grooves and about 4 dorsal grooves. Nucleus is near the centre of the cell. Observed from Carlton River.

***Rhynchobodo* sp.** (Figs 10k-m): Eugelnozoon. One cell observed, about 20 µm long, metabolic with spiral groove and with large inclusions as food vacuoles. Observed from Carlisle beach.

DISCUSSION

As a contribution to the study of the geographic distribution of free-living heterotrophic flagellates, species occurring in a number of freshwater sites in Tasmania (Australia) are reported. Fourty four species are described with uninterpreted records based on light-microscopy and the records include an account of one new species: *Ploeotia tasmanica* sp. n. Of the 44 species, 23 species (*Adriamonas peritocrescens, Amastigomonas debruynei, Ancyromonas sigmoides, Astasia* cfr. *gomphonema, Bodo saltans, Ciliophrys infusionum, Codosiga botrytis, Colpodella vorax, Goniomonas truncata, Gyromonas ambulans, Heteromita globosa, Heteronema exaratum, H. globuliferum, Microcometes paludosa, Neobodo designis, Parabodo caudatus, Petalomonas poosilla, Ploeotia tasmanica* sp. n., *Protaspis simplex, Protaspis obliqua, Reclinomonas Americana, Rhynchomonas nasuta*) are new to Australian freshwater sites, but 11 species (*A. debruynei, A. sigmoides, C. infusionum, H. exaratum, H. globuliferum, N. designis, P. caudatus, P. poosilla, P. simplex, P. obliqua, R. nasuta*) have been reported from Australian marine sites. The majority of species encountered here have also been found at other locations world-wide. The relative lack of novelty provides little support for the existence of endemic biota among this group of organisms, as has been shown to also be the case with marine species. We conclude that the hypothesis that geographical location of discontinuous environments does not influence the communities of heterotrophic flagellates remains unfalsified. This suggests that contiguity of habitats has not been a major determinant in the distribution of heterotrophic flagellates.

The literature to date is not explicit in dealing with geographic distribution of free-living protozoa. The taxonomic literature from Northern Europe and North America has been regarded as largely interchangeable,

suggesting that the communities of organisms are very similar. However, reports of organisms are frequently made in the absence of uninterpreted records - rendering most records largely anecdotal. It is for this reason that this paper contains uninterpreted records, even of familiar species. The literature also contains a number of statements arguing that some groups have a geographically restricted distribution (e.g. Foissner *et al*. 1988; Tyler 1996a, b). The studies which distinguish biological species of protozoa from morphological species do tend to reveal a more restricted distribution of those species (*Euplotes*, *Crypthecodinium*, *Tetrahymena*) (Nanney and McCoy 1967, Beam and Hines 1987, Bowers *et al*. 1998), but such studies have yet to be extended to the heterotrophic flagellates. The issues of cosmopolitanism versus endemism have yet to be fully resolved and probably will require agreement as to what constitutes a species.

We hold that our understanding of the biogeographic distribution of organisms may be attributed not only to biological properties intrinsic to the organisms, but also to factors which are extrinsic to the biology (Lee and Patterson 1998, Patterson and Lee 2000). Among the latter are distortions which might arise from differing taxonomic preferences, incomplete sampling regimes, and incomplete or selective reporting. To address some of the problems arising from taxonomic uncertainty, we have favoured an approach in which we rely heavily on uninterpreted records (Larsen and Patterson 1990; Vørs 1992a, b; Patterson *et al*. 1993; Ekebom *et al*. 1996; Patterson and Simpson 1996; Tong 1997a, b, c; Tong *et al*. 1997, 1998; Lee and Patterson 2000; Al-Qassab *et al*. 2002; Lee 2002; Lee *et al*. 2003). Drawing on the information relating to marine heterotrophic flagellates, Ekebom *et al*. (1996) concluded that there was no case for endemism of these organisms, a perspective sustained by subsequent studies (Lee and Patterson 1998, Patterson and Lee 2000, Al Qassab *et al*. 2002). Ekebom *et al*. sought to rationalise the cosmopolitan distribution by reference to the contiguous nature of the habitat and the high numbers of flagellates. Both factors will favour dispersal mechanisms, and reduce the isolating mechanisms which are likely to lead to endemism. More recently, a rare organism (*Postgaardi mariagerensis*) occupying non-contiguous marine locations has been reported from both northern temperate and Antarctic locations (Simpson *et al*. 1997), suggesting that factors other than population size and contiguity of habitats may be operating.

As freshwater habitats are more short-lived than marine ones, and because they are not contiguous, we have sought to extend our studies to such habitats. Of the 44 species observed here only one is new and all others have been reported from other geographic sites. So, despite claims for microbial endemism in Tasmanian lakes (Bowling *et al*. 1993; Tyler 1996a, b) we are unable to support the case for endemism in freshwater flagellates, at least at the level of the morphological species, as currently recognised. Recently, the other group (ciliates) of protists has been reported from a crater lake in Tasmania by Esteban *et al*. (2000), who conclude that the ciliate fauna of Tasmania is similar to that in Europe and other parts of the world. Their result also does not support the case for endemism in freshwater ciliates.

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ACTA
PROTOZOOLOGICA

Deviata estevesi sp. n. (Ciliophora: Spirotrichea), a new ciliate protist from a restinga lagoon in Rio de Janeiro, Brazil

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Summary. In samples collected from the Cabiúnas Lagoon, located in the north region of Rio de Janeiro, Brazil, we found *Deviata estevesi* sp. n., a spirotrich ciliate protist characterized as: *Deviata* measuring 80-110 × 25-50 µm *in vivo*, body flexible and contractile; narrowed at the anterior end and broad at the posterior end; cortical granules absent; dark coloration under dissection microscope. Cirri arranged in seven rows right of adoral zone of membranelles and four rows left of it. Contractile vacuole located in the left mid-body, away from the margins. Buccal cirrus lacking. Macronucleus usually composed of two small ovoid or elliptical nodules. Two dorsal kineties, with right kinety posterior shortened. Stomatogenesis begins with oral primordium of the opisthe developing from very close to the terminal cirrus of row R3.

Key words: *Deviata estevesi* sp. n., Kahliellidae, lagoon, Macaé, restinga, Rio de Janeiro, South America.

INTRODUCTION

Eigner (1995) has redefined the family Kahliellidae Tuffrau, 1979, as "Euhypotrichina with more than one longitudinal cirral row on right side of body. Neokinetal anlagen develop during morphogenesis". In the same paper, he erected the genus *Deviata* to include the new species *Deviata abbrevescens*, placed within the Kahliellidae, and *D. bacilliformis*, as new combination for *Kahliella bacilliformis* (Gelei, 1954) sensu Gelei (1954) and Berger and Foissner (1987). Eigner also mentioned that the French population of *Kahliella bacilliformis*, presented by Fleury and Fryd-Versavel (1984) could actually be a third species of *Deviata*, because of its ciliary configuration. In subsequent papers, Eigner (1997, 1999) transferred the genus *Deviata* to the family Oxytrichidae (Ehrenberg, 1838), based on morphogenetic studies. Such discussion is not in focus here.

In this paper, we present a morphological study of *Deviata estevesi*, a new species discovered in samples of brackish water with bottom sediment from a coastal lagoon located in Rio de Janeiro (Brazil) and compare it with the remaining congeners. This species is also the first occurrence of *Deviata* reported for Brazilian locations.

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MATERIALS AND METHODS

Study area: the Cabiúnas Lagoon (Figs 1, 2) is a restinga lagoon located in the city of Macaé (Rio de Janeiro), and is part of a complex of coastal lakes in the Parque Nacional da Restinga de Jurubatiba. This is a federal area of ecological preservation, which extends over 44 km along the northwest coast of the Rio de Janeiro State, between the cities of Macaé and Quissamã. Limnological studies on this and the other lagoons in this region were conducted by Esteves *et al*. (1983) and Petrucio (1998).

Methodology: *Deviata estevesi* was discovered in samples of brackish water and sediment from the bottom of the lagoon (1.2 m depth), in July of 2003. The water was dark colored due to the heavy concentration of detritus in suspension and the presence of humic substances. Samples were stored in hermetic flasks and brought to the laboratory the same day they were obtained. Raw cultures in Petri dishes were established by adding crushed rice and wheat grains to aliquots of the samples. After two days, the ciliates were highly abundant and were isolated by means of micropipettes under the dissecting microscope. Morphological studies included *in vivo* observations, protargol-impregnation following the protocol proposed by Dieckmann (1995), and scanning electronic microscopy (SEM) according to Silva-Neto (1994).

Measurements of living specimens and observations on the body outline shape in various angles were made at a magnification of 100-200× on coverless slides. Magnification of 630-1000× with differential interference contrast (DIC) was used to check for the presence of cortical granules. The approximate measures of cirri and adoral membranelles were made at 400-1000× with phase contrast. The biometric data presented in Table 1 was made from observations at a magnification of 1000×, using bright field microscopy. The values were obtained from protargol impregnated interphasic specimens selected according to the criteria proposed by Berger and Hatzidimitriou (1978).

Continuous biometric characters were obtained as following: (i) body length and width are the measurement of the major longitudinal body axis and its central orthogonal line; (ii) length and width of the macronuclear nodules were obtained by measuring each nodule along its major orthogonal axes in respect to their orientation; (iii) the distance between macronuclear nodules corresponds to the distance measured from the internal opposing end of each nodule, parallel to the body length; (iv) the length of adoral zone of membranelles (AZM), corresponds to the distance between the anterior end of the body to the basis of the first proximal membranelle. The statistic procedures were conducted according to Sokal and Rohlf (1981) and calculated with a 99% confidence interval.

The drawings of living specimens are reconstructions based on sketches and notes taken from observations of organisms *in vivo.* Drawings of protargol-impregnated specimens were made with a camera lucida, at 1000× magnification, using bright field microscopy. They are based on the most representative specimens present in the slides. All drawings are shown with the anterior end of the organisms pointing to the top of the page.

RESULTS

Deviata estevesi sp. n.

Diagnosis: Size *in vivo* about: 100 × 40 µm (n=15). Dark, or almost black coloration under dissection microscope. Cortical granules lacking. Cytoplasm filled with

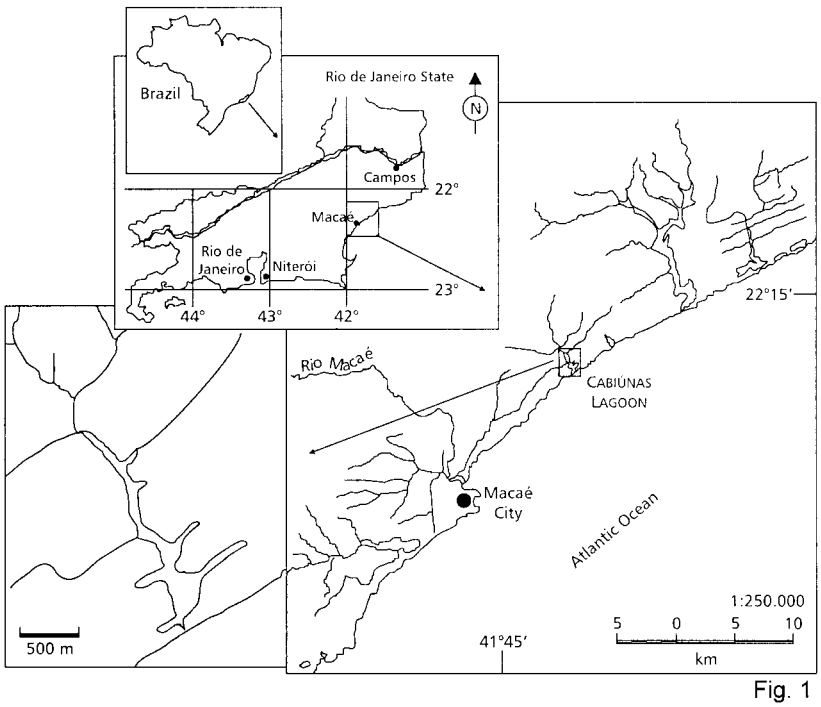
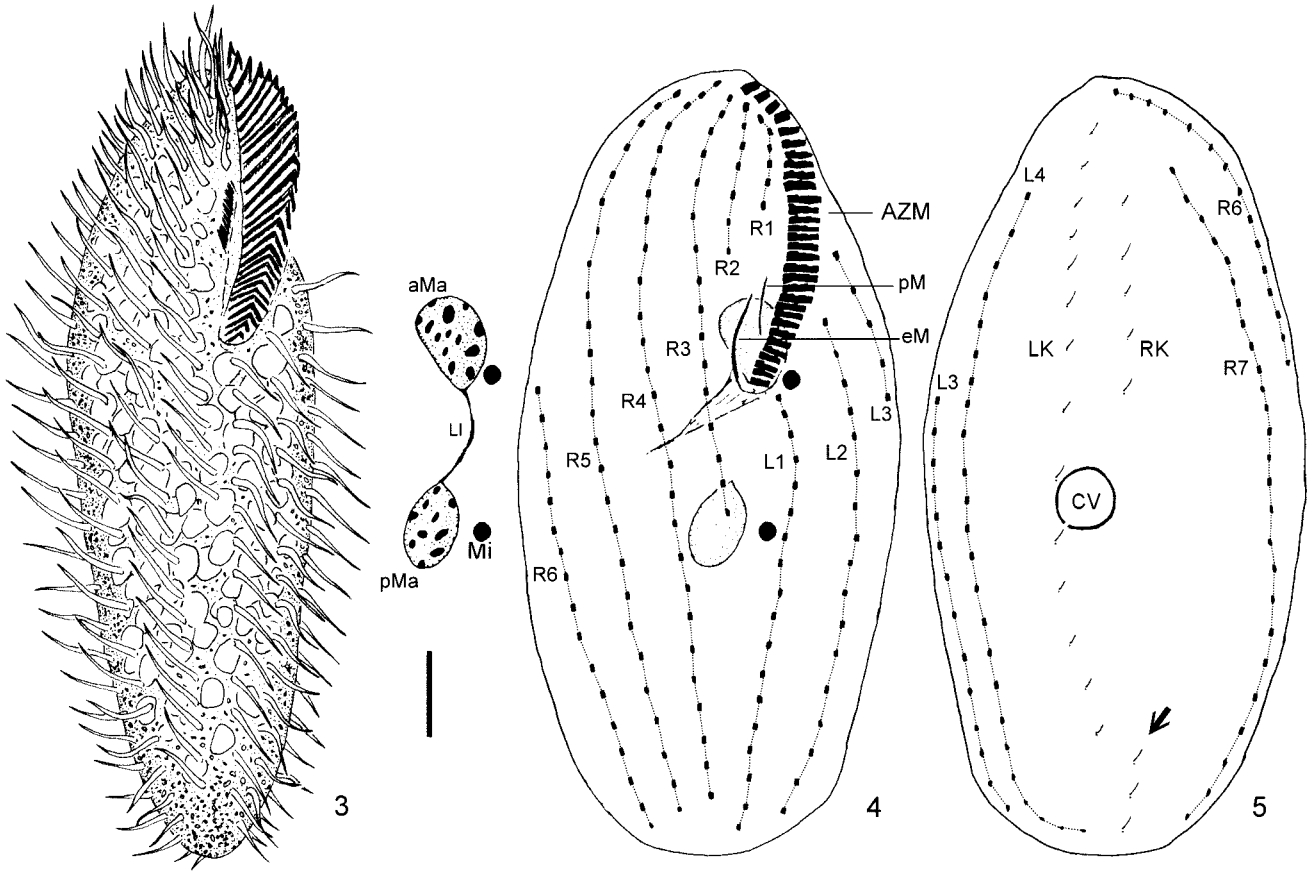


Fig. 1

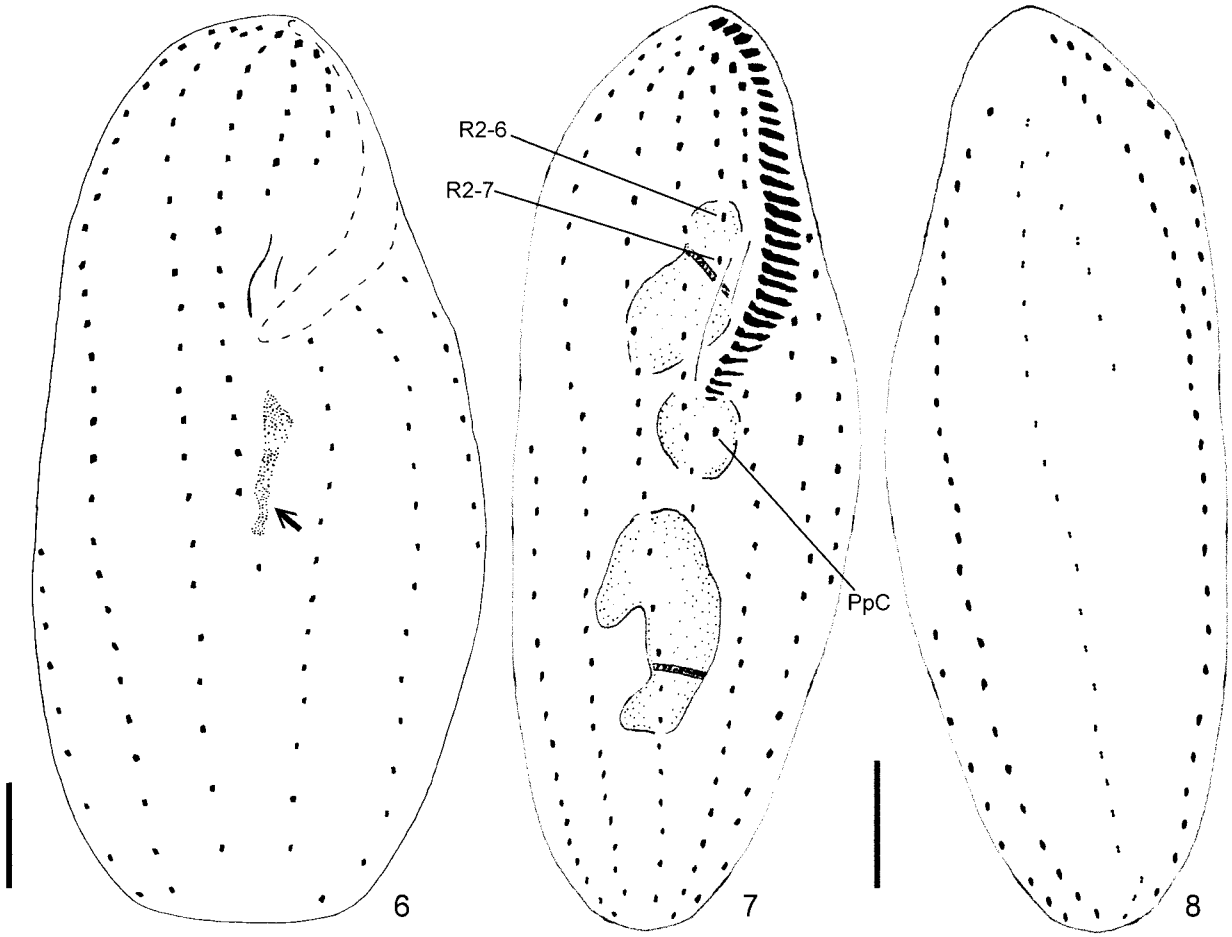
Figs 1, 2. The Cabiúnas Lagoon; **1** - map showing the location of the lagoon along the Brazilian coast (from Branco 1998); **2** - picture showing the location from where the samples containing *Deviata estevesi* sp. n. were collected.



Figs 3-5. *Deviata estevesi* sp. n. **3** - ventral side of living *specimen*; **4** - drawing of protargol impregnated specimen, showing ventral cirral pattern, morphology and relative position of nuclear apparatus; **5** - drawing of protargol impregnated specimen, showing dorsal cirral pattern and dorsal kineties (the cilia on dorsal kineties were reconstructed based on scanning electron micrographs). Arrow points to the short file of cilia located close to the posterior end of body. aMa - anterior macronuclear nodule, AZM - adoral zone of membranelles, eM - endoral membrane, L(x) - cirral rows located left of adoral zone of membranelles, LI - linking isthmus between the two macronuclear nodules, LK - left dorsal kinety, Mi - micronucleus, pM - paroral membrane, pMa - posterior macronuclear nodule, R(x) - cirral rows located right of adoral zone of membranelles, RK - right dorsal kinety. Scale bar 10 µm.

compact crystals measuring about 3-5 µm. Body flexible and contractile, with outline variable, but usually narrowed at the anterior end and broad at the posterior end. Dorsoventral flattened in the anterior region and ellipsoid in cross section below the cell equator (Figs 3, 10, 11). With small, round contractile vacuole located at the mid-body, away from its margins. Cirri arranged in 7 rows right of adoral zone of membranelles and 4 rows left of it. Row R1 with 5-7 cirri; R2 with 6-9 cirri; R3 always ending in equatorial region of body. Buccal cirrus absent. Nuclear apparatus composed of usually two macronuclear nodules and two micronuclei. Two dorsal kineties, right kinety posterior shortened. A short file of dorsal cilia, possibly an extension of the right kinety, is present at the posterior region of body.

Morphological characterization: The cytoplasm is filled with numerous compact, 3-5 µm big, transparent light-greenish crystals that often cause cell rupture during fixation and do not stain with protargol, but remain visible in slides (Figs 3, 13-18, 22). Such crystals tend to be less numerous in specimens of old cultures, possibly due to starvation. The crystals are located about 3 µm distant from the body wall, separated by a "layer" containing a smaller, irregular shaped kind of cytoplasmic granulations (Fig. 18). The contractile vacuole is round and lacks lacunar collector ducts. It is inconspicuously located at the mid-body of the organism, close to its inner dorsal margin, and below the level of the back side of the adoral zone of membranelles (AZM) (Figs 5, 9, 17). Full diastole occurs at intervals of approximately



Figs 6-8. *Deviata estevesi* sp. n. **6** - ventral side of protargol impregnated specimen showing development of oral primordium, pointed by a short arrow; **7** - ventral side of protargol impregnated specimen which shows a postperistomial cirrus and very irregular shaped macronuclear nodules; **8** - same specimen, in dorsal view. PpC - postperistomial cirrus, R2-6 - 6th cirrus of 2nd cirral row right of adoral zone of membranelles, R2-7 - 7th cirrus of 2nd cirral row right of adoral zone of membranelles. Scale bars 10 µm.

10 seconds, followed by a brief systole lasting about 1 second.

The adoral zone of membranelles shows a conspicuous shape for this genus, and is composed of 29-33 membranelles. It occupies about 38% of body length. In addition, as observed in similar species, it terminates at the apex of body, not extending much into the right margin. The membranelles located in the middle region of AZM measure about 5 µm. Paroral membrane anterior to endoral, never intersecting it (Figs 3, 4, 14-16, 29, 30).

All studied specimens show seven cirral rows right of the AZM and four rows left of it. All cirral rows are comprised of very thin cirri, which in the equatorial region, are about 8-12 µm long and formed by four cilia (Fig. 31). Rows R1 and R2 end anterior to the infundibulum. Row R3 ends in the equatorial region of the cell. Row R4 begins anterior to the first cirrus of row R3, extending along the ventral surface to the posterior end of the cell. Row R5 begins to the right of R4, and usually at the level of the first cirrus in R3. The rows R6 and R7 begin in the dorsal region, but R6 twists to the ventral side in the posterior region of the cell (Figs 4, 5).

The first row left of the AZM, L1, begins close to the infundibulum border, posterior to the proximal adoral membranelles and extends to the posterior end of the cell. Row L2 begins to the left of L1, starting from the middle part of the AZM, extending to the posterior region of the cell. Row L3 begins close to L2, and twists to the dorsal surface in the equatorial region. It ends close to the posterior end of the cell, curving inwards to the opposite body margin. Row L4 begins on the dorsal

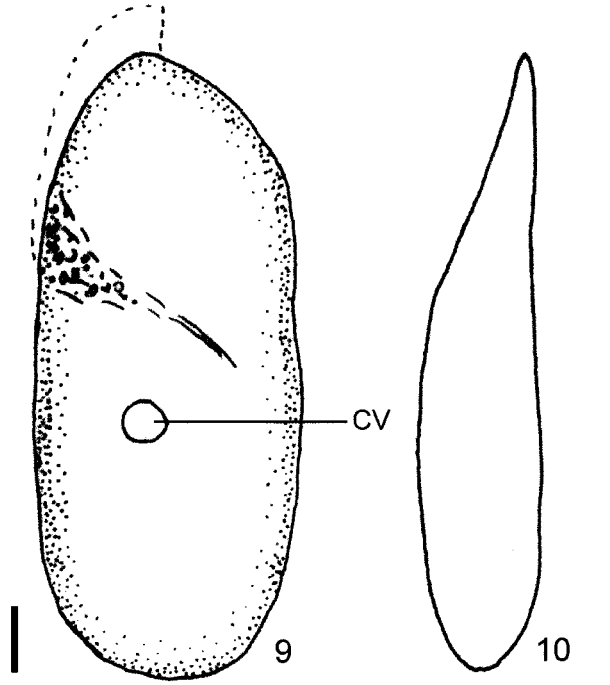
Table 1. Biometric characterization of *Deviata estevesi* sp. n. All measurements are in µm. $\bar{x}$ - arithmetic mean, M - median, SD - standard deviation of the arithmetic mean, SE - standard error of the arithmetic mean, CV - coefficient of variance (in %), Min - minimum value observed within the sample, Max - maximum value observed within the sample, n - sample size.

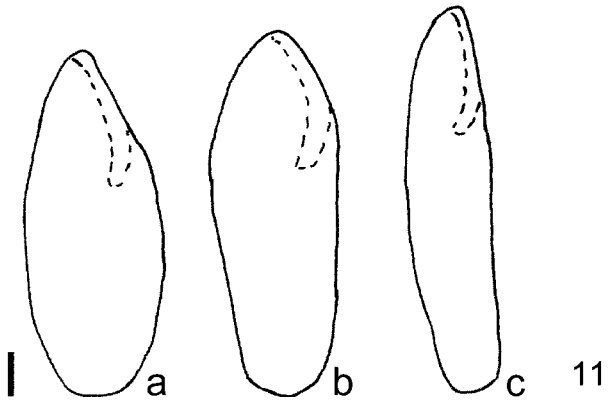
Character	$\bar{x}$	M	SD	SE	CV	Min	Max	n
Body length	86.20	84	7.42	1.49	8.61	75.00	100.00	25
Body width	42.72	44	8.73	1.75	20.42	27.00	60.00	25
Length of AZM	32.50	32.50	2.86	0.61	8.78	28.00	40.00	22
Number of adoral membranelles	30.32	30.00	1.52	0.33	5.03	28	33	22
Number of cirri rows left of AZM	4.00	4.00	0	0	0	4	4	20
Number of cirri in L1	17.90	19.00	1.77	0.46	9.90	14	20	15
Number of cirri in L2	18.20	18.00	2.24	0.58	12.32	14	20	15
Number of cirri in L3	21.88	22.00	1.76	0.43	8.06	19	25	17
Number of cirri in L4	24.47	24.00	3.20	0.78	13.09	20	32	17
Number of cirri rows right of AZM	7.00	7.00	0	0	0	7	7	25
Number of cirri in R1	5.95	6.00	0.50	0.11	8.36	5	7	21
Number of cirri in R2	7.38	7.00	0.86	0.19	11.71	6	9	21
Number of cirri in R3	16.00	16.00	1.92	0.43	12.00	13	20	20
Number of cirri in R4	24.72	24.00	2.52	0.59	10.18	22	32	18
Number of cirri in R5	27.94	28.00	2.14	0.52	7.64	24	32	17
Number of cirri in R6	31.38	31.50	2.22	0.55	7.07	25	35	16
Number of cirri in R7	27.75	27.00	1.96	0.57	7.06	25	32	12
Number of dorsal kineties	2.00	2.00	0	0	0	2	2	20
Number of macronuclear nodules*	2.10	2.00	0.45	0.10	21.30	2	4	20
Length of anterior macronclear nodule*	13.32	13.00	2.10	0.45	15.78	10.00	19.00	22
Width of anterior macronuclear nodule*	8.23	7.5	2.22	0.47	27.03	5.00	12.00	22
Length of posterior macronuclear nodule*	12.41	12.00	1.99	0.41	15.46	9.00	16.00	22
Width of posterior macronuclear nodule*	8.09	8.5	2.22	0.47	27.48	5.00	14.00	22
Distance between macronucelar nodules*	14.09	14.00	3.32	0.71	23.58	9.00	20.00	22
Number of micronuclei	2.16	2.00	0.37	0.07	17.32	2	3	25
Diameter of micronuclei	2.86	3.00	0.31	0.06	10.73	2.00	3.00	25

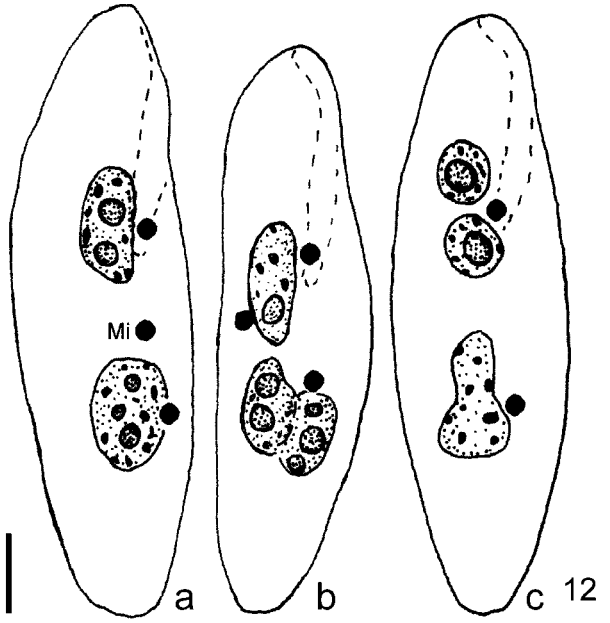
* Measured only in specimens with two not bisected macronuclear nodules.

surface, not twisting to the ventral side. It ends close to the end of L3, curving inwards even more to the opposite margin (Figs 3-5, 21, 23, 24, 29-31). One specimen in a sample of 25 showed a postperistomial cirrus immediately posterior to the infundibulum vertex (Figs 7, 8). This specimen, which has a rather irregular macronucleus, also has the terminal cirrus of row R2 placed close to the anterior right of the endoral membrane. Such cirrus and the one anterior to it are relatively more spaced than the other cirri in this row. On the dorsal side, there are two ciliary rows (kineties) composed of dikinetids (Figs 5, 8, 21, 28, 32). The left kinety begins anterior to the first dikinetid of the right kinety, extending to the posterior region of the organism. The right kinety usually begins close to the anterior region of kinety R7, and is interrupted in the equatorial region of the organism. In the posterior end of body, there is a small file of 3-5 (n=5; $\bar{x}$ =3.40; SD=0.89) dikinetids. Its anteriormost dikinetid is always aligned to the terminal dikinetid of the right kinety. Therefore, this file is possibly the continuation of the right dorsal kinety.

The nuclear apparatus is usually composed of two small, ovoid macronuclear nodules and 2-3 micronuclei. On average, the anterior macronuclear nodule measures 13 × 8.5 µm and the posterior nodule measures 12.5 × 8 µm (n=20). In the specimens with fewer cytoplasmic crystals, the nodules tend to be elliptical, sometimes irregular shaped, and show increased size in relation to the body overall measurements. In some specimens, we noticed that the macronuclear nodules are narrowed in their equatorial region or bisected (Figs 12, 23-26). The macronucleus contains internal bodies that measure 1-5 µm, and the larger of these stain in lighter tonality than the smaller ones (Figs 24, 26). The micronuclei measure about 3 µm in cross section, and are in number of two or three, often adjacent to a macronuclear nodule. The entire nuclear apparatus is located in the middle-left region of body, away from both lateral margins (Figs 4,







20). The anterior macronuclear nodule is usually placed posterior to the proximal region of the AZM. The nodules are linked by a very thin isthmus, as reported by Eigner (1995) for *D. abbrevescens* and by Walker and Goode (1976) for other spirotrich ciliates. This structure could only be seen in a few interphasic specimens, possibly because it stains poorly with protargol.

In a sample of 25 specimens, some other variations of the nuclear apparatus morphology were observed in cells with fewer cytoplasmic crystals: (i) four specimens with three micronuclei; (ii) two specimens with macronuclear nodules narrowed in their equatorial region or bisected; (iii) one specimen with four macronuclear nodules; and (iv) one specimen with a very irregular shaped macronucleus (Figs 23-26). Discoid macronuclear DNA replication bands, characteristic in spirotrich ciliates (Olins and Olins 1994), appear at the anterior half of the anterior nodule and posterior half of the posterior nodule (Figs 23, 26).

Stomatogenesis begins with oral primordium of the opisthe developing from very close to the terminal cirrus of row R3 (Figs 6, 22). The biometric characterization of *Deviata estevesi* is shown in Table 1.

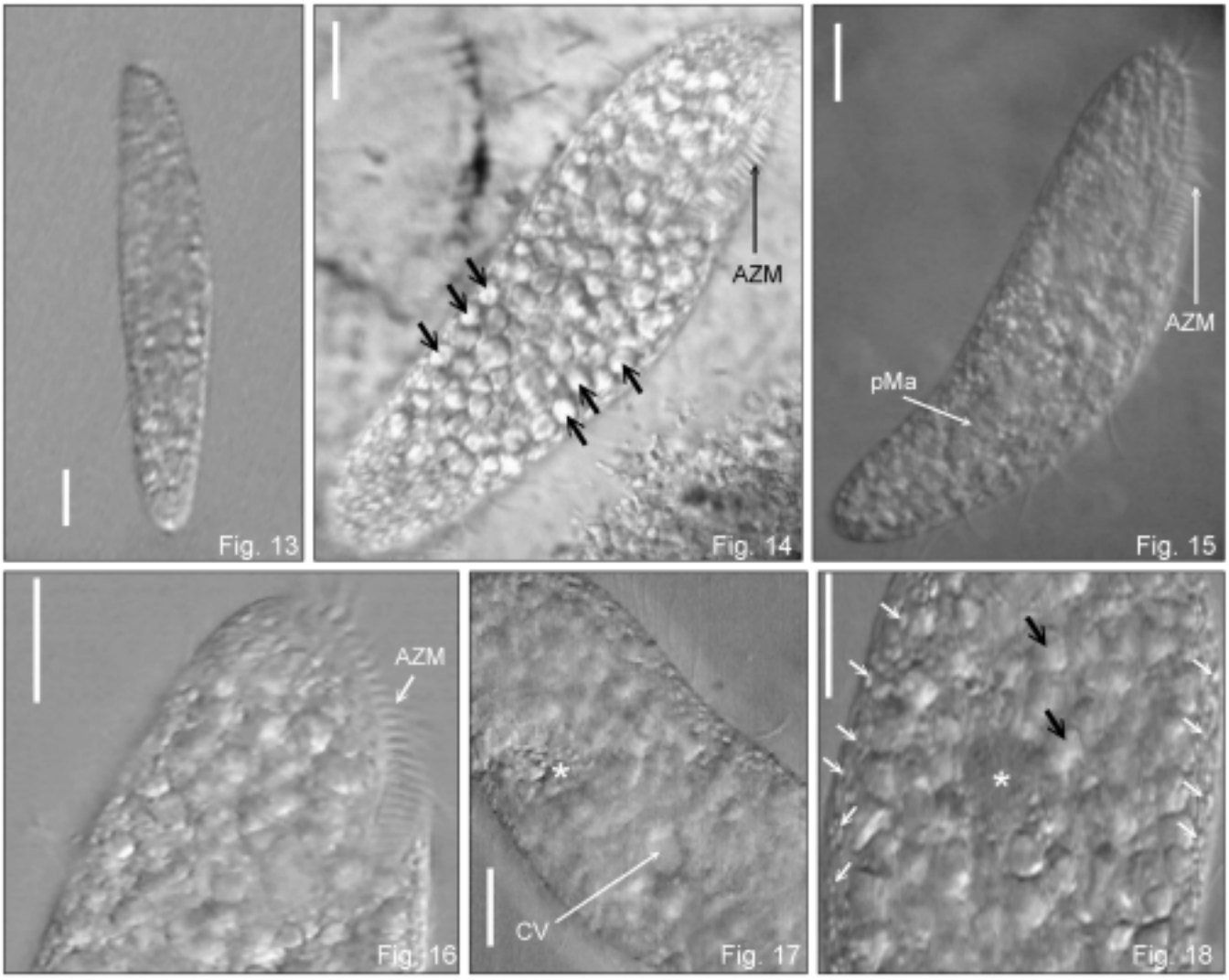
Type specimens: slides containing the holotype (IBZ-UFRJ 0008-6-1 slide) and paratypes (IBZ-UFRJ 0008-7-1 slide) of *Deviata estevesi* were deposited in the collection of Laboratório de Protistologia, Dept. de Zoologia, Inst. de Biologia - CCS, Universidade Federal do Rio de Janeiro (UFRJ).

Etymology: species name in dedication to Prof. Dr. Francisco de Assis Esteves, coordinator of Núcleo de Pesquisas Ecológicas de Macaé - NUPEM.

Type locality: Cabiúnas Lagoon, Macaé - RJ, Brazil. Geographic coordinates of sampling site: S 22° 17'46.7" W 41° 41' 32.3". Water characteristics: pH 7.24; dissolved [O_2] 3.29 mg/dm^3; temperature 22.9°C; conductivity 1875 µS; salinity 1.0‰.

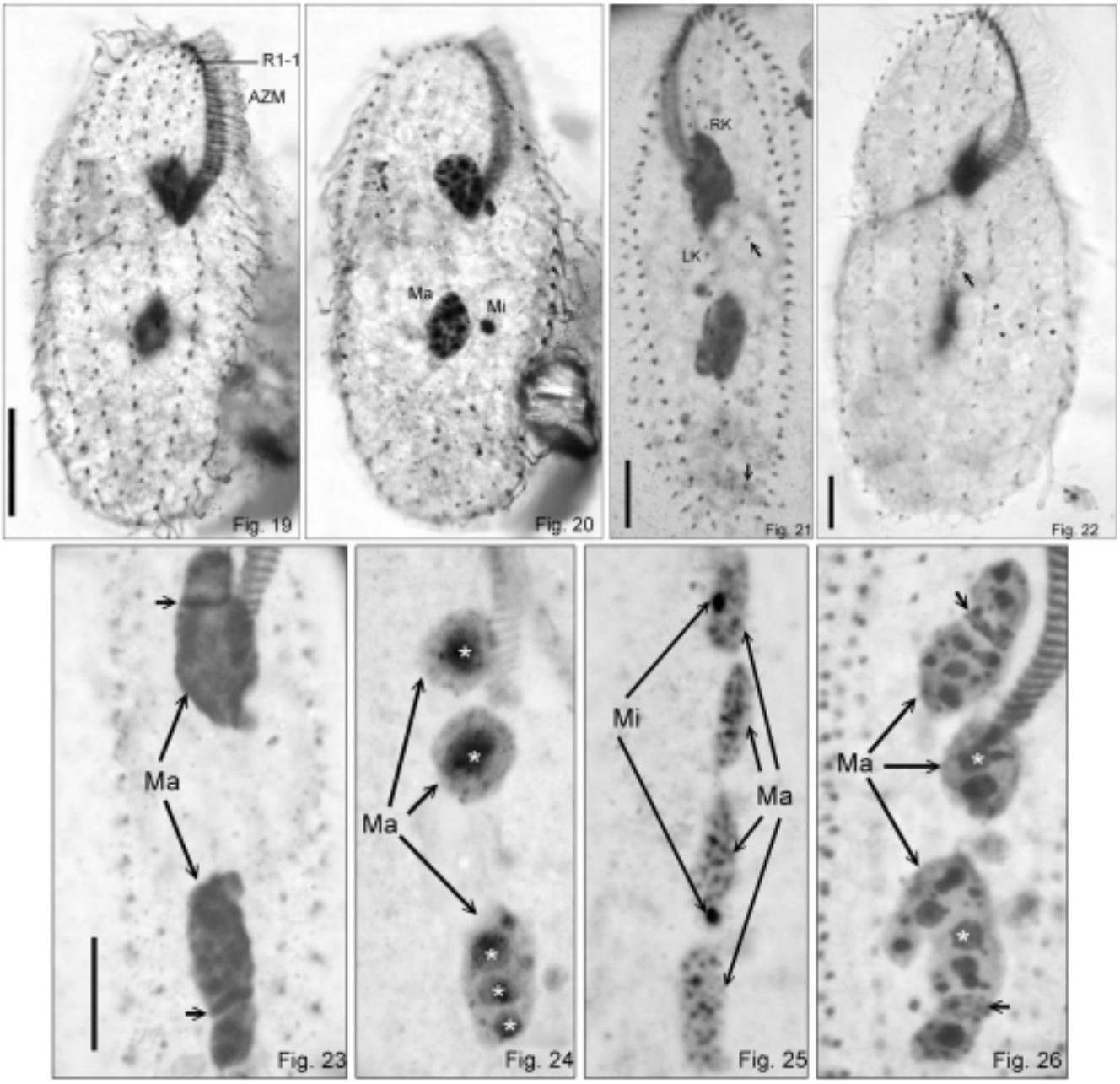
Ecological remarks: *Deviata estevesi* is bacterivorous and occurred simultaneously with *Coleps*

Figs 9-12. *Deviata estevesi* sp. n. **9** - dorsal surface of living specimen squeezed under a coverslip, showing contractile vacuole position; **10** - scheme of specimen in lateral view, from life; **11 a-c** - schematic drawings showing shape variations observed from living specimens under stereoscopic microscope. CV - contractile vacuole. **12a-c** - schematic drawings of anomalous nuclear apparatus present in protargol impregnation slides. **a** - with elliptical, enlarged macronuclear nodules and 3 micronuclei, **b** - specimen showing elliptical anterior and irregular shaped macronuclear nodule and posterior macronuclear nodule, with 3 micronuclei, **c** - macronucleus with bisected anterior nodule and narrowed posterior nodule, 2 micronuclei. Scale bars 10 µm.

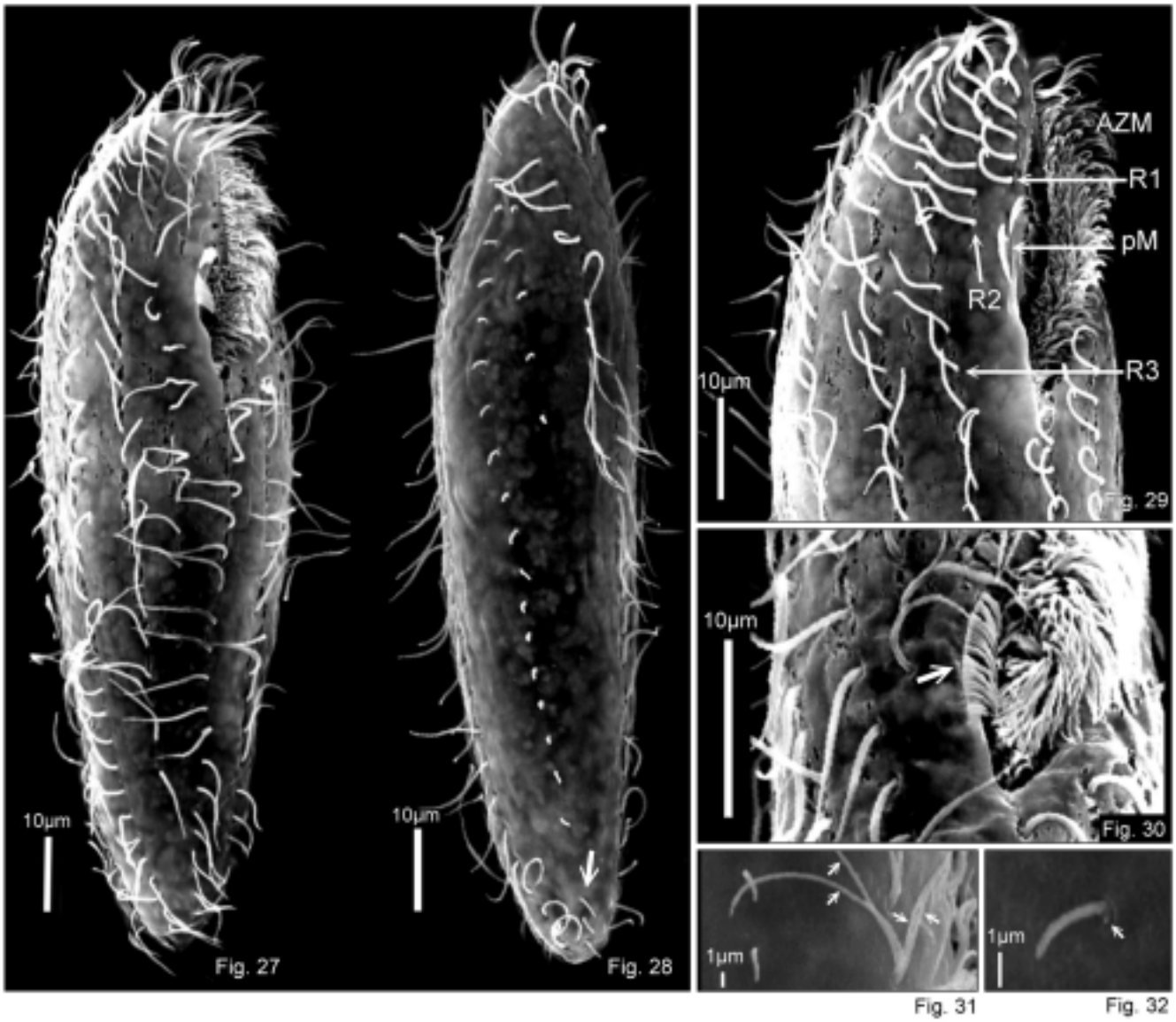


Figs 13-18. Light micrographs of living *Deviata estevesi* sp. n. **13 -** free motile specimen seen in dorsal view; **14 -** specimen seen under a coverslip, showing ventral surface and cytoplasmic crystals, marked by short black arrows; **15 -** specimen with very few cytoplasmic crystals; **16 -** anterior region of ventral surface, showing detail of the adoral zone of membranelles; **17 -** dorsal view of specimen heavily squeezed under a coverslip, showing contractile vacuole, located right below the infundibular region. Asterisk marks the back side of the oral region; **18 -** specimen showing macronuclear nodule (asterisk), cytoplasmic crystals (short black arrows) and layer with cytoplasmic granulations (short white arrows). AZM - adoral zone of membranelles, CV - contractile vacuole, pMa - posterior macronuclear nodule. Scale bars 10 µm.

elongatus Ehrenberg, 1830, *Cristigera* sp., and some scarce specimens of *Brachonella spiralis* (Smith, 1897) and *Saprodinium dentatum* Lauterborn, 1901. In enrichment cultures, *D. estevesi* was the only species left after 6-8 days, with the exception of few specimens of *Cristigera* sp. and numerous specimens of *Chilodonella uncinata* Ehrenberg, 1838, which had excysted. An important observation about *D. estevesi* is that it does not show highly thigmotactic behavior. It sometimes used to glide in the bottom of Petri dishes, but very rarely attached to sediment particles and crawled over them, as seen in other interstitial or bottom-dwelling spirotrichs (Kattar 1970). Eigner (1995) reported that *D. abbrevescens* sometimes stays anchored in the bottom of Petri dishes by its anterior end. Such behavior was not observed in *D. estevesi*, which kept swimming among sediment particles most of the time, rotating around its major body axis. It was not observed to occur in the water column high above the sediment, neither was present in the samples of sediment with water



Figs 19-26. *Deviata estevesi* sp. n. protargol impregnated specimens. **19** - ventral side, showing cirral pattern and adoral zone of membranelles; **20** - nuclear apparatus; **21, 22.** *Deviata estevesi* sp. n. protargol impregnated specimens; **21** - dorsal side, showing dorsal cirral pattern and dorsal kineties. Short arrows indicate the gap between the terminal region of the right dorsal kinety and the small file of cilia located close to the posterior border of cell; **22** - specimen showing oral primordium formation (short arrow). Asterisks mark some of the cytoplasmic crystals, which do not stain with protargol, however, remain visible after the process; **23-26 -** variations of *Deviata estevesi* sp. n. nuclear apparatus found in silver impregnated slides; **23 -** irregular, elongated nodules. Short arrows pointing to the discoid replication bands; **24 -** anterior nodule bisected; **25 -** with four macronuclear nodules; **26** - very irregular- shaped nodules. Asterisks on Figs 24 and 26 mark some large internal bodies which stain in different tonality (see text). AZM - adoral zone of membranelles, Ma - macronucleus, Mi - micronucleus, LK - left dorsal kinety, RK - right dorsal kinety. R1-1 - first cirrus of first cirral row right of adoral zone of membranelles. Ma - macronuclear nodules, Mi - micronuclei. Scale bars 10 µm (21, 23-26); 20 µm (19, 20, 22).



Figs 27-32. *Deviata estevesi* sp. n. scanning electron micrographs. **27** - ventral side; **28** - dorsal side. Short arrow marks the small file of cilia located close to the cell posterior border; **29** - ventral side, close view of anterior region, showing its cirral pattern; **30** - detail of the paroral membrane, marked by short arrow; **31** - detail of cirrus located in the equatorial region, formed by four cilia, which are indicated by short arrows; **32** - dorsal dikinetid, showing cilia and posterior aciliferous kinetosome, marked by a short arrow. AZM - adoral zone of membranelles, pM - paroral membrane, R1, R2, R3 - first, second and third cirral rows right of adoral zone of membranelles, respectively.

collected along the year of 2001, from the margin adjacent to the sand cord that separates the lagoon from the sea (Paiva and Silva-Neto 2004).

DISCUSSION

This species was included in genus *Deviata* because it matches most of the features mentioned in the original diagnosis by Eigner (1995), that is: it shows at least one cirral row right of AZM which ends in the equatorial region of body; parental rows are not preserved after division, which can be noticed by the constant number of cirral rows.

The most obvious difference from the other two congeners is the ventral cirral pattern in the anterior end of body (Figs 3, 4, 19, 21, 29, 30), which is highly constant in the studied population. In *Deviata abbrevescens* and

Table 2. Comparison among *Deviata abbrevescens*, *D. bacilliformis* and *D. estevesi* sp. n.

Character[1]	*Deviata abbrevescens*[2]	*Deviata bacilliformis*[3]	*Deviata estevesi*
Morphology of contractile vacuole	Small, round shaped vacuole, located left-midbody margin.	Slighty elongated vacuole, with lacunar collector ducts, located close to the left margin of body, extending from behind AZM to the proximity of the posterior end of body	Small, round shaped vacule, located at mid-body, away from the margins. (Figs 5, 9, 17)
Body size	90-300 × 16-40 µm ($\bar{x}$ = 172.4 × 27.1 µm; n = 25)	112-145 × 17-25 µm ($\bar{x}$ = 124.1 × 19.3 µm; n = 15)	75-100 ($\bar{x}$ = 86.2 µm) × 27-60 µm ($\bar{x}$ = 42.7 µm); n = 25
Length of AZM	23-32 µm ($\bar{x}$ = 27.7 µm; n = 20)	21-27 µm ($\bar{x}$ = 23.7µm; n = 15)	28-40 µm ($\bar{x}$ = 32.5 µm; n = 22)
Number of adoral membranelles	19-26 (M = 21; n = 20)	18-21 (M = 20; n = 15)	28-33 (M = 30; n = 22)
Number of dorsal kineties	2 (n = 20)	1 (n = 15)	2 (n = 25)
Morphology of macronuclear nodules[4]	2 elongated nodules, measuring about 2/3 of body length. Posterior nodule measures 20-100 ($\bar{x}$ = 50 µm; n = 25) × 4-8 µm ($\bar{x}$ = 5.2 µm; n = 20)	2–4 small, ellipsoid nodules (M = 2; n = 15). Posterior macronuclear nodule measuring 11-34 ($\bar{x}$ = 20 µm) × 4-7 µm ($\bar{x}$ = 5.8 µm); n = 15. Nodules sometimes bisect.	2 (rarely 4) small, variable shaped[5] nodules (n = 20). Posterior nodule measuring 9-16 ($\bar{x}$ = 12.4 µm) × 5-14 µm ($\bar{x}$ = 8.1); n = 22. Nodules sometimes bisect.

[1] Arithmetic mean $\bar{x}$ is shown for continuous characters. For discrete characters, median (M) shown; [2] Data from Eigner (1995); [3] Data from Berger and Foissner (1987); [4] Only the posterior nodule was used for comparison, because biometric data from the anterior nodule of *D. abbrevescens* and *D. bacilliformis* was not provided by the referred authors. Sometimes, *D. estevesi* may present 4 macronuclear nodules. See text; [5] The shape of macronuclear nodules has shown to be highly variable in this species, tending to be elliptical or irregular in specimens from old cultures. See text.

D. bacilliformis, the cirri in the first and second rows right of AZM are organized in sets of distinct, isolated frontal cirri and buccal cirrus. In *D. estevesi*, these rows, named in this paper as R1 and R2, are composed of 5-7 cirri and 6-9 cirri respectively, not forming distinct isolated frontal cirri, but comprising of short rows of that terminate anterior to the paroral membrane distal end. In addition, the buccal cirrus is lacking, except for one paratype, which shows the last cirri of row R2 more widely spaced than the remaining cirri in this row (Fig. 7). The contractile vacuole in *D. estevesi* is located in the mid-body (Figs 5, 9, 17) whereas in the other two congeners, it is located close to the left margin of the cell. Its inconspicuous location observed in *D. estevesi* makes the position of the contractile vacuole another suitable feature for species differentiation. Nevertheless, it does not show lacunar collector ducts, which are also absent in *D. abbrevescens*, but present in *D. bacilliformis*. Another feature of *D. estevesi* that distinguishes it from the remaining congeners is the right dorsal kinety, which is interrupted posteriorly. The short dorsal file composed of closely grouped cilia, present in the posterior end of body is also unique among the three species. Berger and Foissner (1987) studied a population of *D. bacilliformis* from Israel that presents spherical or elliptical colorless granules measuring 2 µm, and 2-5 µm large globules in the cytoplasm. From the drawings present in their paper, we think that these inclusions may be related to the cytoplasmic crystals that we observed in *D. estevesi*. Yet, those are reported as colorless. Table 2 shows a summary with main differences which we consider to be useful for discerning among the three species.

The French population of *Kahliella bacilliformis*, characterized by Fleury and Fryd-Versavel (1984), is also morphologically different from the species we

describe in the present paper due to the arrangement of cirri in its anterior region, rather similar to *D. bacilliformis*, and the presence of 3 dorsal kineties, as well as overall body size. Some morphological variations observed by Fleury and Fryd-Versavel (1984) in *K. bacilliformis* under different physiological conditions seem to match the ones observed in specimens of *D. estevesi* from old cultures, like cell elongation and the increase of the macronuclear nodules number. However, Fleury and Fryd-Versavel (1984) did not mention the occurrence of specimens with irregular shaped macronuclear nodules, such as observed in *D. estevesi* (Fig. 26).

Another kahliellid that must be compared with *D. estevesi* is *Kahliella microstoma* (Dragesco, 1970) Dragesco and Dragesco-Kernéis, 1986, from Yaoundé, Cameroon, which was originally presented by Dragesco (1970) as *Uroleptopsis multiseta* Dragesco, 1970. One strain of this species has the nuclear apparatus, shape of AZM and its proportion relative to the body size somewhat similar to *D. estevesi*. This organism shows one ventral cirral row right of AZM which ends at the equatorial level of the body (Dragesco 1970; p. 98, Fig. 71), thus indicating that it might also belong to *Deviata*, but differently from the other known species, the equatorially shortened cirral row is the second right of the adoral zone of membranelles. The other strain *K. microstoma* from Cameroon, which was also described by Dragesco (1970; p. 99, Fig. 72), lacks an equatorially shortened cirral row right of adoral zone of membranelles and has a distinctly different ventral cirral organization in the anterior region of body. However, Dragesco (1970) considered both as conspecific.

Based on our results, we regard the presented population of *Deviata* as a novel species. A complete study of the morphogenesis is necessary to verify if multiple within anlagen develop. According to Eigner (1995), this trait occurs during the divisional morphogenesis of *D. abbrevescens*, being considered by him as one of the diagnostic features for this genus. Such study will also clarify the precise classification of the first cirrus in row R1, which is always a little displaced to the right of the row itself (Figs 4, 19). It may be possible that this cirrus originates from a different anlage than the one from where row R1 originates. If this is confirmed, this cirrus may be homologous to the second frontal cirrus, right of AZM in *D. abbrevescens*. Another point is the origin of the short file of 3-5 cilia at the posterior region of dorsal surface, which at the present state and due to its position, we believe to be part of the right kinety.

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ACTA
PROTOZOOLOGICA

Trypanosoma (*Megatrypanum) ornata* sp. n., a Parasite of the Eurasian Water Shrew *Neomys fodiens* (Pennant, 1771)

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Summary. Infection with the new trypanosome species *Trypanosoma ornata* sp. n. was detected in water shrews *Neomys fodiens* examined for presence of blood parasites in September 2003 and 2004 in Białowieża Forest (eastern Poland). The prevalence of infection was about 36.3% (4 out of 11 shrews examined). The parasites were described based on light microscopic characteristics, from 100 parasites isolated from blood of four infected animals. The trypanosomes occurred as trypomastigote forms only. Body length ranged from 20.50-38.26 µm (mean 26.66 µm), and width from 1.93-4.12 µm (mean 3.06 µm), with the free flagellum 3.12 ± 1.26 long, sometimes absent. Other morphological features of the species (small kinetoplast, sometimes the vermiform posterior end, the surface of the cell markedly striated with longitudinal "myonemes", short or absent free flagellum) support its affiliation to the *Megatrypanum* subgenus. The study provides the first detailed description of trypanosomes occurring in the Eurasian water shrew *Neomys fodiens*.

Key words: Białowieża Forest, Poland, *Neomys fodiens*, *Trypanosoma ornata* sp.n.

INTRODUCTION

The trypanosomes parasitizing wild mammals in the palearctic zone are not very well known. These are the species belonging to the Stercorarian group, from the subgenera *Megatrypanum*, *Herpetosoma* and *Schizotrypanum*. So far, 25 species of trypanosomes are noted as permanent components of parasitofauna of European mammals (Hoare 1972, Karbowiak *et al*. 2001). However, trypanosomes parasitizing insectivorous mammals in the palearctic zone are poorly known. There is only a single report on the presence of a non-identified *Trypanosoma* in Eurasian water shrew *Neomys fodiens* from England from the beginning of 20th century (Henry 1913). Their pathogenic effects on hosts and life cycles are unknown. The vectors are probably fleas (Hoare 1972).

The goal of our study was description of the blood parasitofauna of shrews, and the morphological and morphomethric characterisation of parasites found. The study is a base for the future research on their possible role as zoonotic foci of diseases.

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MATERIALS AND METHODS

Eurasian water shrew *Neomys fodiens* (Insectivora: Soricidae) is a small (max. 15 g body mass), semiaquatic mammal occurring across most of Palearctic. It lives in wet habitats along freshwater, and aquatic prey usually constitute over 50% of its diet. During its short life (mean life-span 13.5 months), the water shrew produces 2 or 3 litters of 6 or more (up to 15) young (Pucek 1981, Innes 1994, Rychlik 2000).

The material from water shrews was collected in September 2003 and 2004 in Białowieża Forest (eastern Poland, 52°42'N, 23°52'E). The shrews were caught in live-traps placed at permanent trap stations arranged in a rectangular grid or along a line. Traps were placed and set in the afternoon, checked four times per day, and blocked after a final check around midnight.

Blood samples taken from the tip of the tail were examined for the presence of trypanosomes using the microhaematocrit centrifugation technique (8 minutes, 6200 g) (Kingston *et al*. 1992). The flagellates accumulated above the WBCs fraction, and their movements were observed under a light microscope, using magnifications of 10×10 and 10×20 (eyepiece × objective).

Giemsa-stained blood films from the trypanosome fraction were used for morphological and mensural characteristics of trypanosomes. The smears were air-dried, fixed in 100% methyl alcohol (10 minutes) and stained for 1 hour with Giemsa (diluted 1:5 in phosphate buffer, pH 7.2). Slides were rinsed, dried and examined under a light microscope using magnifications of 10×100 and 12×100 (eyepiece × objective) with oil immersion.

For the measurements of parasites, the "Analysis" software, in combination with a video camera and Olympus BX50F4 microscope was used. This method provided possibilities for obtaining results accurate to 0.01 µm. In order to detail, characterize, and compare morphological features of parasites, the terminology commonly adopted by trypanosome researchers (Hoare 1972, Matthews *et al*. 1977, Kingston *et al*. 1992) was employed in the present study. Morphometric measurements were made on 100 parasite specimens, at a magnification of 1200×. Typical trypomastigote forms were selected for measurements. Weakly stained, damaged, or distorted individuals were ignored.

RESULTS

Infection with *Trypanosoma ornata* sp. n. was discovered in the blood of 4 out of 11 water shrews examined, prevalence of infection was 36.3%. Additionally, unidentified *Bartonella* sp. bacterias were detected in 5 shrews (prevalence about 45.4%), but there were no mixed infections. The trypanosomes occurred as pleomorphic trypomastigote forms only. The epimastigote, dividing forms or other developmental stages were not observed. The density of infection was low; only single parasites were observed in the haematocrit capillars and stained blood films.

The trypanosomes were broad, and in many cases had a tapering and pointed, sometimes somewhat vermiform, posterior end. A few specimens were characterized with a less sharp or blunt posterior end. The body was slightly curved, with the narrow undulating membrane located on the external side of the curvature. The surface of the cell was markedly longitudinally striated with dark-blue, "myonemes" (Figs 1, 2). The length of the body was 20.50-38.26 µm (mean 26.66 µm), the width was 1.93-4.12 µm (mean 3.06 µm). The free flagellum was relatively short, and varied from a stub to a short whip. The wide range of free flagellum length was readily noted, it was 1.0-9.18 µm (median length was 2.96 µm, mean 3.12 ± 1.26 µm), in some specimens free flagellum was to short to recognize. A few specimens had a longer flagellum, up to 9.18 µm. The undulating membrane was narrow, 0.55-1.09 µm. The oval nucleus was located in the middle or slightly displaced to the posterior part of the body (range of NI = 0.31-1.20, mean 0.69), parallel the longitudinal axis of body, and close to the margin of undulating membrane. The nucleus was 1.18-6.88 µm (mean 3.78) long, and 0.65-3.01 µm (mean 1.15) wide, in the Giemsa-stained smears it was violet coloured. The kinetoplast was particularly small, oval, 1.20×0.60 µm in size, usually lay close to the margin of the body. It was located near the posterior end of the body or in the middle of the PN distance (KI = 1.31-2.53, mean 1.68) (Figs 1, 2). The morphometric parameters are summarised in Table 1.

Taxonomic summary

Type host: Eurasian Water Shrew *Neomys fodiens* (Pennant, 1771)

Site of infection: Blood.

Trypomastigote form: Broad, slightly curved, with a tapering and pointed or vermiform posterior end; narrow undulating membrane located on the external side of the curvature; the surface of the cell marked with dark-blue, longitudinal "myonemes"; the length of the body 20.50-38.26 µm (mean 26.66 µm), the width 1.93-4.12 µm (mean 3.06 µm), free flagellum relatively short, varies in length from a stub to a short whip, range 1.0-9.18 µm, the median length 2.96 µm, mean 3.12 ± 1.26 µm, in some specimens too short to recognize; the undulating membrane narrow, 0.55-1.09 µm in width; nucleus oval, located in the middle or slightly displaced to the posterior part of the body, 1.18-6.88 µm (mean 3.78) long, and 0.65-3.01 µm (mean 1.15) wide, NI = 0.31-1.20, mean = 0.69; located along to the longitudinal axis

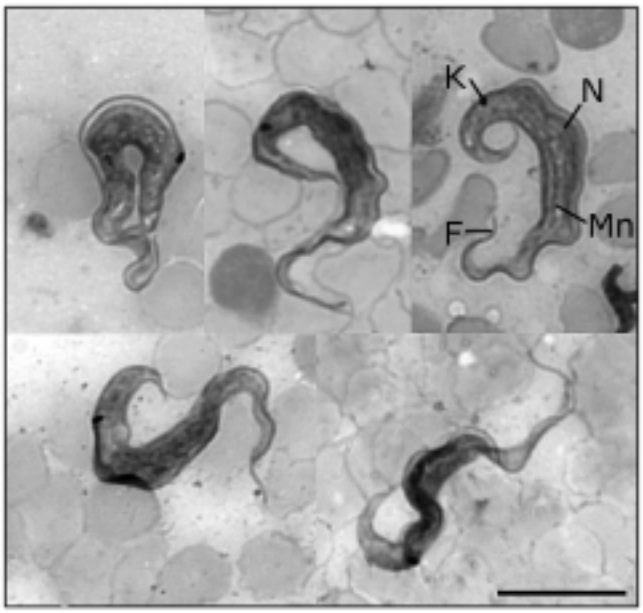

Fig. 1. Photomicrograph of Giemsa stained bloodstream forms of *Trypanosoma ornata* sp. n. from the Eurasian water shrew *Neomys fodiens*. F - free flagellum, K - kinetoplast, Mn - longitudinal "myonemes", N - nucleus. Scale bar 10 µm.

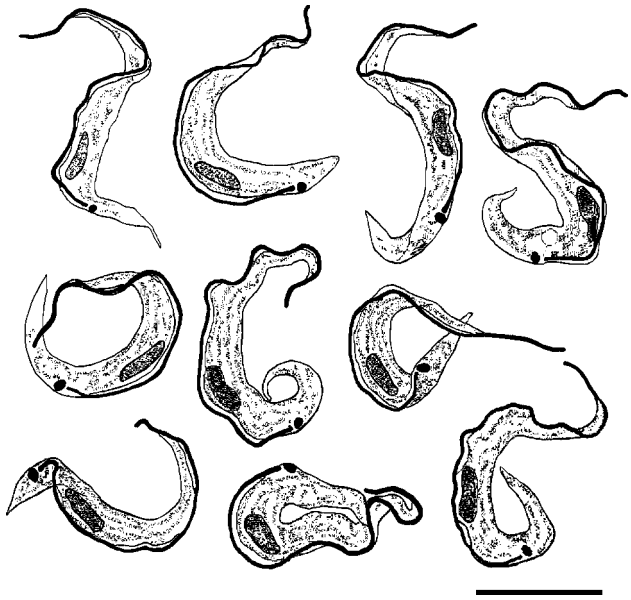

Fig. 2. The trypanosome *Trypanosoma ornata* sp. n. from the Eurasian water shrew *Neomys fodiens*. Scale bar 10 µm.

Table 1. Dimensions (in µm) of *Trypanosoma ornata* sp. n. from the Eurasian water shrew *Neomys fodiens* from Białowieża. PK - posterior end to kinetoplast, KN - kinetoplast to nucleus centre, PN - posterior end to nucleus centre, NA - nucleus centre to anterior end, BL - body length, FF - free flagellum length, L - total length, Nl - nucleus length, Nw - nucleus width, W - width of body on the nucleus level excluding the undulating membrane. Indices: nuclear index NI = PN / NA, kinetoplastic index KI = PN / KN, flagellar index FF:BL. Measurements from 100 trypomastigote specimens.

Parameter	PK	KN	PN	NA	BL	FF*
mean	4.27 ± 1.54	6.48 ± 1.43	10.75 ± 2.30	15.90 ± 2.32	26.66 ± 3.52	3.12** ± 1.26
range	2.62-10.65	2.48-14.35	5.41-18.84	10.97-21.01	20.50-38.26	1.00-9.18

Parameter	L	Nl	Nw	W	NI	KI	FF:BL*
mean	29.43 ± 4.10	3.78 ± 0.85	1.15 ± 0.35	3.06 ± 0.43	0.69 ± 0.16	1.68 ± 0.24	0.10 ± 0.06
range	20.50-40.90	1.18-6.88	0.65-3.01	1.93-4.12	0.31-1.20	1.31-2.53	0.03-0.30

* - if the free flagellum is present; ** - the median is 2.96 µm.

of body, on the side of undulating membrane. The kinetoplast particularly small, oval, 1.20 × 0.60 µm in size, lies close to the margin of the body, near the posterior end or in the middle of the body, KI = 1.31-2.53, mean 1.68.

Type locality: Białowieża National Park, eastern Poland, 52°42'N, 23°52'E.

Deposition of the type slides: The protozoological collection of the Witold Stefański Institute of Parasitology, Polish Academy of Sciences, Twarda str. 51/55, 00-

818 Warsaw, Poland. Contact with corresponding author.

Etymology: After Latin *ornata* = decorated, alluding to the longitudinal striped pattern on the body surface of trypanosomes.

DISCUSSION

According to Hoare (1972) two *Trypanosoma* subgenera, *Megatrypanum* and *Herpetosoma*, are represented in small mammals of Europe. The characteristic features of *Megatrypanum* trypanosomes are: relatively large size; relatively wide body; frequent presence of the filiform "tail" at the posterior end; short or absent free flagellum; small kinetoplast, situated close to the nucleus and far from the posterior end of body (index KI ≥ 2.0); reproduction in epimastigote stage in mammalian host. The diagnostic features of trypomastigotes of *Herpetosoma* are: medium size; slender and curved body; long and pronounced free flagellum; anterior nuclear position or in the middle of the body; large and often rod-shaped kinetoplast, lying nearer to the posterior extremity than to the nucleus (KI = 1.2-2.0, average 1.5) (Hoare 1972).

There are two species of *Trypanosoma* described in insectivorous mammals in Europe: *Trypanosoma talpae* Nabarro, 1907 and *Trypanosoma crocidurae* Brumpt, 1923. *Trypanosoma talpae* is described from the blood of the mole *Talpa europaea* in western Europe, and is included presently in the *Megatrypanum* subgenus (Hoare 1972, Baker 1974, Podlipaev and Krylov 1990). The trypanosomes found in *N. fodiens* differed from *T. talpae* in some features: different localization of nucleus and kinetoplast in the cell (in *T. ornata* sp.n. both lie on the same side of the body, in *T. talpae* both lie on the opposite side of the body), proportionally shorter PK and longer KN distances, and a narrower body in *T.ornata* sp.n. *Trypanosoma crocidurae* is described from the blood of greater white-toothed shrew *Crocidura russula* and common shrew *Sorex araneus* in Belgium, Germany and France (Krampitz 1961, Hoare 1972) as well as from the lesser white-toothed shrew *Crocidura suaveolens* in the former Czechoslovakia (Šebek 1975). This species is included in the *Herpetosoma* subgenus; however, the classification based on the morphological analysis of data given in the descriptions by several authors and collected by Hoare (1972) can be discussed. The size of *T. crocidurae* (about 20 µm by Šebek 1960, 25-32 µm after Hoare 1972) is similar to the medium size of species of the *Herpetosoma* subgenus, but the long PK distance (11 µm) and presence of the filiform "tail" at the posterior end relate this species to *Megatrypanum* subgenus. The only feature of *T. crocidurae* similar to the trypanosomes found in *N. fodiens* in the present study is the filiform "tail" on the posterior end of the body; the other parameters of these two species are different. *T. ornata* sp.n. differ from *T. crocidurae* in different localization of nucleus, bigger size and wider body. Moreover, Šebek (1960) reported high density of infection with *T. crocidurae*, not observed in the case of *T. ornata* sp. n.

It is impossible to compare our results with the record of Henry (1913) on the presence of trypanosomes in *N. fodiens* in England due to the absence of any description.

The trypanosomes found in *N. fodiens* fulfil some of the conditions of subgenus *Megatrypanum*. The morphological features, namely broad body, small kinetoplast, short free flagellum, presence of the vermiform "tail" and "myonemes", the lack of free flagellum in some specimens, the value of KI index in some cases bigger than 2 (till 2.53) (Table 1) are characteristic of *Megatrypanum*. Only few parameters (the body size, KI index smaller than 2.0 in many specimens) (Table 1) support its affiliation to *Herpetosoma* subgenus, the most common in small mammals of Europe (Hoare 1972, Baker 1974, Karbowiak *at al*. 2001).

In conclusion, the morphological and biological characteristics of *Trypanosoma ornata* sp.n. found in *N. fodiens* from Białowieża Forest place this parasite within *Megatrypanum* subgenus. However, only a few specimens of water shrews have been investigated, and the developmental cycle of the parasite, its invertebrate vector and ecology remain unknown.

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ACTA
PROTOZOOLOGICA

Protacanthamoeba bohemica sp. n., Isolated from the Liver of Tench, *Tinca tinca* (Linnaeus, 1758)

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Summary. A new species of amphizoic amoeba, *Protacanthamoeba bohemica* sp. n., isolated from the liver of *Tinca tinca* (Cypriniformes: Cyprinidae) is described. Trophozoites typical of the genus *Protacanthamoeba* Page, 1981 differ distinctly from those of the type species of the genus in having constantly more numerous and much longer acanthopodia. No relevant ultrastructural distinctions were observed. SSU rRNA gene sequence acquired for *P. bohemica* (the first within the genus) have been deposited in the GenBank database under accession number AY960120. A phylogenetic analysis based on SSU rRNA gene sequences assigned *P. bohemica* to the *Acanthamoeba* and *Balamuthia* clades.

Key words: amphizoic amoebae, morphology, phylogeny, *Protacanthamoeba bohemica* sp. n.

INTRODUCTION

Series of studies revealed that free-living amoebae infect fish as well as some aquatic invertebrates, playing parts of innocuous epi- or endobionts or infectious agents of diseases (Sawyer 1976, Johnson 1977, Jones 1985, Dyková and Lom 2004). The reasons why many amoebae can convert from free-living to epi- or endobionts of fishes are enigmatic. The study of amoebae capable of colonising fish tissues is undoubtedly the most important step towards resolving this enigma. In general, the morphological diversity and polymorphism of amoebae with poorly documented descriptions of species sometimes make even generic diagnosis of newly isolated strains extremely difficult. Some genera of free-living amoebae seem to be morphologically well defined, but they were often erected to include one species, the description of which was based on a single isolate. When the type strains of such taxa have not been maintained in culture collections, newly isolated strains identifiable with them gain importance. Studies of such strains ensure that after the progress achieved in the establishment of genera of free-living amoebae at light-microscopical and ultrastructural levels, other objective characters resulting from molecular studies might be added to generic and species diagnoses. This objective is the focus of our study.

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Based on an amoeba strain isolated from the liver of a cyprinid fish *Tinca tinca* (Linnaeus, 1758), we describe a new species of the genus *Protacanthamoeba* that have been represented to date by the type species *P. caledonica* Page, 1981 and the species *P. invadens* (Singh *et* Hanumaiah, 1979) described originally as *Acanthamoeba* sp. To the best of our knowledge, only slides of *P. caledonica* stained with Kernechtrot are deposited as a type material in The Natural History Museum of London, England (Holotype 1980: 12:22.1).

MATERIALS AND METHODS

Strain origin, cultures, sample preparation and morphological studies. The clone designated as TT3H/I was derived from amoeba strain isolated from the liver of *Tinca tinca*, which was collected in Spolský pond, South Bohemia, Czech Republic, in January 1997. The amoebae were cultured routinely on non-nutrient agar (NNA) seeded with an autoclaved suspension of *Escherichia coli* (Kalinina and Page 1992). Comparison of growth was done in cultures kept simultaneously at fluctuating RT, 4ºC, and constant (20ºC) temperature of an incubator. The hanging drop preparations were used to observe and take measurements of trophozoites. The thin layer of 1.5% agar was used to observe and document cysts. Fixation for electron microscopy was done either with 2% osmium tetroxide (selected blocks of agar with submerged trophozoites and cysts) or cacodylate buffered 2.5% glutaraldehyde (fixation of cultures *in situ* on agar plates) followed by postfixation with 1% osmium tetroxide (Dykstra 1993). Both materials, either blocks of agar or peletted material, were embedded in Spurr's resin (Spurr 1969). The ultrathin sections were double stained with uranyl acetate and lead citrate and examined under a Jeol JEM 1010 electron microscope at 80 kV accelerating voltage.

DNA isolation, PCR amplification and sequencing. Total DNA was extracted from the clonal culture of TT3H strain using the DNeasy™ Tissue Kit (Qiagen), according to the manufacturer's protocol. The SSU rRNA gene was amplified by PCR, using a set of universal eukaryotic primers (Medlin *et al*. 1988). Thermal cycling was initiated by heating for 5 min at 95ºC, followed by 30 cycles (each comprising 94ºC for 1 min, 55ºC for 1 min and 72ºC for 2 min) and terminated by elongation at 72ºC for 10 min. The amplified product was purified, cloned into the pCR® 2.1 TOPO cloning vector (TOPO-TA Cloning kit, Invitrogen) and sequenced using the automatic sequencer CEQ™ 2000 (Beckman Coulter) with the CEQ DTCS Dye Kit (Beckman Coulter).

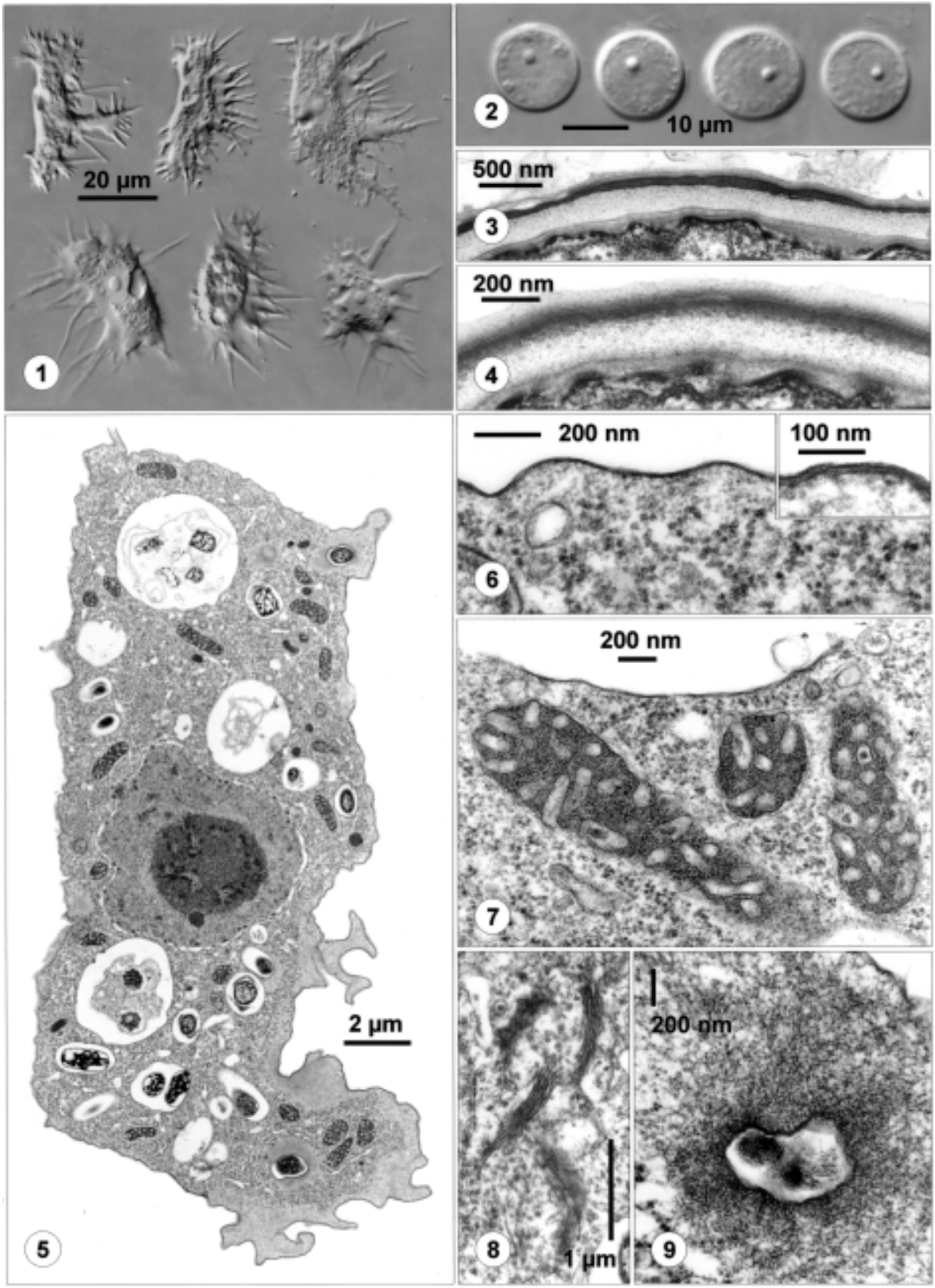
Alignments and phylogenetic analyses. The sequence of the SSU rRNA gene from the studied strain was aligned with two sets of sequences retrieved from GenBank. The first dataset was compiled in order to find out phylogenetic position of the representative of newly sequenced amoeba genus. This dataset included representatives of important lineages of Amoebozoa sensu Bolivar *et al*. (2001) and species that have trophozoites with some morphological similarities. It was completed with representatives of the Metazoa, Fungi, Viridiplantae, Alveolata and Stramenopiles. Of the basal taxons of Eukaryota, *Hexamita inflata* and *Trichomonas vaginalis* were chosen as an outgroup (Arisue *et al*. 2005). In total, 45 sequences were aligned using the Clustal_X program (Thompson *et al*. 1997) with various alignment parameters. Corrections were done by eye using the BioEdit sequence alignment editor (Hall 1999). The alignment consisted of 1518 unambiguous aligned positions with 750 parsimony informative characters. The second dataset reduced to sequences closely related to the sequence of the strain under study consisted of 19 sequences. Alignment comparisons were limited to 1865 sites, from which 477 were parsimony informative.

Phylogenetic analyses of both datasets were performed using the maximum parsimony (MP) and maximum likelihood (ML) methods. They were carried out with the program package PAUP*, version 4.0b8 (Swofford 1999). Search for the MP tree was done heuristically with the random addition of taxa (10 replications). Gaps were treated as missing data. Transition:transversion (Ts:Tv) ratios were set to 1:1, 1:2 and 1:3. Clade support was assessed with bootstraping of 1000 replicates and calculation of Bremer indices (Bremer 1994). For ML analysis, the likelihood ratio test (LRT) implemented in Modeltest v. 3.06 (Posada and Crandall 1998) was used to determine the best model of evolution. Based on the LRT, the ML analysis of the first dataset was performed with the GTR + Ã model of evolution (á shape parameter 0.4036), while for the second dataset, the GTR + Ã + I model of evolution was applied (á shape parameter 0.5241; proportion of invariable sites 0.2691). A bootstrapping of 1000 replicates was employed to estimate clade support.

RESULTS

Light and electron microscopy of trophozoites and cysts. Since their primary isolation, trophozoites cultured on agar plates revealed remarkable features. They formed dense growing zones toward the periphery of the Petri dishes, consisting of distinctly irregular cells that left behind a zone of cyst formation. Trophozoites submerged deeply into NNAs of 1.5, 2.5 and 3% strength. They multiplied slowly and hence subculturing was done usually once in two, to three weeks. To achieve a good harvest of trophozoites, clonal cultures were maintained at 4ºC. At higher temperatures (RT or

Figs 1-9. Light- and electron micrographs of *Protacanthamoeba bohemica* sp. n. **1** - trophozoites as observed in hanging drop preparations with Nomarski DIC; **2** - cysts as seen in Nomarski DIC; **3, 4** - two segments of cyst walls with layers differing in electron density; **5** - overview of a thin section through trophozoite. In addition to three food vacuoles with remnants of phagocytosed material, the cytoplasm also contains phagocytosed bacteria surrounded by a rim of hyaloplasm (see also detail in Fig. 9); **6** - the cell surface of trophozoite; **7** - mitochondria with tubular cristae; **8** - cisternae of Golgi complex in a parallel arrangement; **9** - hyaloplasmic microfilaments accumulated around phagocytosed material.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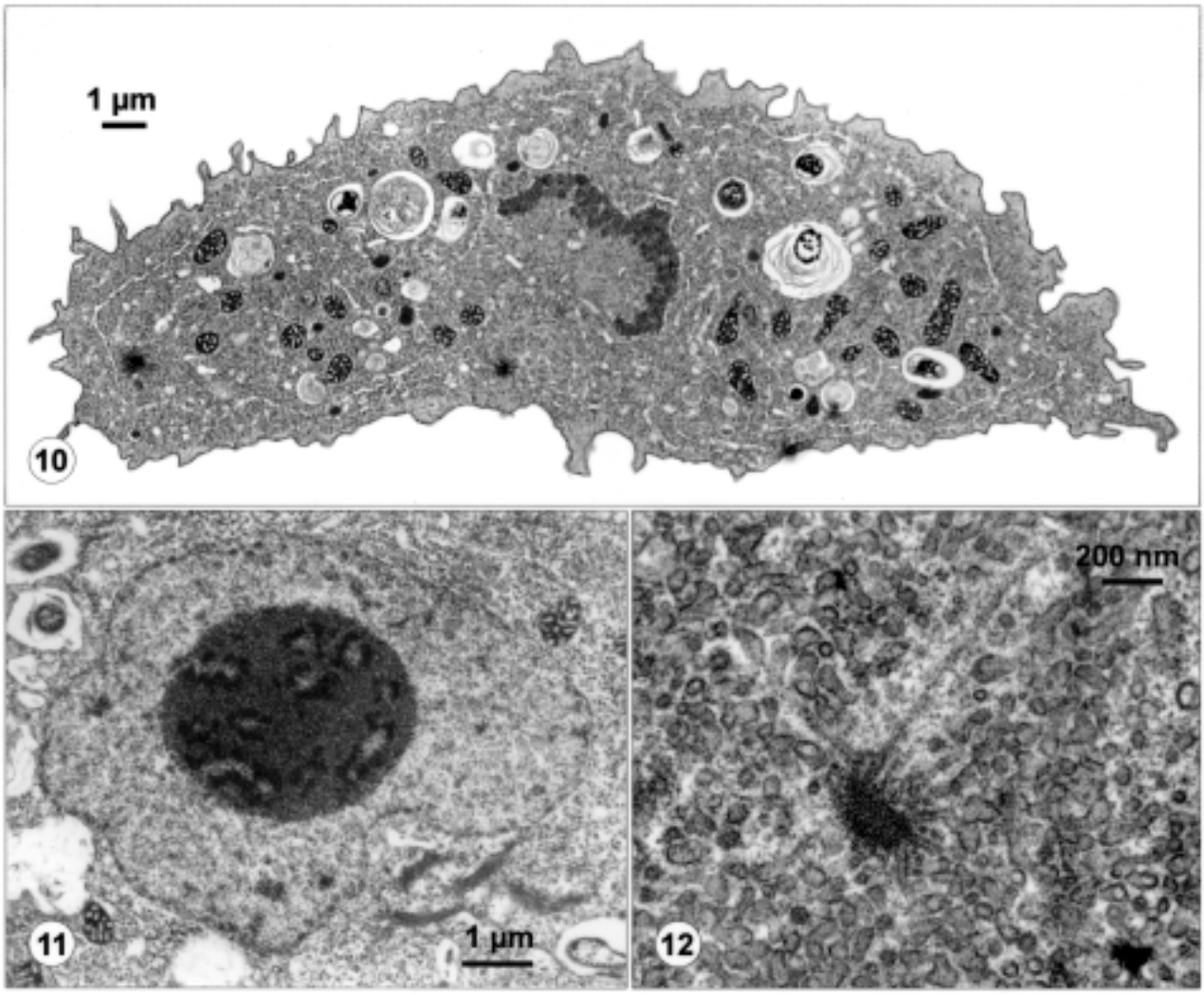

Figs 10-12. Details of ultrastructure of *Protacanthamoeba bohemica* sp. n. **10** - part of the nucleus (lacking nuclear envelope) and remnants of a nucleolar mass; **11** - the heterogeneous appearance of nucleolus; **12** - centriole-like body with radiating microtubules.

20°C), the encystment of trophozoites was extremely fast. Cysts were spherical with a smooth appearance. When observed in hanging drop preparations, polymorphic trophozoites had distinctive acanthopodia extending from the zone of hyaloplasm (Fig. 1). There was no signifficant difference from acanthopodia, but they were more numerous and much longer than observed in *Acanthamoeba* strains previously isolated from fish. In addition, one extremely long acanthopodium frequently predominated. Long furcated acanthopodia characterised our strain irrespective of agar concentrations and temperatures applied, invariably during long-term culturing. Floating forms were rounded and had several pseudopodia usually equal or slightly longer than the diameter of the cell. The plasma membrane of trophozoites was thin (12-14 nm) and the cell coat was not detected in ultrathin sections despite of the two fixation protocols applied (Figs 5, 6). The cytoplasm contained bean-shaped mitochondria with branching tubular cristae (Fig. 7). The stacks of Golgi cisternae were located in the vicinity of nucleus (Fig. 8). In addition to ingested microorganisms, the cytoplasm of trophozoites contained parallel arrays of endoplasmic reticulum and aggregates of microfilaments (Fig. 9). They were observed in hyaloplasmic regions, usually accumulated around partly phagocytosed material (Fig. 10). A somewhat spherical nucleus with numerous nuclear pores (visualised after osmium tetroxide fixation by electron dense material located in

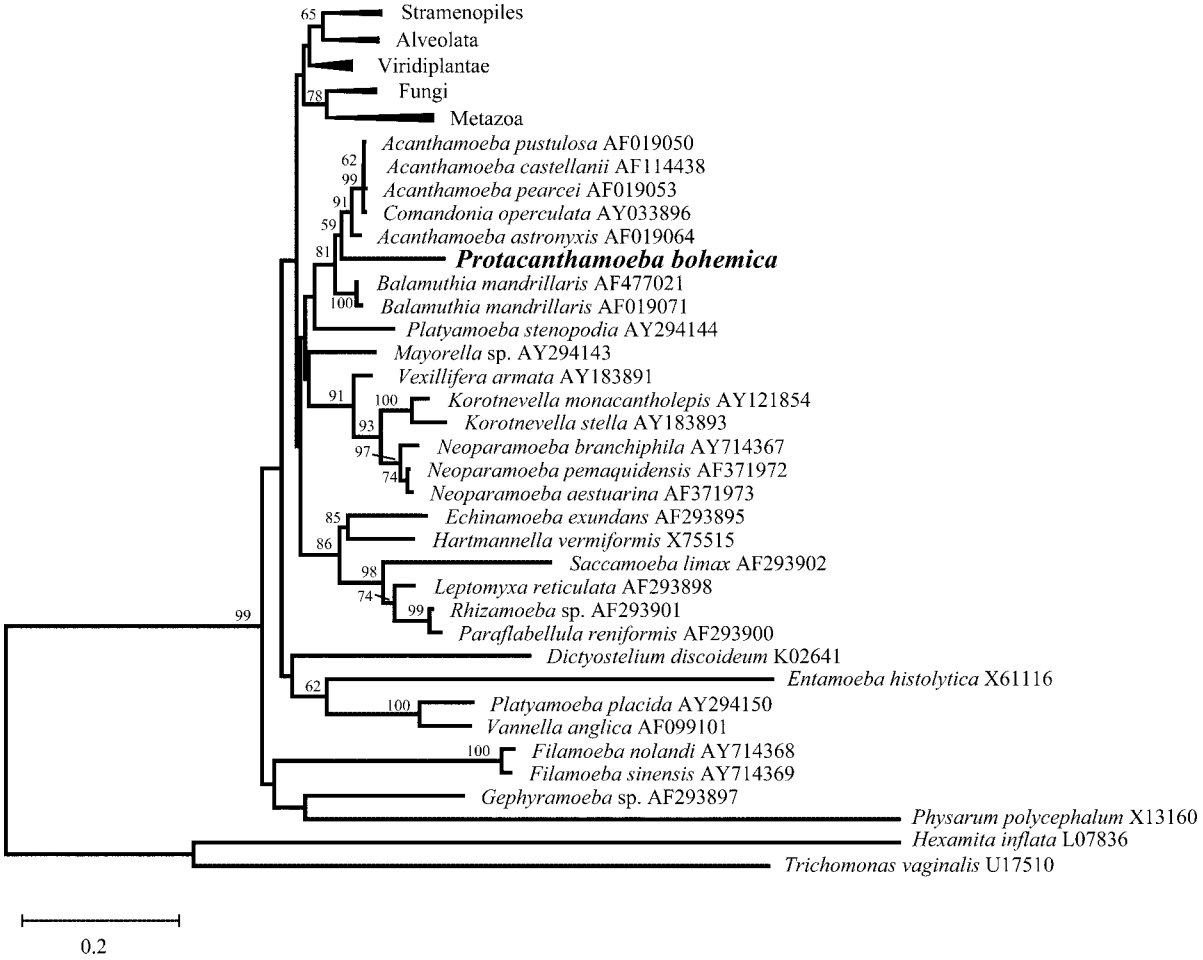

Fig. 13. Maximum likelihood tree (- ln = 19757.4333) of the SSU rRNA sequences. The distance scale is given under the tree. Bootstrap values are given for the nodes gaining more than 50% support. Stramenopiles = *Labyrinthuloides minuta* (L27634), *Fucus distichus* (AB011423); Alveolata = *Oxytricha nova* (X03948), *Gymnodinium fuscum* (AF022194); Viridiplantae = *Zea mays* (AF168884), *Equisetum ferrissii* (AF313576), *Chara polyacantha* (AF032742); Fungi = *Saccharomyces cerevisiae* (J01353), *Chytridium confervae* (M59758), *Mycale fibrexilis* (AF100946); Metazoa = *Xenopus laevis* (K01373), *Hydra circumcincta* (AF358080).

the perinuclear cisterna) contained a nucleolus mostly of heterogeneous appearance (Figs 5, 11). The disappearance of the nuclear envelope and rearrangement of the nucleus (Fig. 10.) were observed together with a centriole-like body and microtubules projecting from it (Fig. 12). Smooth, solid walls of spherical uninucleated cysts already observed in Petri dish cultures were also found in their native preparations (Fig. 2.) and when the silver staining method (Pussard and Pons 1977) was applied. Ectocysts were smooth and no cyst pores, irregularities, arms or separations of endocysts were observed. Three to four layers could be recognised in the walls of mature cysts (Figs 3, 4). In addition to poorly defined amorphous material, a dense region with membranes parallel to the cell surface formed the outermost layers of cysts. Dense amorphous and finely granular translucent layers followed these two layers. When only electron-density was used as a marker, cyst wall structure could be interpreted as subdivided into outer, middle, and inner layers. The cytoplasm of the mature cyst was dense. In addition to the nucleus and mitochondria, spherical lipid bodies and several membrane-bound dense bodies were observed in ultrathin sections.

On the basis of light microscopical and ultrastructural features of trophozoites and cysts, the strain under study was assigned to the genus *Protacanthamoeba* Page, 1981 as a representative of the new species *P. bohemica* sp. n.

Protacanthamoeba bohemica sp. n.

Origin of type material: Liver of tench, *Tinca tinca* (Linnaeus, 1758) (Cypriniformes: Cyprinidae) collected

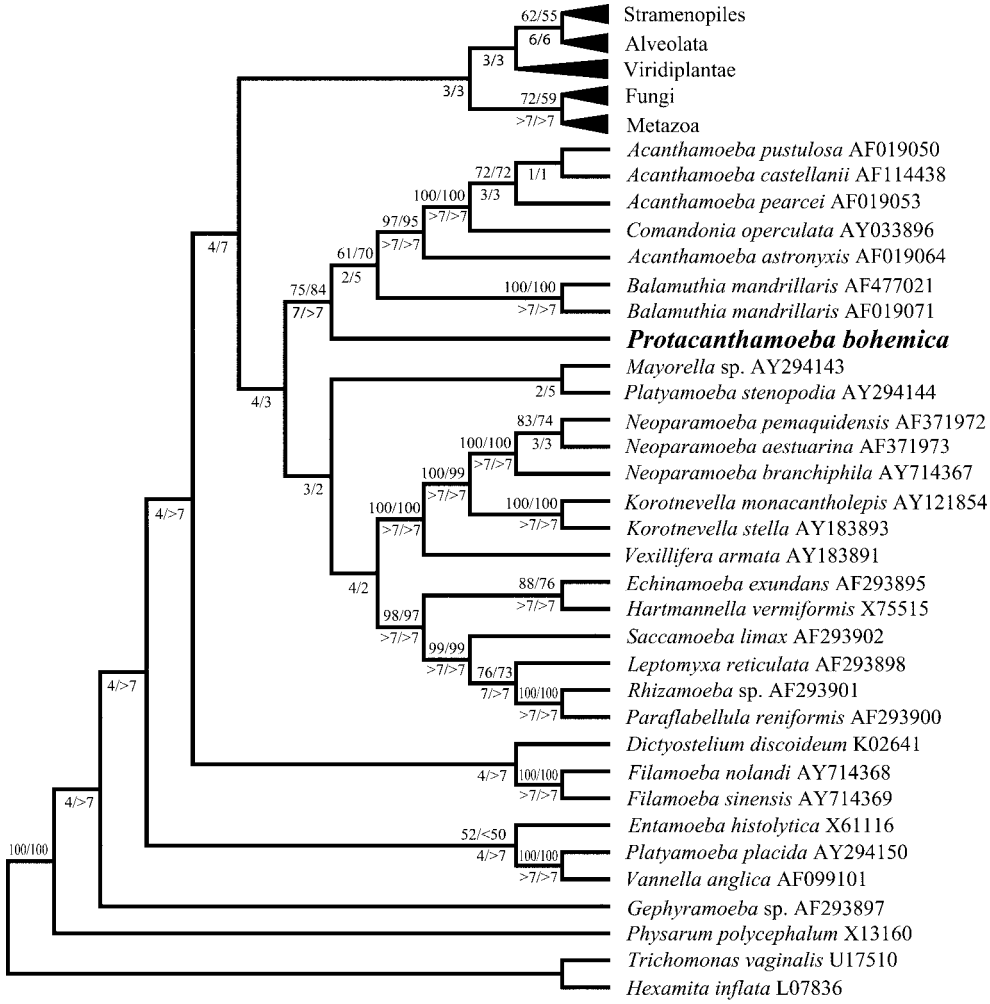

Fig. 14. Maximum parsimony tree of the SSU rRNA sequences (Ts:Tv = 1:2, 5915 steps, one most parsimonious tree). Bootstrap values (for nodes gaining more than 50% support) for MP Ts:Tv = 1:2 and MP Ts:Tv = 1:3 are given above the lines. Bremer indices for MP Ts:Tv = 1:2 and MP Ts:Tv = 1:3 are shown below the lines. See the legend to Fig. 13 for species representing: Stramenopiles, Alveolata, Viridiplantae, Fungi and Metazoa taxons.

from Spolský pond, South Bohemia, Czech Republic, January 1997.

Type material: Cryopreserved clonal culture of the strain denominated TT3H, deposited at the Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice; photosyntypes (light micrographs), nos. 12540-12565 and transmission electron micrographs, nos. 15484-15491; 15637-15640; 15834-15841.

Etymology: The species name is given according to the geographic origin of the fish host (South Bohemia).

Diagnostic summary: Greater dimensions of trophozoites attached to and slowly moving on the under side of coverslips in hanging drop preparations 20-40 µm; acanthopodia (produced from a broad hyaline zone) slender, flexible, often furcate with pointed tips, 5-16 µm in length; nuclear diameter 5-7 µm; diameter of smooth-walled spherical cysts lacking pores 13-17 µm; floating stages with slender pseudopodia. At the light microscopical level, *P. bohemica* differs from the type species of the genus in having trophozoites with more numerous and substantially longer acanthopodia. Smooth walled cysts lacking pores have a smaller range of their diameter. Details of ultrastructure are identical with those described for *P. caledonica* by Page (1981).

Molecular phylogeny based on SSU rRNA gene sequences. The SSU rRNA gene sequence obtained for *P. bohemica* (GenBank accession number AY960120) consists of 2028 bp. The GC content is 55.87%. The

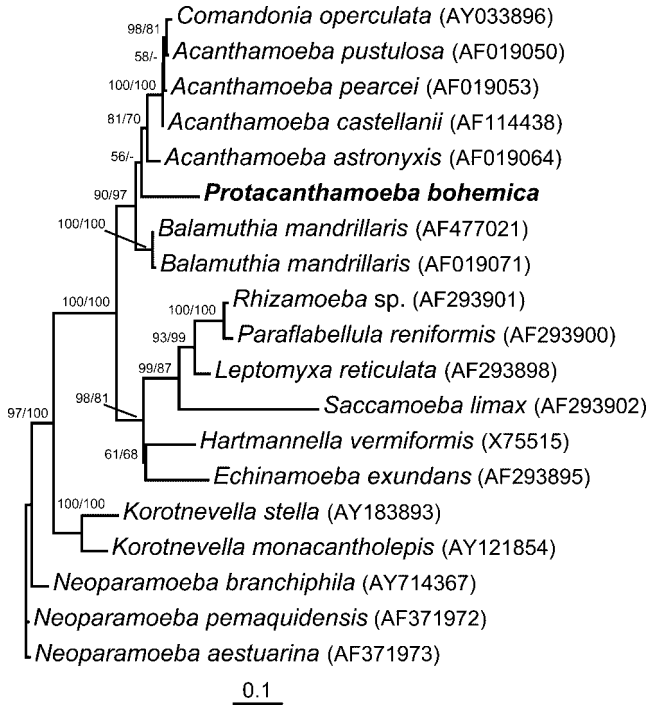

Fig. 15. Maximum likelihood tree (GTR + Γ + I model; -ln = 9278.7826) of the SSU rRNA sequences. Accession numbers of the sequences are in brackets. The distance scale is given under the tree. Bootstrap values (ML and MP Ts:Tv = 1:2) are given for the nodes gaining more than 50% support.

genetic similarities computed from the second alignment (comparing more sites than the first one) revealed homology with the highest identity for *P. bohemica* and *Acanthamoeba* spp. (about 90%) and *Balamuthia mandrillaris* (89 %). For comparison, identities within *Acanthamoeba* sequences were more than 96 % and identities of *B. mandrillaris* and *Acanthamoeba* sequences were about 94%. The sequence of *P. bohemica* clustered together with sequences of *Acanthamoeba* spp. and *B. mandrillaris* in all performed analyses except for MP - Ts:Tv = 1:1. In the alignment of the first dataset, the integrity of the *Acanthamoeba* + *Balamuthia* + *Protacanthamoeba* cluster was supported with a high bootstrap value in ML, MP - Ts:Tv = 1:2 and MP - Ts:Tv = 1:3 (Figs 13, 14). Strong nodal support for this cluster resulted also from the calculation of Bremer indices in both MP - Ts:Tv = 1:2 and MP - Ts:Tv = 1:3 (Fig. 14). *P. bohemica* formed the third lineage associated with *Acanthamoeba* spp. and *B. mandrillaris* within one clade. In comparison with *Acanthamoeba* spp. and *B. mandrillaris* sequences, the branch of *P. bohemica* was relatively long. In both datasets, the ML analysis revealed a closer relation of *P. bohemica* to *Acanthamoeba* spp. than to *B. mandrillaris*, but bootstrap support was low (Fig. 15). MP analyses placed *P. bohemica* in a sister position to *Acanthamoeba* spp. and *B. mandrillaris*, but the clustering of the latter two was not strongly supported as well. Mutual relations among the *Acanthamoeba* spp. + *P. bohemica* + *B. mandrillaris* clade and other amoebozoan lineages differed depending on the type of analysis applied.

DISCUSSION

The comparison of the newly described species *P. bohemica* with *P. caledonica* (the type species of the genus) could be based on relevant, well-documented features. Although a thorough description was not available for *P. invadens* (Singh and Hanumaiah 1979), the second species of the genus, critical comparison could be utilized of *P. caledonica* and *Acanthamoeba invadens* Singh *et* Hanumaiah, 1979, completed by Page (1981) who finally transferred this species to the genus *Protacanthamoeba*. Page (1981) considered the trofozoites of *P. invadens* identical with *P. caledonica* and using the available documentation, he calculated the diameter of cysts of *P. invadens* to be 20-27 µm. The growth at 42ºC and pathogenicity in mice when introduced intracerebrally or intranasally discriminated *P. invadens* from the other two *Protacanthamoeba* spp. Contrary to the thermophily of *P. invadens*, *P. bohemica* grew best at low temperatures around 4ºC.

Although the amoebae SSU rDNA data have been greatly expanded to date, and several evolutionary important lineages were identified within the Gymnamoebia sensu Peglar *et al*. (2003), representatives of numerous morphologically well-defined genera have not thus far been subjected to phylogenetic analyses. This situation is the same for genus *Protacanthamoeba*. Results of our phylogenetic analyses demonstrate a close relationship of *P. bohemica* with *Acanthamoeba* spp. and *Balamuthia mandrillaris*. This result supports to a certain extent close relationships of the genera *Acanthamoeba* and *Protacanthamoeba* proposed by Page (1981) and reflected in the etymology of the name he proposed for the latter genus.

The question as to whether *P. bohemica* is more closely related to *Acanthamoeba* spp. or *Balamuthia mandrillaris* is presently not resolved, but its close relation to both *Acanthamoeba* spp. and *B. mandrillaris* has been well established. MP analyses and comparison of genetic distances supported a closer relation of

Acanthamoeba spp. to *B. mandrillaris*, but ML analysis placed *P. bohemica* and *Acanthamoeba* spp. together.

Phylogeny based on SSU rRNA gene sequences is congruent with the trophozoite morphotype common to *Acanthamoeba* and *Protacanthamoeba* species. Taking into account substantial difference of *Protacanthamoeba* and *Acanthamoeba* cysts, and the diagnostic importance given to cyst morphology within the genus *Acanthamoeba*, the foresight of F. C. Page has to be admired.

The fish-isolated strain of *P. bohemica* is the second representative of the genus described based on both light microscopical and ultrastructural data. Moreover, it is the first that could be included in phylogenetic analyses addressing amoebozoan diversification.

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ACTA
PROTOZOOLOGICA

Ultrastrutural Morphology of *Myxobolus testicularis* sp. n., Parasite of the Testis of *Hemiodopsis microlepis* (Teleostei: Hemiodontidae) from the NE of Brazil

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Summary. *Myxobolus testicularis* sp. n. was found within cyst-like structures in the testis of the freshwater fish *Hemiodopsis microlepis* Kner, 1859 (Teleostei, Hemiodontidae) in close contact with the testis stroma. The fish were collected in the Poty River, near the city of Teresina (State of Piauí, Brazil). Large spherical to ellipsoidal plasmodia up to 0.5 mm were filled with disporic pansporoblasts and mature spores. The plasmodial wall presented a sinuous outline surrounded by several fibroblasts and a continuous layer of numerous collagen fibrils. The plasmodia contained different proliferative stages. Early sporogenic cells were present in the cortical zone. Immature and mature spores were located more internally. Ellipsoidal spores measured 8.6 ± 0.5 µm (n = 30) length, 7.2 ± 0.5 µm (n = 15) width, and 2.7 ± 0.4 µm (n = 12) thickness. The anterior end of the spores contained two equal pyriform polar capsules measuring 3.5 ± 0.3 µm (n = 15) length, 1.7 ± 0.4 µm (n = 10) width, each with an isofilar polar filament with 5-6 turns obliquely to the longitudinal axis. The wall of the polar capsule was filled with a hyaline substance contrasting with the very dense internal matrix. The spores consisted of two equal valves forming the wall. The spore wall was surrounded by a dense and uniform layer with variable thickness (up to 0.6 µm) consisting of a complicated network of anastomosed microfibrils closely adhered to the wall. More externally, in the plasmodial matrix, the fibrils formed a looser network. Based on the ultrastructural morphology and specificity to the host species, we propose the creation of a new species, named *Myxobolus testicular*is sp. n.

Key words: fish parasite, *Myxobolus testicularis* sp. n., Myxozoa, ultrastructure.

INTRODUCTION

Numerous descriptions of myxosporeans have been reported in fish from different geographic areas (Landsberg and Lom 1991, Lom and Dyková 1992). Myxozoa Bütschli, 1882 comprises more than 1200 available species commonly found in fish (Lom and Dyková 1992). Among them, the genus *Myxobolus* Bütschli, 1882, with 744 species described, is the largest group of the Myxobolidae family (Eiras *et al*. 2005), and has been reported as an important pathogen in freshwater fishes (Kent *et al*. 2001).

Although considerable information has already been collected on South America myxozoans, the available descriptions are based, almost exclusively, on light microscopical records and diagrammatic drawings of the spores (Aragão 1919, Nemeczek 1926, Penido 1927,

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Pinto 1928, Walliker 1969, Kent and Hoffman 1984, Molnár and Békési 1993, Cellere *et al*. 2002). At the present, nineteen Brazilian *Myxobolus* species have been described from different host fishes (Walliker 1969; Kent and Hoffman 1984; Molnár and Békési 1993; Gioia and Cordeiro 1996; Casal *et al.* 1996, 2002; Molnár *et al.* 1998; Adriano *et al.* 2002; Azevedo *et al.* 2002; Cellere *et al*. 2002). However, very few represent ultrastructural studies on *Myxobolus* sp. from Brazil, of which only three species have been described (Casal *et al*. 1996, 2002; Azevedo *et al*. 2002).

The present study gives ultrastructural details of the developmental stages, including spores of a new myxosporean infecting the Amazonian teleost *Hemiodopsis microlepis* Kner, 1859 species. The morphological characteristics and taxonomic position of the new species are discussed.

MATERIALS AND METHODS

Forty specimens (27 males and 13 females) of *Hemiodopsis microlepis* Kner, 1859 (Teleostei: Hemiodontidae) known by the Brazilian common name "Flexeiro", were collected from Poty River (05º05´21"S/ 42º48´07"W), near the city of Teresina (State of Piauí), NE Brazil. The fishes were killed by over-anaesthetising in MS 222 solution (3-aminobenzoic acid ethyl ester) (Sandoz Laboratories) and dissected. Small fresh fragments of testes, ovary, gills, kidney and liver were excised and examined under light microscope with Nomarski differential interference contrast (DIC) optics. The infected testes were observed by DIC for spore measurement. For ultrastructural studies, small fragments of infected tissues were excised and fixed in 3% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2) for 12 h at 4ºC, washed in the same buffer for 12 h at 4ºC and postfixed in 2% OsO_4 buffered with the same solution for 4 h at the same temperature. After dehydration in a graded ethanol-series followed by two changes of propylene oxide (4 h in each), samples were embedded in Epon. Semithin sections were stained with methylene blue-Azur II. Ultrathin sections were cut with a diamond knife, following double-contrast with both aqueous uranyl acetate and lead citrate and observed in a JEOL 100CXII TEM operated at 60 KV.

RESULTS

The only organs parasitized were the testes. No other organs studied by light microscopy were parasitized. The majority of the parasitized male fish had 2-3 cyst-like structures up to 0.5 mm in diameter in different locations in contact with the seminiferous lumen (Figs 1, 2). The plasmodia contained different proliferative stages with early sporogenic cells in the cortical zone and immature and mature spores more internally (Figs 2, 3). Mature spores were extremely numerous (several thousands). After rupture of the cysts some free spores were observed (Fig. 1 insert). The plasmodial wall was surrounded by a single thin layer of dispersed fibroblasts and numerous collagen fibres (Fig. 3). Externally, this layer appeared in contact with the seminiferous lumen. (Fig. 2).

Myxobolus testicularis sp. n. (Figs 1-10)

Type host: *Hemiodopsis microlepis* Kner, 1859 (Teleostei: Hemiodontidae) (Brazilian common name "flexeiro").

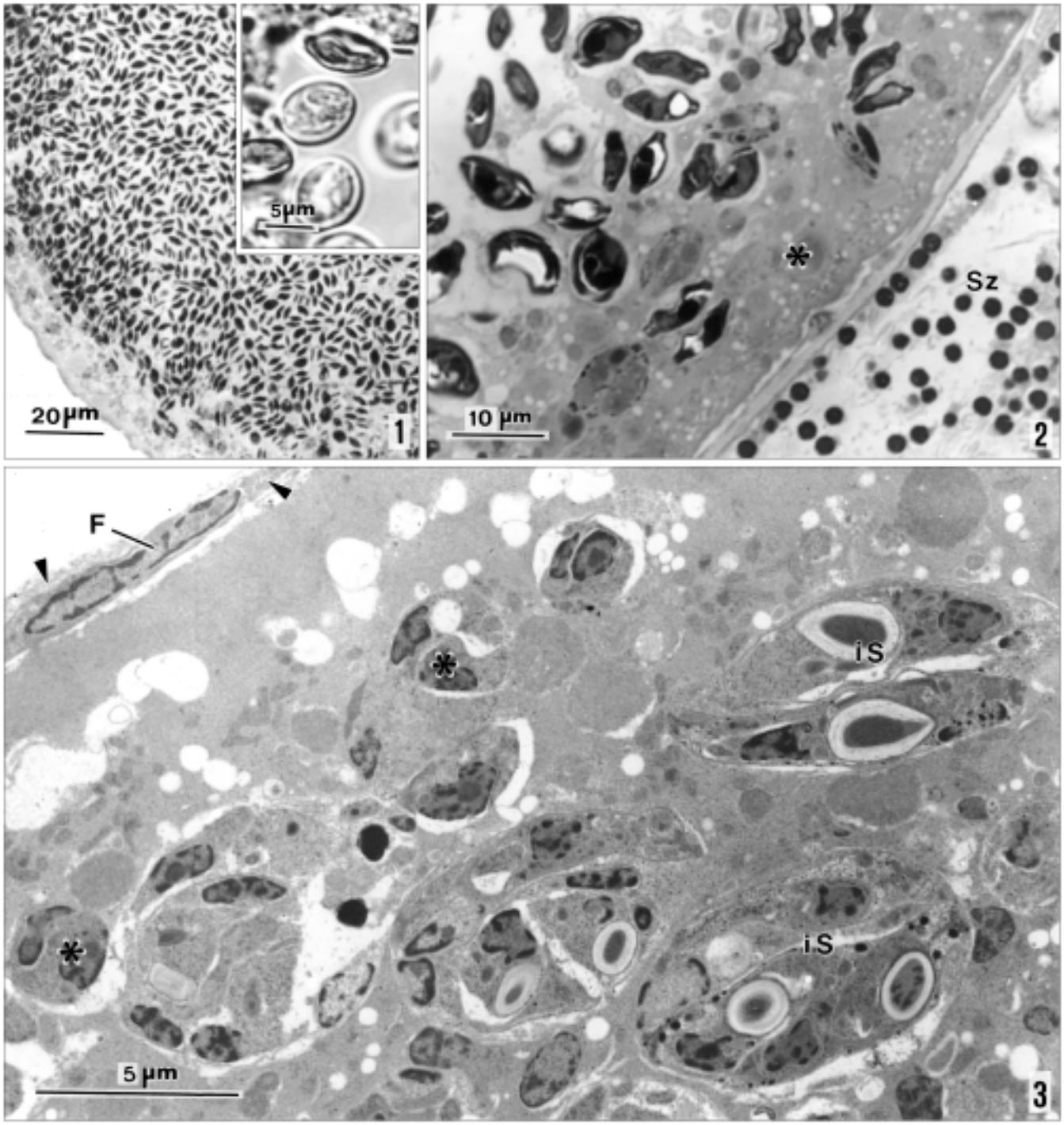
Site of infection: Plasmodium in contact with the seminiferous lumen.

Prevalence of infection: Seven of 40 (17.5 %) of the total fish or 7 of 27 (25.9 %) male fish were parasitized.

Type locality: Poty River near the city of Teresina (State of Piauí), NE Brazil (Latitude: 05º05´21"S; Longitude: 42º48´07"W).

Description of spores: The spores were typical of the genus *Myxobolus* Bütschli, 1882 because they were rounded in valvular view and biconvex in sutural view, and the shell valves were smooth and without projections. They measured 8.2-9.1 (8.6 ± 0.5) µm (n = 30) in length, 6.7-7.5 (7.2 ± 0.5) µm (n = 15) in width and 2.4-3.0 (2.7 ± 0.4) µm (n = 12) in thickness (Fig. 1 insert, 5-7). The spores consisted of two equal dense valves adhering together along the suture line forming the wall (Figs 5-8). Two equal smooth polar capsules, located symmetrically to the spore axis, measured about 3.3-3.8 (3.5 ± 0.3) (n = 15) × 1.3-2.0 (1.7 ± 0.4) (n = 10) µm each and contained a polar filament with 5-6 turns in a single layer surrounded by a dense matrix (Figs 6, 7). Sporoplasm containing two nuclei was surrounded by irregular patches of dense matter (Fig. 5). The spore wall was smooth and contained a dense and uniform layer with variable thickness (up to 2 µm) of numerous anastomosed microfibrils closely adhered to the wall (Figs 5-8). More externally, these microfibrils formed a more diffused network (Fig. 5). The layer of microfibrils was absent in early sporogenic cells. It appeared during the spore maturation stage and attained maximum development in the mature spore (Fig. 4). A schematic drawing of the ultrastructure of the spore (Fig. 9) was made from serial ultrathin sections and another was obtained by DIC observations (Fig. 10).

Type specimens: One slide with paratypes and one slide with semithin sections of the cyst containing spores and surrounding tissues were deposited in the Interna-



Figs 1-3. *Myxobolus testicularis* sp. n. **1** - semithin section of a cyst-like structure, showing numerous spores; **1 insert** - some free mature spores released from the cyst-like structure observed in DIC optics; **2** - semithin section of the periphery of the cyst-like structure, showing some spermatozoa (Sz) in close contact with the wall. Near the periphery of the plasmodium some early sporogenic cells (*) are present; **3** - ultrathin section of the periphery of the cyst-like structure showing different life cycle stages (* - young sporogenic stages, iS - immature spores). In the wall, one fibroblast (F) and numerous collagen fibres (arrowheads) are present.

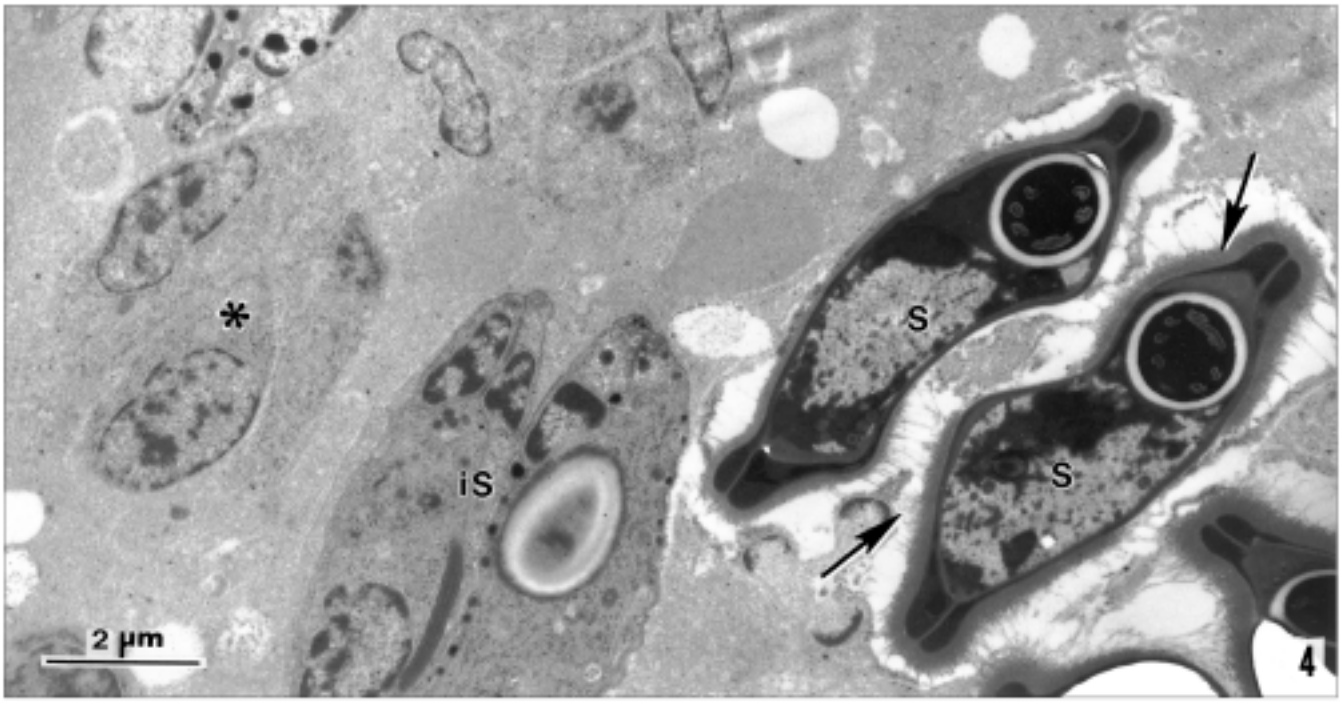


Fig 4. *Myxobolus testicularis* sp. n. ultrathin section showing a young sporogenic stage (∗), two immature spores (iS) and two mature spores (S). Surrounding the mature spores, the microfibrils are observed (arrows).

tional Type Slide Collection at Smithsonian Institution, Washington, D.C. USA (USNM number 1076956)

Etymology: The specific epithet "*testicularis*" derives from the name of the infected organ of the host.

DISCUSSION

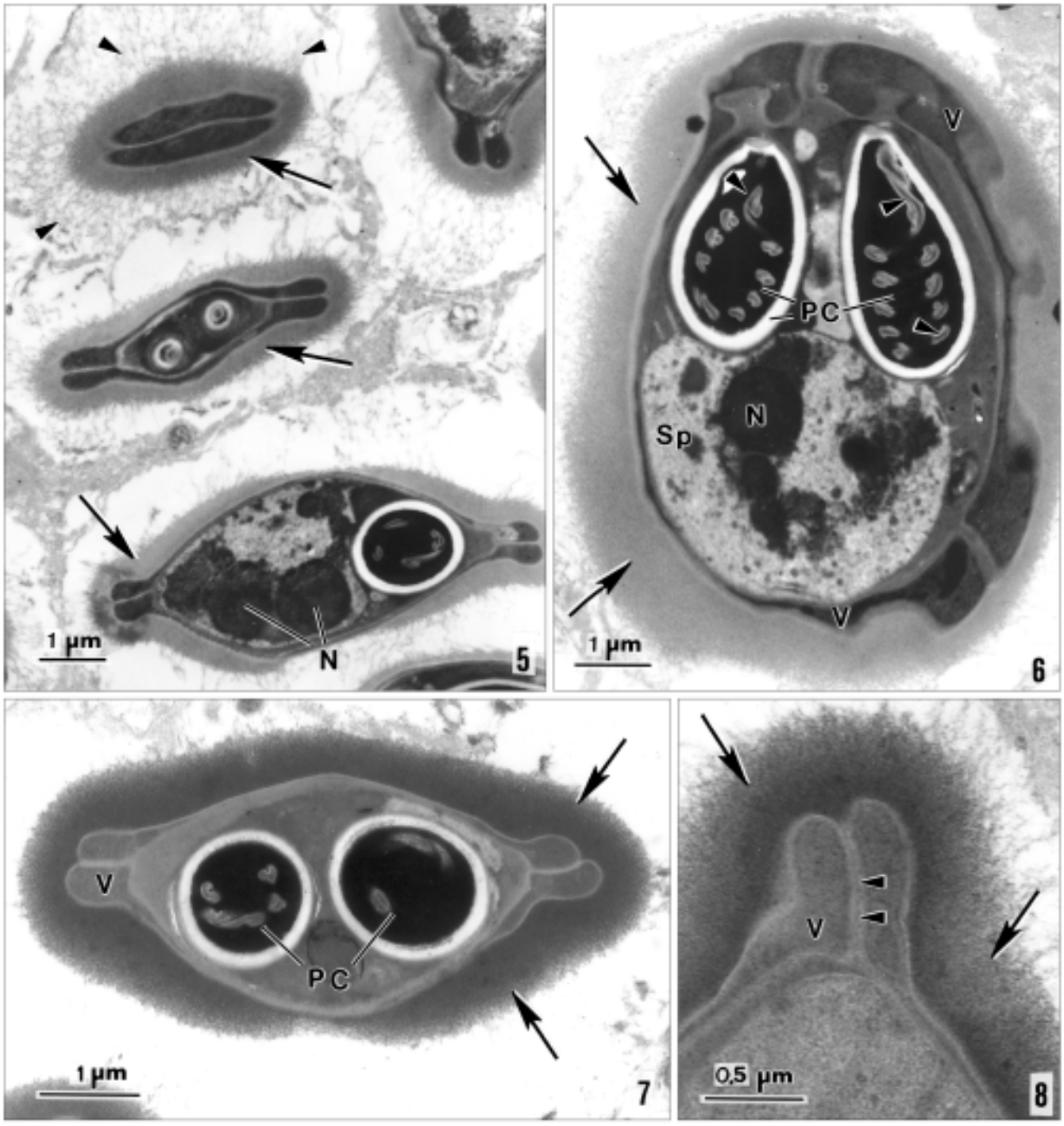
The light and ultrastructural morphology of the spores described in the present work, corresponds to those of genus *Myxobolus* (family Myxobolidae) (Lom and Noble 1984, Lom and Dyková 1992).

When comparing these results with those obtained for the different *Myxobolus* spp., with equal polar capsules, previously described mainly from Brazilian host fishes (Table 1), both *Myxobolus kudoi* Guimarães et Bergamin, 1938 and *Myxobolus lutzi* Aragão, 1919 show similarities with our results. The spores of *M. kudoi* and its polar capsules have very similar dimensions to those of *M. testicularis* sp. n. (Table 1). Despite the fact that the only information available on *M. kudoi* represents diagrammatic drawings of the spores, the morphology seems quite dissimilar from that of *M. testicularis* sp. n. In *M. kudoi*, the spores appear to be pyriform shaped, while in *M. testicularis* sp. n. they are almost circular with small differences in the length and width diameters. It is particularly difficult to establish a comparison between the spores of *M. testicularis* sp. n. and *M. lutzi* because the only data available on the later is the medium length and the width of the spore (Table 1) and a diagrammatic drawing. In spite of their similar spore dimensions, a close analysis of the diagrammatic drawing of *M. lutzi* reveals that is clearly pyriform shaped unlike *M. testicularis* sp. n., which is rounder.

Myxobolus sp. are very common parasites of freshwater fishes. In most of the fishes studied, one or more *Myxobolus* species were found. Although there have been very few studies on the host specificity of *Myxobolus* sp., new data indicates that most species are strictly host specific or only capable of developing in closely related fishes (Molnár *et al.* 1998). These studies support the distinction between *M. testicularis* sp. n. that appears in the testes of *Hemiodopsis microlepis* (Teleostei: Hemidontidae), *M. kudoi* which parasitizes the body skin except the fins of "*Nematognatha* sp."

Table 1. Characters and dimensions (in µm) of the spores and the polar capsules in species of *Myxobolus* found in Brazil which show only one type of spore and equal polar capsules.

Myxobolus sp.	Host tissue	Spore			Polar Capsules			References
		Length	Width	Thickness	Length	Width	Number of coils	
M. lutzi	Testes	10	7	-	-	-	-	Aragão 1919
M. chondrophilus	Gills	6	4.5	-	-	-	-	Nemeczek 1926
M. associatus	Kidney	15	10	-	-	-	-	Nemeczek 1926
M. pygocentris	Intestinal contents	15-16	9-11	-	9-11	3-4	-	Penido 1927
M. cunhai	Intestinal contents and cloaca	9-11	4-6	-	-	-	-	Penido 1927
M. noguchii	Gills (?)	13.6	8.5	-	6.8	2.2	-	Pinto 1928
M. stokesi	Subcutaneous tissue of the snout	8.5	5.3	-	3.4	1.7	-	Pinto 1928
M. kudoi	Skin of the body except the fins	8.5-8.9	6.5-7.3	-	3.5-4.2	1.3-2.0	-	Guimarães and Bergamin 1938
M. pfeifferi	Esophagus, stomach, intestine, liver, gall-bladder, kidney, muscle, gills, ovary and fins	8.2	5.6	-	3.8	1.9	-	Thélohan 1895
M. colossomatis	Connective tissues of various organs	11.8	6.9	3.7	6.0	2.1	7-8	Molnár and Békési 1993
M. braziliensis	Gills	10.17	5.28	3.69	5.30	1.43	9-11	Casal *et al*. 1996
M. macroplasmodialisis	Abdominal cavity	11	8.5	5.2	4.5	2.8	6	Molnár *et al.* 1998
M. maculatus	Kidney	21	8.9	7.5	12.7	3.2	14-15	Casal *et al*. 2002
M. porofilus	Visceral cavity	5.7	4.8	-	1.6	1.1	3	Adriano *et al*. 2002
M. testicularis	Testes	8.2-9.1 (8.6 ± 0.5) (n = 30)	6.7-7.5 (7.2 ± 0.5) (n = 15)	2.3-3.1 (2.7 ± 0.4) (n = 12)	3.3-3.8 (3.5 ± 0.3) (n = 15)	1.3-2.0 (1.7 ± 0.4) (n = 10)	5-6	Present study



Figs 5-8. *Myxobolus testicularis* sp. n. **5** - ultrathin section of some spores sectioned at different levels, showing the spore organization with special emphasis on the surrounding microfibrils (arrows). More externally, a loose network of anastomosed microfibrils (arrowheads) is seen. In the spore section located below, two nucleus (N) are observed in the sporoplasm; **6** - ultrathin longitudinal section of a spore in frontal view (slightly oblique), showing all spore elements arrows - surrounding microfibrils, V - valves, PC - polar capsules each with a coiled polar tube (arrowheads), Sp - sporoplasm, N - nucleus; **7** - ultrathin transverse section at the polar capsules (PC) level, showing their circular transverse section, the equal valves (V) and the surrounding microfibrils (arrows); **8** - ultrastructural detail of the two equal valves (V), the sutural line (arrowheads) and the complex network of anastomosed microfibrils (arrows).

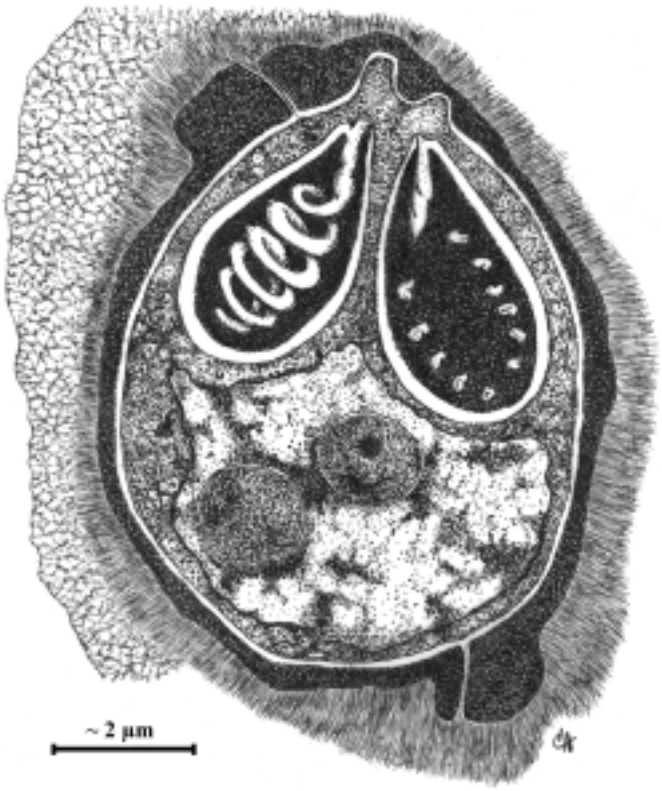


Fig. 9. Semischematic drawing of a slightly oblique longitudinal section of a mature spore showing the morphology of *Myxobolus testicularis* sp. n. emphasising the spore wall ornamentation which consists of a network of numerous anastomosed microfibrils attached to the spore wall periphery.

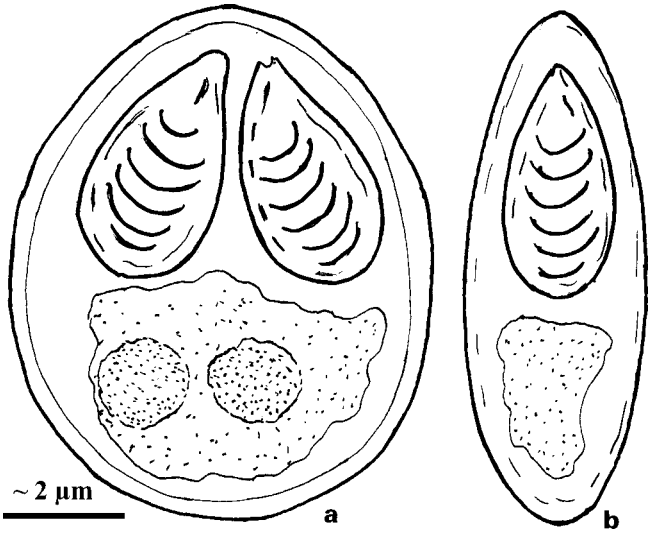


Fig. 10. Semischematic drawing of *Myxobolus testicularis* sp. n. in frontal view (**a**) and lateral view (**b**).

(Pisces) (Guimarães and Bergamin 1938), and *M. lutzi* which appears in the testes of *Poecilia vivipara* (Teleost). The British Museum (Natural History) considers that "*Nematognatha* sp." probably refers to catfish.

Our results show several morphological differences between *M. testicularis* sp. n. and other *Myxobolus* spp. described from South America mainly in the dimension and shape of the spores and polar capsules, as well as in the number, position and structure of the coils in the polar filament. Moreover, another difference between the present species and those previously described, is in the complicated network of irregular and anastomosed surrounding microfibrils forming projections in the spore wall towards the periphery of the spore. This feature has never been observed before in other *Myxobolus* sp. from South America. Nevertheless, in *Henneguya pilosa* the spores present similar fibrils to our results (Azevedo and Matos 2003). These fibrils were only observed by TEM and not by DIC because they were not distinguishable from the spore wall.

Based on these differences, and host specificity we are able to justify the establishment of a new species, named *Myxobolus testicularis* sp. n.

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ACTA
PROTOZOOLOGICA

Stomatophora cloptoni sp. n. (Apicomplexa: Eugregarinida) from the Seminal Vesicles of an Indian Earthworm

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Summary. In a survey of the endoparasitic acephaline gregarines in the district South 24 Parganas of West Bengal, India seminal vesicles of earthworm *Eutyphoeus orientalis* (Beddard) were found to be infested with a new species of the genus *Stomatophora* Drzhevetskii, 1907. *Stomatophora cloptoni* sp. n. is spheroidal and measures 95.0-135.0 (110.0 ± 9.0) µm in diameter. A conical sucker 40.0-62.0 (55.0 ± 1.5) µm is present marked by eight furrows dividing the lateral margin of the sucker into 8 lobes. Nucleus spherical, 18.0-24.0 µm (20.0 ± 2.0) µm in diameter and placed very close to the sucker. Gametocysts are ovoid and measure 59.0-86.0 × 58.0-68.0 µm and oocysts biconical with almost bluntly pointed ends and measure 13.0-19.0 × 10.0-12.0 µm.

Key words: Acephaline gregarines, earthworm, India, seminal vesicle, *Stomatophora cloptoni* sp. n.

INTRODUCTION

Aseptate gregarine fauna have been reported from various parts of the world including India. But especially in India the search is far from complete. In an attempt to investigate the occurrence of acephaline gregarines in the oligochaete worms, seminal vesicles of earthworm *Eutyphoeus orientalis* (Beddard) collected from the South 24 Parganas district of West Bengal was infected with an undescribed species of *Stomatophora* Drzhevetskii, 1907, in their seminal vesicles. Little work has been done in India on the representatives of the genus *Stomatophora* parasitizing the oligochaete worms. Only seven species of the genus *Stomatophora* (Hesse 1909; Pradhan and Dasgupta 1980; Roychoudhury and Haldar 1984; Bandyopadhyay *et al*. 2001, 2004) have so far been described from India. In this paper taxonomic description of one new species of acephaline gregarines of the genera *Stomatophora*, Drzhevetskii, 1907 as well as comparisons with previously described species, are provided.

MATERIALS AND METHODS

Earthworms were collected and taken to the laboratory. They were dissected while alive and their seminal vesicles were carefully removed. These were placed on clean glass with a drop of 0.5% NaCl solution. A thin film of the seminal fluid was drawn out on a slide

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covered with a cover slip for examination of live protozoans under a phase contrast microscope. The content of seminal vesicles was semidried and fixed in Schaudin's fluid (20 min). The fixed smears were stored in 70% ethyl alcohol for removal of mercuric chloride. The slides were then passed through a descending series of alcohols (5 min each) and stored in distilled water. These were transferred to a 3% iron alum solution and stained with Heidenhain's haematoxylin solution (20 min). Differentiation (over night) was done with 1% iron alum solution. The slides were then washed thoroughly, dehydrated in an ascending series of alcohol, cleared in xylene and mounted in Canada balsam. Photographs were taken with an Olympus camera. In each case minimum and maximum values are given, followed in parentheses by arithmetic mean, standard deviation and number of sample size. All measurements are in µm. Method of describing shapes of planes and solids follows the guidelines of Clopton (2004).

RESULTS AND DISCUSSIONS

Stomatophora cloptoni sp. n. (Figs 1-11)

Phylum**:** Apicomplexa Levine, 1977; Order: Eugregarinida Leger, 1900; Family: Monocystidae Bütschli, 1882; Subfamily: Monocystinae Bhatia, 1930.

Diagnosis: Diameter of body (DB) = 95.0-135.0 (110.0 ± 9.0); diameter of nucleus (DN) = 18.0-24.0 (20.0 ± 1.7); diameter of sucker (DS) = 40.0-62.0 (55.0 ± 1.5); length of gametocyst (LG) = 59.0-86.0 (65.0 ± 1.9), width of gametocyst (WG) = 58.0-68.0 (61.0 ± 1.7); length of oocyst (LO) = 13.0-19.0 (17.0 ± 2.0); width of oocyst (WO) = 10.0-12.0 (10.8 ± 1.9); DB: DN = 5.5:1; DB:DS (1.69:1); DN:DS= 1:0.36; LG:LO = 3.8:1; WG:WO = 5.6:1. Number of measurements made: 34

With the characters of genus *Stomatophora* Drzhevetskii, 1907, as given by Levine (1988); "gamonts spherical or ovoid; sucker petaloid, with radiating sides, oocysts biconical with a flattened button at each end, attached to each other end to end in long chains inside gametocyst". Gamont elliptoid, flattened and compressed between its two poles. Sucker resembles a plate or disc marked by eight furrows and divided into eight lobes. In a few immature gamonts five or six petals are observed. Young gamonts slightly elliptical in shape. Ectoplasm about 1µm in thickness, without any external processes. Endoplasm coarsely granular. Granulation almost uniform. Sometimes a few patches of haematoxylin positive granules distributed at certain portions of the endoplasm. Sucker looks most prominent part of the body. Sucker appears a shallow cup-like depression, almost at the centre of the body. In a few young gamonts a very small mucron seen at centre of depression. Outer margin of aperture of sucker almost rounded. Nucleus almost spherical and always seen closely associated with sucker. Karyosome diffused and sometimes one or two vacuoles seen inside nucleus. Gametocysts almost rounded. Oocysts biconical with a flattened button at each end.

Taxonomic summary

Type material: *Stomatophora cloptoni* sp. n.

Type host: *Eutyphoeus orientalis* (Beddard)

Symbiotype: Host AH - 03/22/2002 deposited in the Museum of the Department of Zoology, University of Kalyani, Kalyani 741235, West Bengal, India.

Site of infection: Seminal vesicles.

Type locality: Dimond Harbour, South 24 Parganas, West Bengal, (22.11°N, 88.14°E).

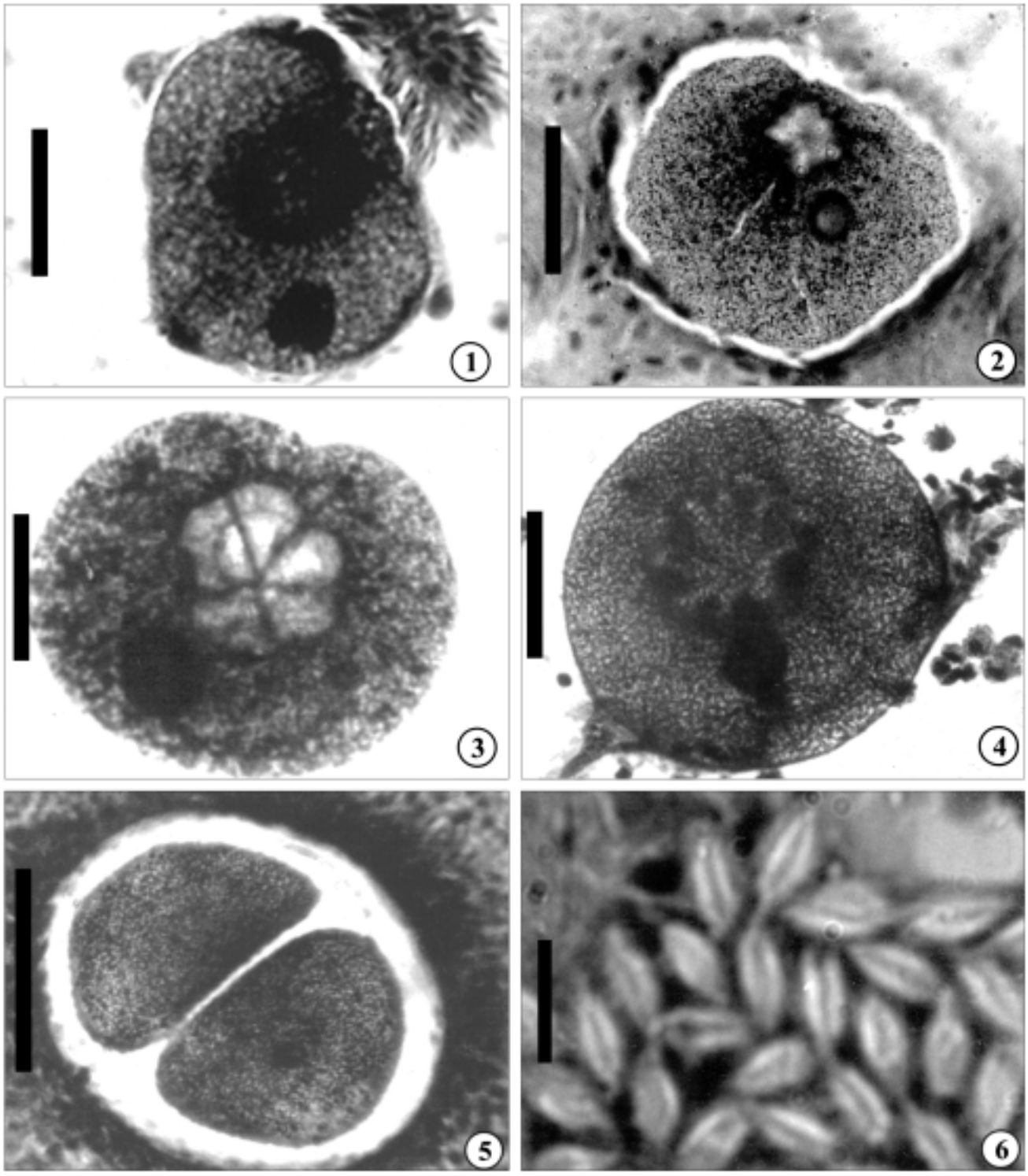
Prevalance: 7 out of 44 (15.9 %).

Type material: Holotype, slide EO/05/2004, and paratypes, slides EO/01/2004, EO/02/2004, EO/04/2004 EO/06/2004, EO/07/2004 are in the collection of the Parasitology Laboratory, Department of Zoology, University of Kalyani, Kalyani 741235, West Bengal, India. Slide EO/03/2004 bearing some paratype materials are in the collection of the Museum of Zoological Survey of India (ZSI), Calcutta - 700016 (Catalogue No. 2506).

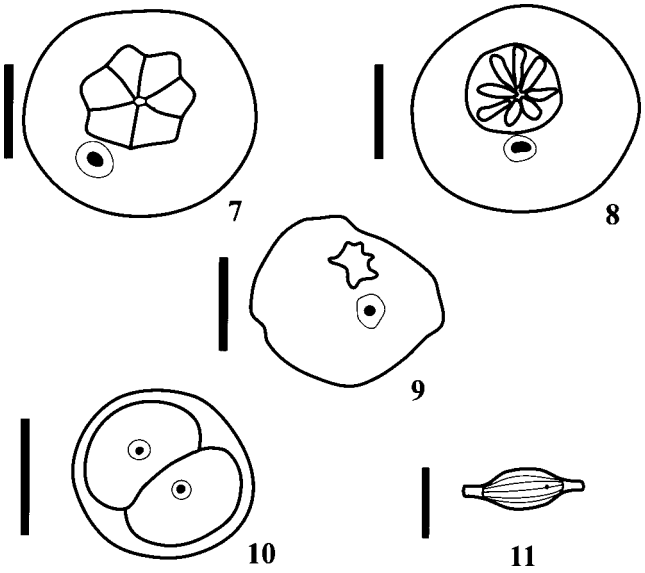
Etymology: The specific epithet "*cloptoni*" is given after the name of Dr Richard E. Clopton of Department of Natural Science, Peru State College, USA, for his outstanding contribution in the field of apicomplexan biology.

Remarks

Spherical body with a cup-like sucker near the centre justifies the inclusion of the present form under the genus *Stomatophora* Drzhevetskii, 1907. At present there are ten described species of *Stomatophora*. No stomatophorid gregarines has been reported from the present host species, *Eutyphoeus orientalis* (Beddard). Considering the shape of gamonts, the present species can be compared with *S. globa* Pradhan et Dasgupta, 1980, *S. diadema* Hesse, 1909 and *S. coronata* (Bhatia, 1924). The size of the gamonts of *S. cloptoni* (95.0-135.0) is comparatively much greater than *S. globa* (30.6-97.2). The sucker of *S. globa* has a superficial similarity with the present species in having invisible mucron and long prolongation radiating outwards which are variable in number. But in *S. cloptoni* the prolongations radiating outwards are always eight in number and the sucker assumes a cup-like appearance. The nucleus of the gamonts of *S. globa* is rounded and karyosome is eccentric and well visible. In *S. cloptoni* although the



Figs 1-6. Photomicrographs of different stages of life cycle of *Stomatophora cloptoni* sp. n. **1**, **3** - young gamonts; **2** - side view of gamont; **4** - mature gamont; **5** - gametocyst; **6** - oocysts. Scale bars 50 µm (1-5); 10 µm (6).



Figs 7-11. Camera lucida drawings of different stages of life cycle of *Stomatophora cloptoni* sp. n.; **7** - young gamont; **8** - mature gamont; **9** - side view of gamont; **10** - gametocyst; **11** - oocyst. Scale bars 50 µm (7-10); 10 µm (11).

nucleus is rounded the karyosome is situated near periphery. The size of the sucker of *S. globa* is comparatively smaller than *S. cloptoni* (12.6-39.6 *vs*. 40.0-62.0 in *S. cloptoni*). The sucker in *S. globa* is lacking any depression and any radiating sides. But the sucker in *S. cloptoni* is having a cup like depression with eight radiating sides. The maximum size of the gamonts of both *S. diadema* (105) and *S. cloptoni* (110.0) do not differ significantly. The whole body of the gamonts of *S. diadema* is marked by furrows and divided into a number of irregular lobes. But the body of gamonts of *S. cloptoni* does not have such type of furrows. In *S. diadema*, a prominent mucron is present whereas the new species under discussion does not have any prominent mucron in most of the mature gamonts. The size of the gametocysts of *S. diadema* is significantly larger (130-170) than that of *S. cloptoni* (59.0-86.0) but the size of the oocysts of *S. diadema* (8-12 × 3-4) is significantly smaller than the oocysts of *S. cloptoni* (13.0-19.0 × 10.0-12.0). The size of the gamonts of *S. coronata* (Bhatia 1924) is comparatively smaller than that of *S. cloptoni* (60.0-80.0 *vs*. 95.0-135.0 as in *S. cloptoni*). The shape of the body in *S. coronata* is also different, when it is compared with *S. cloptoni*. The main characteristic feature of *S. coronata* is the possession of the sucker which is an epimeritic structure slightly elevated off from the body. But in the new species under discussion the sucker does not elevate off the body. The body of *S. coronata* is widely expanded at the anterior end, so as to make it resemble a top or a flower vase. But in *S. cloptoni* the shape of the body is never expanded anteriorly and as a result the flower vase like structure has not been noticed. Another significant difference is the change of structure during movement. In *S. coronata*, during movement, a sharp constriction appears round the middle of the body, causing it to assume an hour glass like shape, and later it may acquire a more regular outline again. But in case of the new species obtained from *Eutyphoeus orientalis* (Beddard) no sign of constriction appears during movements. In *S. coronata*, the sucker is surrounded by a well developed petaloid crown marked by epicytal striations. In case of *S. cloptoni*, sucker is not surrounded by any type of petaloid epicytal striations. The size of the oocyts also differs in both cases. The size of the oocysts in *S. coronata* is also smaller (9-11 by 5-6 and 7-7.5 by 3) than that the new species (13.0-19.0 × 10.0-12.0).

Considering all these differences the parasite described has been given a separate species status under the genus *Stomatophora* Drzhevetskii, 1907 and is designated as *Stomatophora cloptoni* sp. n. in this paper.

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Short Communication

Is *Amoeba proteus* Myosin VI Immunoanalogue a Dimeric Protein?

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Summary. Myosin VI is an unusual actin-based motor protein moving, unlike other known myosins, towards minus end of actin filaments. This implies a unique role of this protein in cell migration and intracellular transport. There are serious concerns whether myosin VI heavy chains may dimerize as its amino acid sequence contains heptad repeats responsible for dimerization but both native and recombinant myosins VI were found to be monomeric proteins. Recently, we have detected 130-kDa myosin VI immunoanalogue in *Amoeba proteus* that also exhibits many features characteristic for mammalian myosins VI (Dominik *et al*. 2005). It seemed interesting to check whether it is a monomeric or dimeric protein. Using a zero-length crosslinker, N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDC), we have shown that under our experimental conditions the 130-kDa band corresponding to myosin VI immunoanalogue disappeared with the concomitant accumulation of about 260-kDa protein band. Similar results have been obtained when the coiled-coil skeletal muscle myosin rod was subjected to the EDC-crosslinking. These data indicate that the heavy chains of *A. proteus* myosin VI immunoanalogue may form dimers. These results also suggest that *in vivo* myosin VI immunoanalogue may play a role of an active transporter that requires dimerization and processivity.

Key words: *Amoeba proteus*, crosslinking, dimerization, myosin VI immunoanalogue.

Abbreviations: EDC - N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide

INTRODUCTION

Myosins are actin-based molecular motors that hydrolyze ATP and convert its chemical energy into mechanical power. They are ubiquitously expressed as multiple isoforms in all eukaryotic cells, including protozoans and plants. They are composed of one or two heavy chains, and one or more light chains non-covalently attached to the heavy chain. Myosin heavy chains follow the common organization pattern, with an N-terminal globular motor domain containing nucleotide and actin-binding sites; a neck domain containing at least one IQ motif [light chain(s) binding site]; and the most variable C-terminal tail domain (Sellers 1999). Based on the diversity of amino acid sequence of motor domains, myosins have been divided into at least 18 distinct families (classes) (Berg *et al*. 2001). The classic, two-headed myosins, also called conventional myosins, form class II, and other myosin families are referred to as

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unconventional myosins. The tail regions of conventional as well as some of the unconventional myosins (for example myosins V, VI VII and X) contain specific sequence pattern (known as the heptad repeats) that enables the molecules to form stable coiled-coil and dimerize. Nonmuscle myosins are involved in various cellular functions, e.g. cell motility (myosins I, II, VI and X), intracellular transport of particles, vesicles and organelles (myosins I, V, VI and VII), endo- and exocytosis (myosins I, V and VI), cell contacts formation (myosins II and VII), cytokinesis (myosins II and VI), and transcription (myosin I).

In this study we have focused on class VI myosin, which is the only myosin known to date that moves towards the minus (pointed) end of actin filaments (Wells *et al*. 1999, Walker *et al*. 2000). It is involved in vesicle transport, cell migration and membrane tension maintenance (Buss *et al*. 1998, Sellers 1999, Hasson 2003, Frank *et al*. 2004). It has been postulated that myosin VI may play an important role during both endo- and exocytosis, but its exact role still needs to be established. In all cell types examined, myosin VI is associated with uncoated endocytic vesicles and carries these vesicles away from actin-rich sub-plasma membrane regions (Aschenbrenner *et al*. 2003). Yoshida *et al*. (2004) showed that myosin VI may be responsible for cell migration, because inhibit myosin VI expression in high-grade ovarian carcinoma cells impeded cell spreading and migration *in vitro*. Mutations in the myosin VI gene lead to deafness in mice (for references see Rędowicz 2002) and humans (Melchionda *et al*. 2001, Ahmed *et al*. 2003, Mohidin *et al*. 2004). In the deaf mice (*Snell's waltzer* syndrome), the major phenotype is manifested in the disorganized stereocillia of inner ear hair cells, but other defects can also be found. For example, in fibroblasts of *Snell's waltzer* mice the Golgi complex size and protein secretion are significantly reduced as compared with wild-type cells (Warner *et al*. 2003). Also, it has recently been shown that severe defects are found in hippocampus of these animals: there are less synapses, abnormally short dendritic spines, symptoms of astrogliosis, and the hippocampal neurons display a significant deficit in the stimulation-induced internalization of one type of glutamate receptors (Osterweil *et al*. 2005).

The heavy chain of mammalian myosins VI has a molecular weight of about 140 kDa with a motor domain containing an unique 53 amino-acid long insert followed by a short neck with one IQ motif binding calmodulin, and a tail region. This C-terminal domain is believed to consist of a helical domain with the heptad repeats and a globular domain, responsible for a cargo binding and/or protein-protein interaction. It has been a general belief that myosin VI forms dimers *via* its coiled-coil region (Fig. 1). Surprisingly, Lister *et al*. (2004) showed that both expressed and native proteins were monomeric. However, they did not exclude the possibility that *in vivo* myosin VI may function both as a monomer and a dimer.

Recently in a highly motile free living *Amoeba proteus*, we have found a novel 130-kDa myosin VI-immunoanalogue (Dominik *et al*. 2005). Sequence comparison of several peptides derived from this protein with the other known myosins revealed a considerable homology to both human and invertebrate myosins VI. In migrating cells, myosin VI immunoanalogue was distributed in the entire cytoplasm as the punctate structures corresponding to membranous vesicular compartments where it colocalized with dynamin II; the colocalization was even more evident in pinocytotic cells. Blocking the endogenous protein with anti-human myosin VI antibody caused the inhibition of the rate of amoebae migration. To further characterize this novel ameboid myosin VI isoform and elucidate whether its heavy chains form dimers, we performed the crosslinking experiments using the zero-length crosslinker, EDC. The obtained data indicate that under our experimental conditions *A. proteus* myosin VI immunoanalogue forms dimers.

MATERIALS AND METHODS

***Amoeba proteus* culture and preparation of high speed supernatant.** *Amoeba proteus* (strain Princeton) was cultured at room temperature in the standard Pringsheim medium [$Ca(NO_3)_2 \cdot 4H_2O$ - 0.848 mM, $MgSO_4 \cdot 7H_2O$- 0.081 mM, $Na_2HPO_4 \cdot 2H_2O$ - 0.112 mM, KCl - 0.112 mM, $FeSO_4 \cdot 7H_2O$ - 0.007 mM, pH 6.8 - 7.2]. Amoebae were fed on *Tetrahymena pyriformis* twice a week and always used for experiments on the third day after feeding. Cells were collected by centrifugation at 2000 × g, washed three times with the ice cold TBS buffer (Tris buffer saline: 150 mM NaCl, 25 mM Tris pH 7.5), homogenized at 4°C in two volumes of a homogenization buffer containing 0.5% Triton X-100, 150mM NaCl, 10 mM KCl, 2 mM $MgSO_4$, 20 mM phosphate buffer pH 7.0, 1 mM EGTA, 2 mM ATP, 0.5 mM PMSF, 12% sucrose, and a set of protein inhibitors ("Complete" tablets, Roche). After centrifugation at 100 000 × g for 1 h the high-speed supernatants (HSS) were collected and subjected to crosslinking experiments.

Preparation of rabbit skeletal muscle myosin rod. Rabbit skeletal muscle myosin rod was prepared by the method described by Margossian and Lowey (1982). Briefly, myosin suspension (~10 mg/ml) in 0.2 M ammonium acetate and 2 mM EDTA, pH 7.2, was

digested at room temperature for 7 min with 0.03 mg/ml papaine dissolved in 5 mM cysteine pH 6.0 and 2mM EDTA. The reaction was stopped by 1mM iodoacetate, and the suspension was spun at 60 000 × g for 90 min. The insoluble precipitate was dispersed in solution A containing 0.6 M KCl and 0.05 M potassium phosphate pH 7.0, and three volumes of ice cold 95% ethanol were then added. The suspension was vigorously stirred for 3 h at 4°C and then spun at 20 000 × g for 30 min. The pellet was redispersed in solution A, and dialyzed overnight against the buffer in the coldroom. The supernatant was collected and further clarified by centrifugation at 70 000 × g for 90 min, and dialyzed against 30 mM KCl and 10 mM potassium phosphate, pH 7.0. The precipitated protein was collected by centrifugation at 20 000 x g for 30 min and then homogenized in two volumes of a homogenization buffer containing 0.5 M NaCl, 10 mM MOPS, pH 7.0, 0.5 mM PMSF, and a set of protein inhibitors. The homogenate was dialyzed overnight at 4°C against the same buffer, and centrifuged at 50 000 × g for 1 h. The supernatant was subjected to further experiments

The protein concentration of amoeba HSS and myosin rod preparation, measured by microbiuret method, was similar and was estimated to be about 5 mg/ml.

EDC crosslinking. Amoeba HSS and rabbit myosin rod were both mixed with *N*-ethyl-*N*'-(3-dimethylaminopropyl)carbodiimide (EDC, Sigma, USA) to its final concentrations of 5 mM and 50 mM. The mixtures were incubated at room temperature for 2 h, and 10 µl aliquots were collected every 30 min and immediately mixed with the Laemmli buffer to stop the reaction. The proteins were separated using 6 % SDS-polyacrylamide gels (Laemmli 1970), and then either stained with Coomassie Brillant Blue, or transferred to a nitrocellulose membrane (Towbin *et al*. 1979). After the transfer, the membrane was blocked for 1 h at room temperature in TBS containing 5% non-fat milk powder, 0.2% Triton X-100 and 0.05% sodium azide followed by 2-h incubation with 1:200 dilution of polyclonal anti-myosin VI antibody directed against the C-terminus of human myosin VI (M 5187, Sigma-Aldrich, USA). The primary antibody was detected using a 1:10 000 dilution of anti-rabbit antibody conjugated with alkaline phosphatase (A 3687, Sigma-Aldrich, USA). The colour reaction was developed using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma, USA) as the substrates.

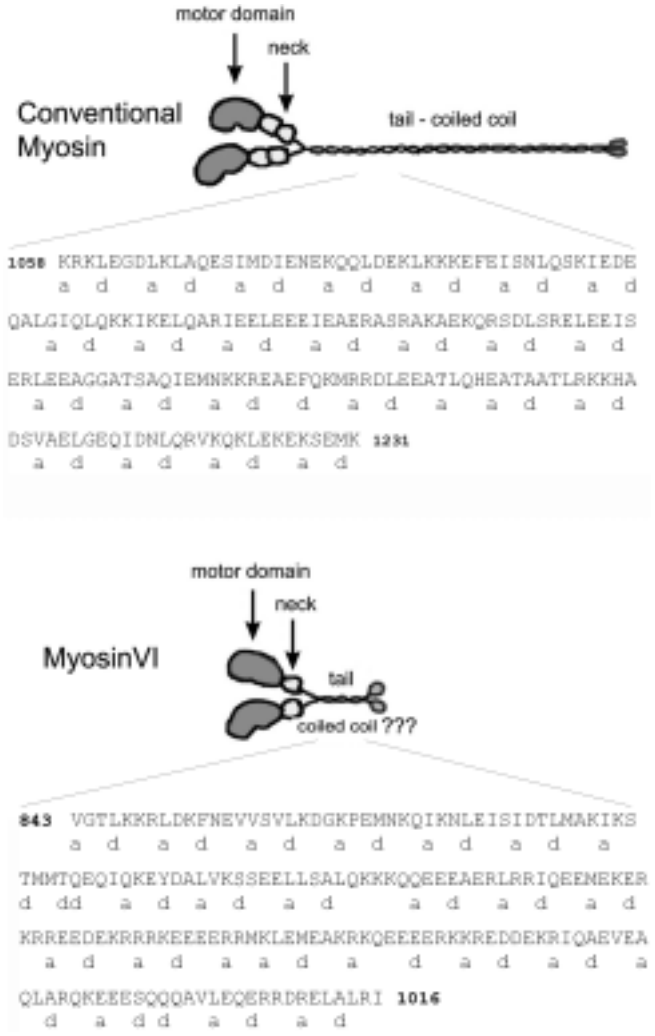

Fig. 1. Scheme of skeletal muscle myosin and myosin VI domain organization, with aminoacid sequences of the fragment (skeletal muscle myosin) or entire (myosin VI) coiled-coil region. a, d - heptarepeats hydrophobic residues, crucial for the coiled-coil formation.

RESULTS

In order to test whether the novel *Amoeba proteus* myosin VI-immunoanalogue is a monomeric or dimeric protein, the crosslinking experiment was performed using a zero-length crosslinker, EDC (Fig. 2). This reagent activates carboxyl groups and couples them with primary amines such as lysine to generate peptide bonds thus producing a zero-angstrom-long cross-link. That ensures that only proteins directly interacting with each other are crosslinked and those that are only in close proximity are not coupled. As a positive control, rabbit skeletal muscle myosin rod that is known to almost entirely consist of heptad repeats, and form stable coiled-coil (Fig. 1) and filaments was used. It should also be explained here that the diffusive appearance of the bands corresponding to cross-linking products resulted from the known negative effects of EDC on protein resolution.

Amoeba proteus high speed supernatant (HSS) (Fig. 2A) and rabbit skeletal muscle myosin rod (Fig. 2B) were treated at room temperature with both 5 and 50 mM EDC for 30, 60, 90, and 120 min, as indicated in the figures. The formation of the cross-linking products was monitored using the SDS-PAGE gels and, in the case of amoebae proteins, Western blots. *A. proteus*

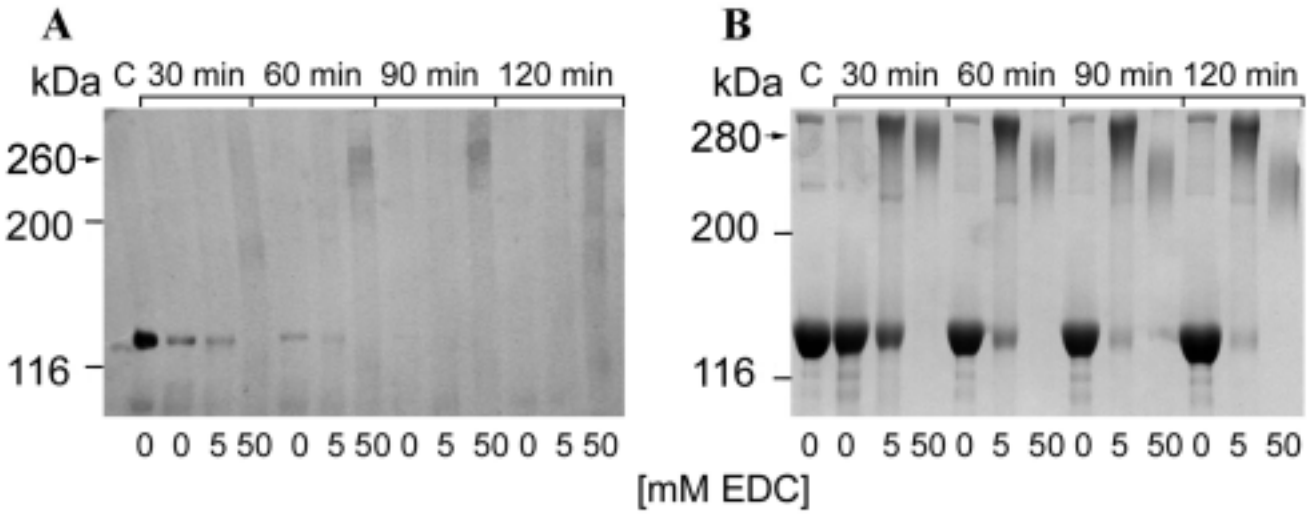

Fig. 2. Time course of EDC-crosslinking. **A** - *Amoeba proteus* high speed supernatant (HSS); **B** - rabbit skeletal muscle myosin rod; were subjected to crosslinking with no (0), 5 mM or 50 mM EDC as indicated in the figure. Myosin VI immunoanalogue and its crosslinking product were detected with polyclonal anti-human myosin VI antibody, and muscle myosin rod and its crosslinking product were visualized with Coomassie Brillant Blue staining. C, HSS and myosin rod not treated with EDC; 30 min, 60 min, 90 min, 120 min, times of incubation with EDC. Arrows points to the crosslinking products

myosin VI immunoanalogue was visible in not-treated HSS as the about 130-kDa band (Fig. 2A, lane C). During the course of the experiment, the band corresponding to myosin VI immunoanalogue diminished and a new band, corresponding to 260 kDa, appeared (Fig. 2A, arrow). This molecular mass seems to be consistent with that of putative dimers of myosin VI immunoanalogue heavy chain. The presence of the band was much more evident when the higher EDC concentration was used (Fig. 2A). Interestingly, while the 260-kDa cross-linking product was stable after its first appearance after 60 min, the intensity of the initial 130-kDa band was gradually decreasing even when the incubation was performed without EDC (Fig. 2A, lanes 0). It can be explained in terms of higher susceptibility of non-crosslinked protein to non specific proteolytic cleavage, even in the presence of protease inhibitors in the solution; crosslinking within the coiled-coil region may protect the protein from the protease attack.

Myosin rod not treated with EDC (Fig. 2B) was visible as the one band corresponding to about 140 kDa and remained stable during the 2 h incubation at room temperature. Addition of EDC, 5 or 50 mM, to myosin rod preparation resulted in the appearance of a new protein band of about 280 kDa (which corresponded to the known molecular weight of a rod dimer) with the concomitant and gradual disappearance of the 140-kDa myosin rod band (Fig. 2B, arrow).

These data indicate that 130-kDa myosin VI immunoanalogue heavy chains may be crosslinked with each other, similarly to muscle myosin rod.

DISCUSSION

Myosins form a diverse superfamily, both structurally and functionally. They are involved in panoply of cellular processes that are vital for cell and organism survival such as - among others - cell migration, muscle contraction, cytokinesis, endo- and exocytosis, hearing and vision (see Rędowicz 2002). It is a general belief that the multiplicity of functions fulfilled by these actin-based molecular motors is realized *via* their structural diversity, and in particular *via* variability of the C-terminal tail domains (Sellers 1999, Berg *et al*. 2001). For example, in the amino acid sequence of the heavy chains of several myosins' (namely myosins II, V, VI, VII, VIII, X, XI, XII and XVIII) tails the heptad repeats responsible for coiled-coil formation were found, indicating that these heavy chains may dimerize (Berg *et al*. 2001). While it has been definitely confirmed that myosins II and V are dimeric both *in vitro* and *in vivo*, the

dimerization of other myosins' heavy chains still remains an open question.

And so is the case of myosin VI, which heavy chain contains approximately 100-amino acid-long heptad repeats region that was predicted, using COILS software (Lupas *et al*. 1991), to form coiled-coil (Fig. 1). Based on this, the assumption has been made that myosin VI is a dimeric protein (Kellerman and Miller 1992, Sellers 1999). This supposition was taken into consideration whilst creating recombinant fragments of myosin VI for structure-function relationship studies (De La Cruz *et al*. 2001, Morris *et al*. 2003). Surprsingly, Lister *et al*. (2004) showed, using size exclusion chromatography and EDC crosslinking that both expressed and native myosin VI preparations contained only one 140-kDa heavy chain. However, it should be emphasized that the cross-linking experiments were carried out in high salt and Tris buffer, pH 8, conditions not suitable for EDC coupling (see Pierce catalog and handbook). Since then no other experimental data supporting this observation have been presented, however, the detailed analysis of myosin VI amino acid sequence made by the authors revealed that the putative coiled-coil sequence contained some proline residues probably breaking the helix and a central charged region that would probably favour formation of intramolecular salt bridges rather than a coiled-coil (Lister *et al*. 2004). Our results showed that the 130-kDa heavy chain of *Amoeba proteus* myosin VI immunoanalogue, a novel protein that might be the first ameboid myosin VI isoform (Dominik *et al*. 2005), could be crosslinked by EDC, even in a phosphate buffer known to reduce the efficiency of EDC reaction, to an about 260-kDa product, most probably corresponding to the heavy chain dimer. This seems to indicate that myosin VI immunoanalogue may form dimers, what excludes the possibility that it belongs to the monomeric myosin I or other (III, IV or IX) families. However, it cannot be excluded that the heavy chains of myosin VI immunoanalogue are not only crosslinked with each other but also with other protein with the similar molecular weight, possibly its binding partner.

Kinetic properties of mammalian myosins VI seem to depend on the state of its dimerization. It was showed in studies performed on fragments or full-length dimeric myosin VI that the protein had a high duty ratio (De La Cruz *et al*. 2001) and moved processively along actin filaments with a large step size of 30-36 nm (Rock *et al*. 2001). On the other hand, Lister *et al*. (2004) demonstrated that monomeric myosin VI was a nonprocessive motor with a 18-nm step size undergoing a large conformational change upon addition of ATP.

The idea that both monomers and dimers of myosin VI may function *in vivo* is intriguing and may, in fact, explain the mechanisms of its functioning within the cell. Such functions as intracellular transport of vesicles and organelles require a processive dimeric motor, while processes such as maintaining tension, clustering transmembrane receptors or tethering membranes and vesicles to actin filaments would benefit more from a nonprocessive monomer (Buss *et al*. 2004). Our data indicate that under experimental conditions applied here, *A. proteus* myosin VI immunoanalogue may exist as a dimer, but most probably *in vivo* there is an equilibrium between monomers and dimers. Dimerization of myosin VI immunoanalogue may favour its role of an active transporter, translocating cargo to other compartments within these gaint cells. This idea seems to be additionally strenghtened by the assumption that myosin VI immunoanalogue may be the only one minus-end driven molecular motor in this microtubule-lacking environment.

Up to the date, there are no reports addressing when and where myosin VI exists as a dimer or monomer, and what factors control its dimerization. It has been proposed that during endocytosis monomers could be recruited to plasma membrane "hot spots" and/or clathrin-coated pits to achieve the concentration sufficient to initiate a spontaneous dimerization through its helical tail domain (Buss *et al*. 2004). This suggestion is based on the report showing that dimerization of kinesin Unc104/KIF1A on the lipid vesicles surface caused its processivity, and that motor dimerization could be used to control intracellular transport by this class of kinesins (Tomishige *et al*. 2002). However, it is also quite possible that association with the binding partners (e.g. Dab2, SAP95 or other yet unknown proteins) or phosphorylation/dephosphorylation of the heavy chain could contribute to dimerization process (Buss *et al*. 2004).

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