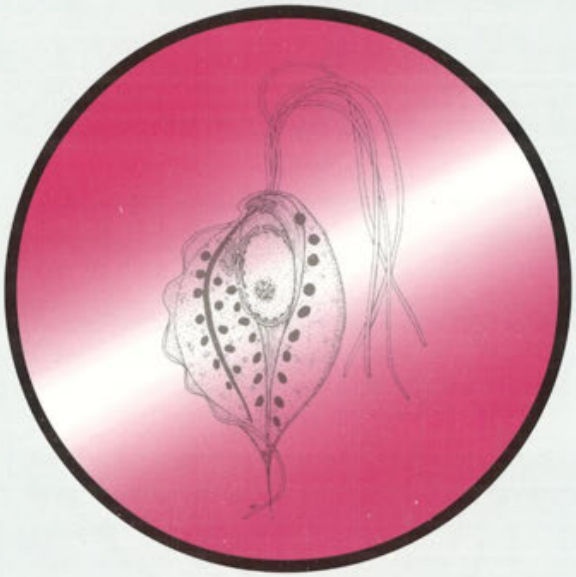
ACTA PROTOZOOLOGICA



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Professor Dr. Igor Borissovich Raikov (1932-1998)

On 27 October 1998, two months before his 66th birthday, Prof. Igor B. Raikov passed away in St. Petersburg (Russia). Friends, colleagues and students have lost in him an extraordinary personality and an untiring researcher in protozoology.

Igor Borissovich, as he was called in Russia, was born on the 30th of December 1932 in St. Petersburg (at that time Leningrad). His father, Prof. Boris E. Raikov (1880-1966), was a prominent Russian historian in biology and a Member of the Academy of Pedagogical Sciences. His mother, Antonina N. Raikova, was also a biologist. Thus, from his childhood, the little Igor was cultured and reared in a "biological medium". No wonder that biology became the subject of choice for his whole life.

Prof. Boris E. Raikov was a strict mentor and brought up his only son (he also had an older daughter from his first marriage) in the best traditions of high Russian society, whose primary demands involved speaking fluently at

least three foreign languages apart from a perfect knowledge of the mother tongue. All this much facilitated Igor's future fruitful scientific collaboration with protozoologists all over the world.

In 1949 Igor Raikov started his education at Leningrad University where he majored in protozoology under the supervision of the eminent zoologist Prof. Valentin A. Dogiel, at that time Chair of Invertebrate Zoology. His diploma was dedicated to the nuclear apparatus of an aberrant marine ciliate. After the death of his tutor Igor Raikov continued his research under Prof. G.I. Poljansky, who in his turn had also been student of V.A. Dogiel. In 1957 Igor Raikov was granted the candidate degree (Ph. D.) at the University and was invited to the newly organized Institute of Cytology, affiliated with the USSR Academy of Sciences, first as a junior and then a senior scientist in the Laboratory of Cytology of Unicellular Organisms chaired by G.I. Poljansky. In 1968 Igor Raikov granted the doctoral degree (D. Sc.) based on his monograph "Karyology of Protozoa" at the Institute of Cytology. Since then, karyology became his pre-eminent interest and remained so until the end of his life. Dr. Raikov always retained enduring in comparative and evolutionary karyology of lower eukaryotes (protists). Self-criticism and correctness characterized his personality and his work. He wrote about 250 papers, the majority published abroad. He and his students elaborated a fascinating phenomenon of nuclear dualism in ciliates. They demonstrated that the macronucleus in karyorelictid ciliates was incapable of dividing, although it retained the transcriptional apparatus providing protein synthesis in the cell. Apart from an examination of the structure and future fate of such "paradiploid" (according to his terminology) macronuclei during the division and conjugation of ciliates, Igor Raikov paid special attention to the mode of division of the polygenomic macronuclei in the ciliate *Nassula ornata.* His artistic skill in doing experiments always deserved admiration.

Later on, his research was extended to include studies into the pattern of mitosis in protists. Of great interest was his discovery (in collaboration with his French colleague Prof. J. Mignot) of meiosis in *Arcella vulgaris*, a fresh-water rhizopod.

In addition to his never-ending interest in experimental work, Igor Raikov was famous for his comprehensive theoretical reviews: on the macronucleus of ciliates (1969); on nuclear phenomena during conjugation and autogamy in ciliates (1972); on the evolution of macronuclear organization (1976); on primitive never-dividing macronuclei of some lower ciliates (1985); on the nuclear genome of Protozoa (1989); on the diversity of forms of mitosis in Protozoa (1994); on meiosis in protists (1995); on the dinoflagellate nucleus and chromosomes (1995); on the structure and genetic organization of the polyploid macronucleus of ciliates (1995). In these and other related publications the problems of protozoan karyology were discussed in terms of modern cell and molecular biology.

But a true scientific memorial of our great colleague has been his comprehensive creation, in the form of three monographs, dedicated to the protozoan nucleus. Its first version "Karyology of Protozoa" appeared in 1967 to be followed by the second monograph, also in Russian, titled "The Protozoan Nucleus. Morphology and Evolution" (1978). In 1982, the world-known Springer-Verlag published the extended version of the latter translated into English. The monographs became true reference books, badly needed by those dealing with cell biology, karyology, cytogenetics and other close disciplines, and brought their author a great international recognition.

Apart from his special interest in karyology, Igor Raikov was readily engaged in solving other questions of general importance. When studying the psammofilic ciliate fauna of the basins populated by the objects of his karyological research, he first observed and described peculiar symbiotic relations between the ciliate *Kentrophorus fistulosus* and sulphur bacteria. The latter made some kind of a paling on the dorsal part of a ciliate and were often used by their hosts as an easily available ready made food.

In co-authorship with G.I. Poljansky he wrote a series of papers on the role of polymerization and oligomerization of homologous organelles in evolution at the cellular level.

Due to his high international scientific reputation Prof. Raikov was awarded numerous honors, including memberships in Groupement des Protistologues de Langue Francaise (GPLF) and in the Deutsche Akademie der Naturforscher Leopoldina. He spoke at numerous International and Regional meetings on protozoology. In 1989, the Organizing Committee of the VIIIth International Congress on Protozoology, held in Tsukuba, Japan, invited him as a plenary speaker on the problem of protistan karyology. Igor Raikov was much pleased when he was provided an opportunity to work on some marine biological stations, especially in Besse-en-Chandesse, Roscoff, or Banyuls-sur-Mer which reflected his obvious frankophilia. In addition to the field and laboratory work, he found a strange fascination in lecturing and being a guide and tutor to students. He was invited to read lectures for students at the Universities of St. Petersburg, Clermont-Ferrand and Tübingen.

Igor Raikov was a member of the Editorial Boards of some International journals: "Acta Protozoologica","Archiv für Protistenkunde", "European Journal of Protistology" and "The Journal of Eukaryotic Microbiology". In 1988 he became Chair of Laboratory of Cytology of Unicellular Organisms at the Institute of Cytology RAS and soon became Professor (1989). In 1991 he was elected a Member of the Russian Academy of Natural Sciences. For years he was an active member of the Scientific Councils of the Institute of Cytology RAS and of Zoological Institute RAS in St. Petersburg. In 1993, after the death of Prof. Poljansky, Igor Raikov was elected President of the Russian Society of Protozoologists. For several years in the past he was a member of the IUBS Commission on Protozoology, serving as a representative of his national Society. As a successor of his great predecessors, V.A. Dogiel and G.I. Poljansky, Igor Raikov followed the best traditions of the Chair of Invertebrate Zoology at the St. Petersburg University and had many students not only in his own country, but also abroad. He was an attentive guide and mentor to research students and his younger colleagues. He encouraged his students and later the staff of his Laboratory to follow their own inclinations, rather than telling them what to do, and gave his colleagues a great deal of freedom. He never dominated or criticized, but always tried to encourage and help. His great organizing talent was especially obvious during the magic time of white nights in Leningrad (now St. Petersburg) in 1969, when the IIIrd International Congress of Protozoology was held in this wonderful city on the Neva. Prof. Poljansky was President of the Congress, and Igor Raikov, who served Secretary General, was truly the heart and soul of the meeting. And this is still remembered by those who attended the Congress.

Prof. Raikov is survived by his wife, Dr. Ekatherina V. Raikova, a leading research scientist of the Institute of Cytology RAS, and his daughter, Dr. Olga I. Raikova, a senior research scientist of Zoological Institute RAS.

The family, friends, colleagues and students sorrow the premature loss of this unique personality.

Tamara V. Beyer and Sergei O. Skarlato

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Review Article

Modulation of Endocytotic Activity of Paramecium by the β-Adrenergic Ligands

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Summary. An overall effect of β-adrenergic ligands on *Paramecium* endocytosis has been summarized: this includes results obtained using light and electron microscopy combined with cytophysiological and biochemical observation and fluorescent mapping. Modulation of *Paramecium* endocytotic activity enabled to create a model cell to follow uptake and retention of photosensitizers used in photodynamic therapy of tumors. Indication on a putative existence of β-adrenergic receptor system in *Paramecium*, based on molecular investigation, has also been reviewed.

Key words: β-adrenergic antagonists, catecholamines, fluid phase uptake, forskolin, *Paramecium aurelia*, PCR, phagocytosis, phorbol ester, photodynamic therapy, Southern hybridization.

INTRODUCTION

Endocytosis is one of the most important physiological processes that take place in living organisms. Nutrients, hormones, growth factors, antibodies, enzymes, lipoproteins and pathogenic substances get into the cell *via* this process (Steinman *et al.* 1983, Goldstein *et al.* 1985, Holtzman 1989). Recent years have brought an increase in studies on endocytosis, which is a result of two following facts: (i) - gaining of the evidence that endocytosis can be treated as a process of continuous membrane flow from the surface of the cell to its inside and back again (Steinman *et al.* 1983); (ii) - discovery of the receptor-mediated endocytosis, which had an unusual importance in explaining molecular mechanism of some metabolic diseases (Goldstein *et al.* 1985) and which was honored with the Nobel's Prize in 1985.

Protozoan cells (Ciliata) have been a subject of studies on endocytosis for many years (Allen 1974; Fok and Shockley 1985; Wyroba 1986a, b; Quinones-Maldonado and Renaud 1987; Allen *et al.* 1992; Christensen *et al.* 1998) and important findings concerning this process including the mechanism of non-lysosomal acidification of endosomes, originated from these studies (Allen and Fok 1983). Morphological and ultrastructural details of endocytosis are well known (Steinman *et al.* 1983, Holtzman 1989, Weigel and Oka 1998, Claus *et al.* 1998). However, the mechanism regulating the process of endocytosis in this unicellular, free-living protozoan cell has not been clearly established.

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We review here cytophysiological evidence indicating that membrane β-adrenergic system controlling the process of endocytosis exists in the cells of *Paramecium aurelia*. This finding makes experimental modulation of endocytotic activity easy to achieve, creating an interesting model useful in many biological studies.

INHIBITION OF ENDOCYTOSIS BY β-ADRENERGIC ANTAGONISTS

β-adrenergic receptor antagonists show an inhibiting effect on *Paramecium* endocytosis. This process was studied at the level of:

A - a light microscopy as phagocytosis of latex (Giordano *et al.* 1985, Wyroba 1986b, 1989a)

B - an electron microscopy, observing:

(i) - endocytosis of ruthenium red, which accompanies labeling of the surface of *Paramecium* living cells (Wyroba 1984a);

(ii) - endocytosis of cationized ferritin (Wyroba 1988)

C - a fluorescence microscopy, analyzing the internalization of cycloheptaamylose - dansyl chloride complex (CDC), which occurs during labeling of the surface of living, starved cells of *Paramecium* (Giordano *et al.* 1985).

Inhibition of phagocytosis of latex beads

β-blockers inhibit phagocytosis of latex beads in *Paramecium* cells (Wyroba 1989a). This process is reversible and dose- and time-dependent but does not depend on the size (0.5 - 4.5 μm) of the particles uptaken or cell culture age. This effect has been observed when β-blocker was administrated prior to latex addition (Fig.1) as well as in the case of simultaneous addition of both compounds. Starved cells were more sensitive to the antagonists than the non-starved ones of the same age (Wyroba 1986a, 1989a).

The extent of inhibition of phagocytosis was dependent on lipophilicity of applied β-blocker and was the highest for the most lipophilic blocker among the tested ones, that is 1-propranolol, which completely blocks the formation of phagosomes at 75 μM concentration. The level of inhibition was the lowest for hydrophilic atenolol (not exhibiting the membrane stabilizing effect), which - when applied at above concentration - causes a decrease in a number of phagosomes formed by 85%. The full inhibition of phagocytosis can be observed at higher doses of atenolol (Wyroba 1989a).

In the case of simultaneous incubation of cells with β-blocker and latex phagocytotic activity was decreased to 7%, 12% and 30% of the control for 75 μM propranolol, alprenolol and atenolol respectively. Then the process of restoration of phagocytic activity takes place and its duration depends directly on concentration of antagonist (Wyroba 1986a, 1989a).

The stronger inhibiting effect of lipophilic blockers (propranolol and alprenolol), in comparison with hydrophilic atenolol, may be ascribed to the fact that ligand-binding site in β-receptor molecule is situated within the 7th hydrophobic transmembrane region of the receptor (Strader *et al.* 1987, 1989).

The effect of β-adrenergic receptors antagonists on *Paramecium* cells has not been previously observed. However, there is a controversy concerning the effect of β-blockers on *Tetrahymena* cells: Ricketts (1983) reported that dichloroisoproterenol inhibited phagosomes formation at 700 μM concentration, while Fok and Shockley (1985) observed it at 40 μM. In addition, Quinones-Maldonado and Renaud (1987) claimed that either alprenolol or propranolol do not affect phagocytosis in *Tetrahymena* at micromolar concentration, but do inhibit it at millimolar concentration, which suggests that this may be an unspecific cellular response due to membrane stabilizing effect of β-blockers.

Inhibition of endocytosis of ruthenium red and cationized ferritin.

Labeling of a surface of living cells may be associated with endocytosis of applied marker, which was observed during analysis of starved *Paramecium* cells that had undergone Luft's procedure, i.e. simultaneous fixation and staining with ruthenium red in order to carry out ultrastructural studies (Wyroba 1984a). This dye does not penetrate an intact cell, but it appeared to be internalized by starved protozoans (probably due to its incomplete solubility in water solutions). In addition to characteristic staining of the surface coat of cell membrane, the small vesicles (0.2 - 0.6 μm) filled with electron-dense product of cytochemical reaction were observed in such cells (Wyroba 1984a).

X-ray microanalysis carried out in electron microscope revealed that the vesicles contain both ruthenium and osmium (Wyroba 1989b) thus confirming that they are formed during the procedure of simultaneous fixation and staining, which lasts several seconds. *Paramecium* cells pretreated with β-blocker (at the concentration inhibiting latex phagocytosis) prior to Luft's procedure did not show the presence of internalized dye (Wyroba 1986a).

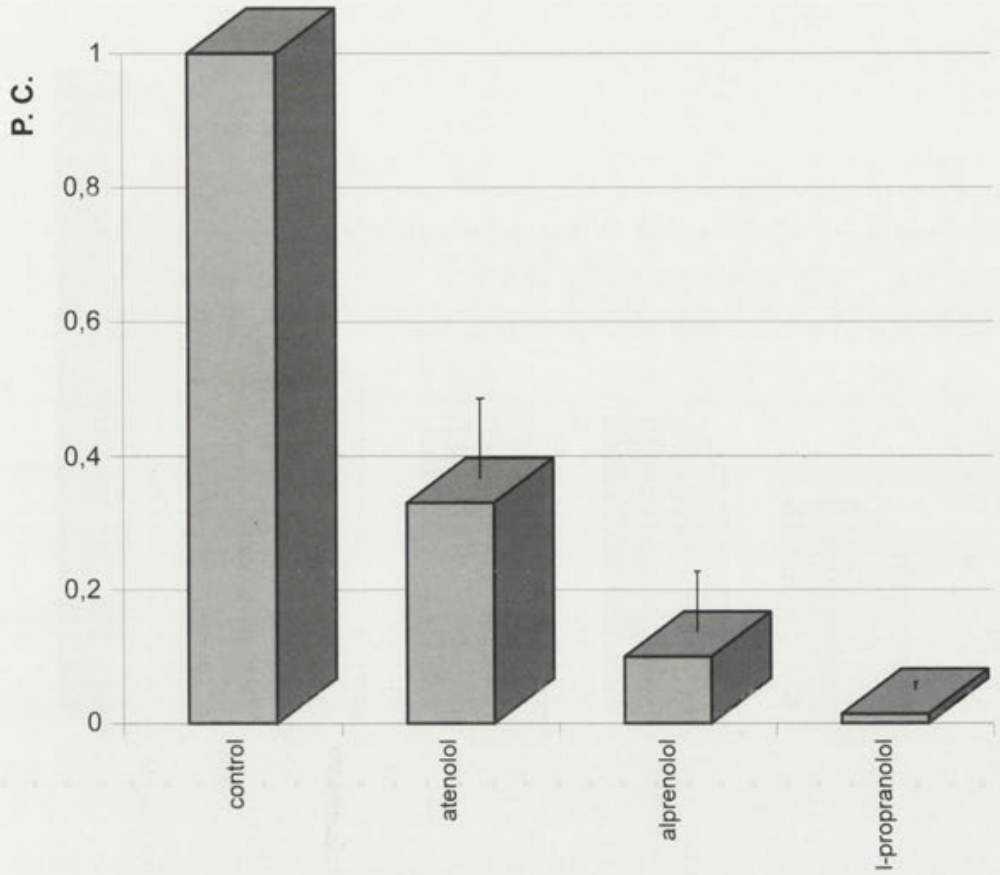

Fig. 1. Inhibition of *Paramecium* phagocytosis with β-adrenergic antagonists. The cumulated data on uptake of latex particles after pretreatment (for 20 min) with the 70 μM β-adrenergic blockers are expressed as P. C. (phagocytic coefficient) based on the results presented in Wyroba (1989a)

It should be pointed out that intracellular membrane pool, necessary for phagosome formation - that exists in Ciliata in a form of discoidal vesicles (Allen 1974) - was observed both in cells pre- and treated with β-antagonists, bringing an evidence that the effect of these blockers is limited to the cell surface (Wyroba 1986a,b).

Cationized ferritin penetrates cells *via* adsorption pinocytosis (van Deurs *et al.* 1982). Our results indicate that β-antagonist - dichloroisoproterenol inhibits endocytosis of cationized ferritin but does not affect its binding to the surface of the cell and to the parasomal sacs (Wyroba 1988) which are important mediators in endocytotic pathway (Allen *et al.* 1992).

Inhibition of internalization of cycloheptaamylose - dansyl chloride complex (CDC)

CDC, which does not enter the living cells, was used for fluorescent labeling of the surface of protozoan cells (Wyroba *et al.* 1981b) to follow dynamic properties of membrane (Wyroba *et al.* 1983, 1987; Wyroba 1984b). However, it was noticed that a minute amount of CDC was internalized during this process by starved *Paramecium* cells. This phenomenon was invisible until UV irradiation of dried samples occurred which resulted in an appearance of fluorescing endosomes. These endosomes (2.3 - 4.5 μm in diameter) emit fluorescence in other spectrum region than CDC labeled cell membrane. Using extracellular tests (Giordano *et al.* 1985), we showed that the product of identical spectrum characteristics of that recorded from the endosomes might be obtained only by dansyl chloride irradiation with UV. Dansyl chloride by itself is not a fluorescent substance. Living starved cells uptake CDC probably in a form of microcrystals, which may be due to a weak solubility of this compound in water solutions. Subsequently, in endosomes the CDC complex was cleaved to cycloheptaamylose and dansyl chloride, the latter one underwent photochemical reaction and gave a product

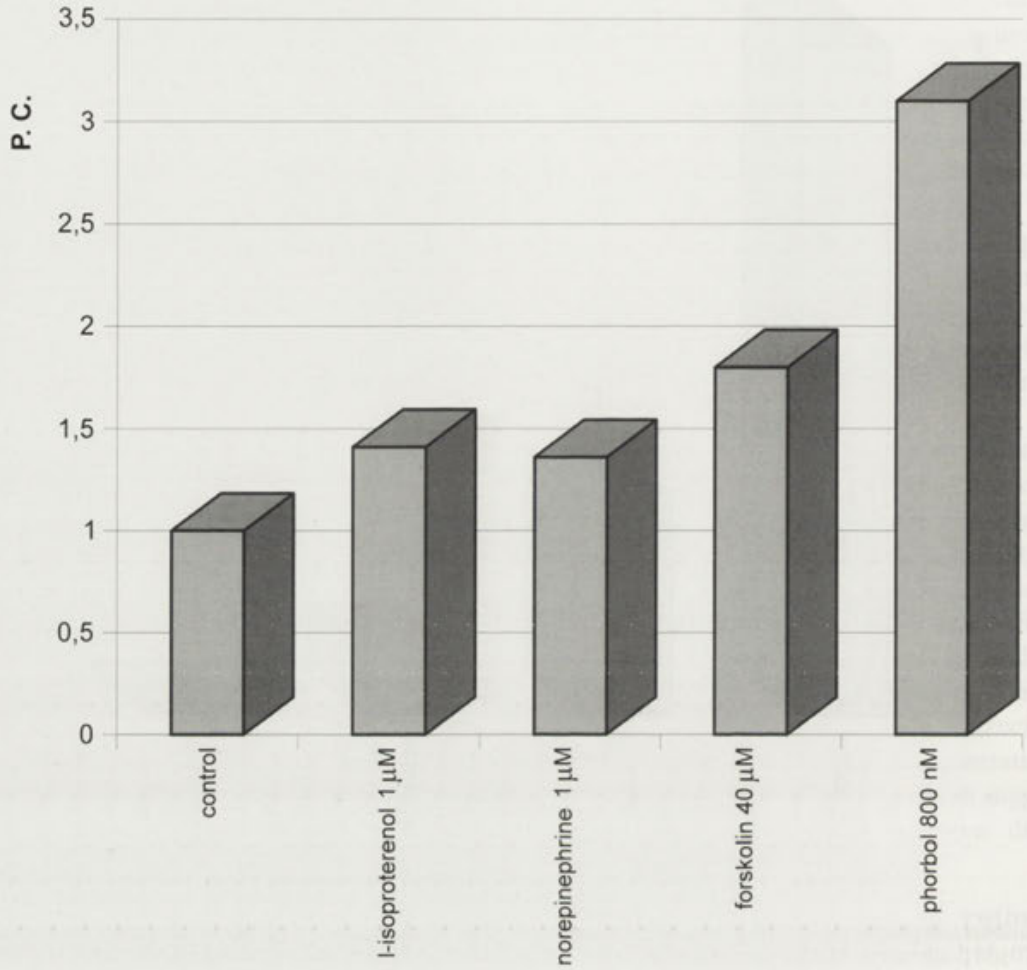

Fig. 2 Effect of catecholamines, phorbol ester and forskolin on uptake of latex particles. The cumulated data of *Paramecium* cells exposed to the drug and latex beads for 10 min. Data expressed as in Fig. 1 (based on the chosen results presented in Wyroba 1987, 1989c)

fluorescing in other spectrum region. CDC internalization process may be inhibited by β-adrenergic antagonist (dichloroisoproterenol) at concentration in which it blocked latex phagocytosis (Giordano *et al.* 1985).

STIMULATION OF *PARAMECIUM* PHAGOCYTOSIS BY β-ADRENERGIC AGONISTS, PHORBOL ESTER AND FORSKOLIN

It has been found that catecholamines at physiological concentrations, forskolin and phorbol ester stimulate phagocytosis in *Paramecium aurelia* cells (Wyroba 1987, 1989c) (Fig. 2). Norepinephrine and isoproterenol at 10^{-8} - 10^{-3} M enhance *Paramecium* phagocytotic activity after a few min incubation with latex particles. The maximal stimulation was observed at 10^{-6}M isoproterenol (41%) and norepinephrine (36%) within 10 min after catecholamine addition. Prolongation of incubation caused a slow decrease in endocytotic activity and its approaching to the control value (Wyroba 1989c), indicating a decrease in sensitivity to agonist - a phenomenon known as receptor desensitization (Lefkowitz *et al.* 1983).

Interaction with isoproterenol was stereospecific towards isomer (-) in comparison with tested (+) isoproterenol, which is in accordance with characteristics of cellular response in the higher eukaryotes, where stereoselective interaction of catecholamines with their receptors has been observed (Lefkowitz *et al.* 1983).

Incubation of the cells with cyclic AMP dibutyrate (db cAMP) caused a similar enhancement of phagocytic coefficient as in the case of stimulation with isoproterenol

or norepinephrine (Wyroba 1989c). It has been reported that cyclic AMP belongs to the attractant stimuli in chemosensory transduction in *Paramecium* cells (Yang *et al.* 1997). These cells respond to cAMP throughout their growth cycle and van Houten (1994) suggested that paramecia might use cAMP as a food cue rather than a social cue. There is almost no data concerning either presence of endogenous catecholamines or their effect in *Paramecium* apart from the reports dated of 1919 and 1936 (Wichterman 1986). A small amount of norepinephrine and epinephrine was discovered in *Tetrahymena* in which dopamine was a major catecholamine found (Goldman *et al.* 1981). There is a discrepancy in a literature concerning the catecholamine effect on *Tetrahymena* phagocytosis: Csaba (1985) as well as Quinones-Maldonado and Renaud (1987) reported that it enhanced phagocytic activity whereas Rothstein and Blum (1974) did not notice any effect.

Forskolin - diterpene activator of adenylate cyclase (Seaman *et al.* 1981) exerts a 2-fold higher stimulating effect on *Paramecium* phagocytotic activity than catecholamines: in 10 min phagocytotic coefficient reaches the values of 1.88 for 40 μM (Fig.2) and 1.75 for 1 μM of forskolin, respectively (Wyroba 1987).

Yet the most potent stimulator of *Paramecium* endocytosis has been the phorbol ester (PMA): its effect is concentration-dependent and almost a 3-fold increase in a number of digestive vacuoles was observed. The effect of phorbol esters lasts much longer than observed influence of catecholamines or forskolin. Phorbol esters have been thought to exert their effect through activation of protein kinase C by substituting diacylglycerol, produced physiologically from the breakdown of phosphatidyl inositol bisphosphate. The second product of this breakdown, inositol trisphosphate, exerts its action by elevation of cytosolic calcium concentration (Nishizuka 1984, Berridge 1987). The application of calcium ionophore A_{23187} was an attempt to mimic this process and did not cause any change in *Paramecium* phagocytotic activity (Wyroba 1987).

A role of inositol trisphosphate in *Paramecium* is not known and none of protein kinases discovered in this protozoan is activated by diacylglycerol (Gundersen and Nelson 1987, Son *et al.* 1993, Carlson and Nelson 1995, Kim *et al.* 1998). Therefore, stimulation of *Paramecium* endocytosis by phorbol esters should be explained by the reported effect of phorbol ester on modulation of the β-adrenergic receptor-linked adenylate cyclase (further discussed in: Wyroba 1987).

Both, forskolin and PMA may enhance catecholamines-induced cellular response in *Paramecium* - such an effect was also observed in higher eukaryotes. This process has been tested by adding these drugs to protozoans, which were pre-incubated for 5 min in presence of isoproterenol and latex beads. PMA induced almost a 2-fold increase in phagocytic activity (which had already been elevated by isoproterenol) and forskolin caused a 1.5-fold increase, respectively (Wyroba 1987).

Stimulating effect of catecholamines can be decreased or completely inhibited by simultaneous addition of β-blockers such as propranolol or alprenolol (Wyroba 1989c).

When cells previously blocked with antagonist were exposed to PMA or forskolin a striking increase in phagocytic activity was observed in comparison with the control cells: almost a 5-fold in case of PMA and a 3-fold increase after forskolin application (Wyroba 1987).

FLUORESCENT LOCALIZATION OF β-RECEPTOR SITES IN *PARAMECIUM* CELL

For localization and visualization of β-receptor sites in *Paramecium* cells dansyl analogue of 1-propranolol - DAPN was used (Wyroba 1989c). DAPN binds specifically to β-adrenergic receptors of turkey erythrocytes membranes - a classic biological material which had been used to study and isolate this receptor (Lefkowitz *et al.* 1983; Strader *et al.* 1987, 1989). At the end of 1970s DAPN was used for β-receptors localization in brain sections after intravenous injection (Atlas and Levitzki 1977, Atlas and Melmed 1978). DAPN was applied to free-living *Paramecium* cell and it seems that it was the only case of such application of this analogue.

β-receptors localization was analyzed in the starved cells not exhibiting any autofluorescence (Wyroba *et al.* 1981a). β-receptor sites were visualized on the cell membrane, forming characteristic 'dotted' cell pattern in fluorescence microscopy. If DAPN binding occurred prior to latex beads addition (as phagocytotic stimulus), β-receptor sites were detected in the membrane surrounding the newly formed phagosomes and at the bottom of the gullet where they are emerged (Wyroba 1989c).

It should be noted that the above characteristic fluorescent pattern of the cells labeled with DAPN has nothing to do either with *Paramecium* autofluorescence (Wyroba *et al.* 1981a) or with the living cell membrane dansylation

(Wyroba *et al.* 1981b). Moreover, DAPN emission maximum is at 520 nm and does not overlap either with autofluorescence maximum (430 nm) or with the spectrum from the CDC labeled membrane (450 nm) (Wyroba *et al.* 1981a,b).

The specificity of β-receptors mapping in *Paramecium* cell with DAPN has been checked by using protozoans pretreated with two propranolol isomers: d-propranolol did not influence DAPN binding while l-propranolol, a stereospecific β-receptor antagonist, inhibited the binding.

β-adrenergic receptors have not been localized so far in the free-living eukaryotic cells. Scarce data concerning the higher animals cells indicate homogenous distribution of these receptors on the plasma membrane (Zemcik and Strader 1988). Using a receptor antiserum von Zastrow and Kobilka (1992, 1994) and von Zastrow *et al.* (1993) found out that in transfected human 293 cells the β-adrenergic agonist isoproterenol induced redistribution of receptors from the surface of cells into small punctuate accumulations which were detected in cells within 2 min of agonist addition. This process is related to the desensitization of receptor in the presence of an agonist (Lefkowitz *et al.* 1983, Gagnon *et al.* 1998, Seibold *et al.* 1998). On the other hand, in the absence of agonist, receptors tagged with monoclonal antibody remain in the plasma membrane for over an hour (von Zastrow and Kobilka 1994).

β-adrenergic receptors belong to the group of the surface membrane receptors involved in a signal transduction from the environment to the cell. These receptors are coupled to G proteins, which are mediators between receptor and effector - in this case adenylate cyclase. Catecholamines binding to β-adrenergic receptor stimulate adenylate cyclase, which subsequently leads to generation of cAMP - the second messenger (Lefkowitz *et al.* 1983, 1986; Daaka *et al.* 1997).

All the adrenergic receptors are integral membrane proteins. The most striking characteristics of their structure is the presence of 7 hydrophobic transmembrane regions (Lefkowitz *et al.* 1983, 1986; Clark *et al.* 1989, Strader *et al.* 1989). β-adrenergic receptor is an evolutionary old protein: its gene arose probably as a result of duplication of the gene encoding for muscarinic cholinergic receptor - the oldest one among known receptors, dated for more than 600 millions years to which β-adrenergic receptor is highly homologous. β-adrenergic receptor belong to the group of such conservative proteins as hemoglobin and cytochrome c (Gocayne *et al.* 1987).

Many different functions of vertebrates are under β-adrenergic control (Rehnmark and Nedergard 1989, Safrany and Shears 1998), but it has not been found that adrenergic ligands modulate endocytosis process in the higher eukaryotes (Lefkowitz *et al.* 1983).

PARAMECIUM EXHIBITING A MODIFIED ENDOCYTOTIC ACTIVITY AS A MODEL CELL FOR CYTOPHYSIOLOGICAL STUDIES

Paramecium cells were used for the studies on localization, uptake and retention of chemical compounds applied in photodynamic therapy (PDT) of human tumors.

Thomas J. Dougherty, one of the inventors of this method, wrote in his review of 1989 that the principle of photodynamic therapy was based on the application of chemical compounds, which absorb light in order to initiate photoreaction in biological systems. This method comes out from the observation made 90 years ago, when *Paramecium* cells were exposed simultaneously to the light and acridine dyes (Dougherty 1989).

Photodynamic therapy is based on the irradiation of the tumor - loaded with a sensitizer, with a chosen wavelength of visible light - which initiates photochemical process and leads to the tumor necrosis (Dougherty 1989, Zhou 1989). The sensitizers used in the medical treatment of patients (Patrice *et al.* 1990) have been so far hematoporphyrin derivatives (HpD), known also as Photofrin-I and their purified and more active form, Photofrin-II (Mironov *et al.* 1990). The compound is a mixture of polyhematoporphyrin ethers and esters, whose degree of oligomerization probably ranges between the dimer and the hexamer states (Dougherty 1989, Zhou 1989). This hydrophobic fraction of HpD, preferentially taken up by cancer cells, determines their photodynamic destruction. At a molecular level, porphyrin photosensitization gives rise to singlet oxygen, which may oxidatively react with a number of specific sites in cells and tissues. As a consequence, cells undergo irreversible damage at the level of several subcellular targets, especially the cell membrane and mitochondria. The mechanism of destruction of cancer tumor is complex (Zhou 1989) and it is beyond the scope of this paper.

Sensitizers are carried into living cells by serum proteins, especially by low density lipoproteins (LDL), which are responsible for attached porphyrins distribution to cancer tissues (as much as 140 Photofrin-II particles may

be coupled with one LDL particle) (Zhou 1989). Mammalian cells take up LDL by endocytosis mediated by LDL receptors. LDL receptors loaded with low density lipoproteins are internalized *via* clathrin coated pit pathway (Goldstein *et al.* 1985). Tumor tissues have greater number of LDL receptors than normal cells and, moreover, have increased catabolism of LDL (Zhou 1989).

First phase of studies on *Paramecium* was checking whether LDLs are internalized by these cells and whether this process may be inhibited. Using FITC-labeled LDL, it was shown that LDLs are quickly internalized by *Paramecium* into many endosomes and that this process does not take place in 1-propranolol pretreated cells (Croce *et al.* 1990). Then we have found that HpD is accumulated by protozoans and, what is most important, modulation of endocytotic activity of these cells influences both porphyrin localization and course of internalization and retention.

The results of our studies over localization, uptake and retention of HpD (Wyroba *et al.* 1988, Croce *et al.*1990) may be summarized as follows: (i) - it has been directly documented for the first time that LDL and HpD get into living cells as a complex because their presence has been detected in the same endosomes; (ii) - HpD is localized in *Paramecium* cells both in the endosomes and in a cytoplasm, where it gives rise to a diffused fluorescence; (iii) - inhibition of *Paramecium* endocytotic activity with β-adrenergic blocker (1-propranolol) resulted in the appearance of only diffused fluorescence in cytoplasm, with spectral characteristics of HpD monomers; (iv) - a stimulation of *Paramecium* endocytotic activity with forskolin increased a number of endosomes which contained HpD aggregates.

These results suggest that two pathways of HpD internalization exist, depending on a degree of its aggregation: HpD oligomers get into *Paramecium* cells as a result of an active endocytotic process, most probably through receptor endocytosis, which is mediated by LDL; whereas HpD monomers cross the cell membrane passively and/or are taken up with fluid phase (Wyroba *et al.* 1988, Croce *et al.* 1990).

Biochemical results concerning an uptake of fluid phase marker - horseradish peroxidase by *Paramecium* - may prove the existence of the second pathway (Wyroba 1990, 1991). In fact, it has been found, using a spectrophotometrical assay of fluid phase monitoring, that in the cells pretreated with 1-propranolol an uptake of peroxidase was elevated (Wyroba 1991). We obtained also the same results observing intracellular accumulation of HpD free monomers in the cells treated with β-antagonists, which suggests that those monomers are taken up by *Paramecium* with fluid phase (Croce *et al.* 1990).

Our further experiments have shown that *Paramecium* cells may be useful to study uptake and retention of other photosensitizers such as phtalocyanines and rose bengal (Croce *et al.* 1992a,b).

As comes out from the above data, protozoan cell exhibiting an experimentally modulated endocytotic activity proved to be an interesting and useful experimental model. Studies on mammalian cells bring a lot of difficulties in this case due to the fact that under experimental conditions inhibiting endocytosis - such as decrease in temperature or presence of inhibitors of glycolysis and oxidative phosphorylation - many other essential functions may be affected (Holtzman 1989).

In addition to a simplicity in modulation of endocytosis process in *Paramecium* with β-adrenergic ligands, our model has other advantage because it allows to carry out a spectral analysis in a strictly specified cell microregion, enabling to characterize fluorescence from both endosomal compartment and cytoplasm.

MOLECULAR STUDIES

Molecular studies have been performed using DNA hybridization analysis and PCR to search for putative counterpart of β-adrenergic receptor in *Paramecium* (Wyroba 1996, Surmacz and Wyroba 1997, Surmacz *et al.* 1997, Wyroba and Surmacz 1997, Wyroba *et al.* 1998, Wiejak *et al.* 1998).

Different oligonucleotide probes were designed for Southern hybridization based on the sequence of the cloned β-receptors (Dixon *et al.* 1986, 1987). These probes - synthesized according to protozoan codon usage (Caron and Meyer 1985) - were directed to the following regions: (i) - transmembrane region (TM) 3 of the receptor including Asp 113 involved in beta-antagonists and agonists binding (Strader *et al.* 1987, 1989) - probe No 4; (ii) - TM 4 - probe No 8, synthesized according to the sequence (within 15 bases stretch) *Canis familiaris* ADRB2, 5'-primer (GenBank, Accession # L77384) (iii) - TM 6 (probe No 9) synthesized according to the sequence (within 18 bases stretch) *Canis familiaris* ADRB2, 3'-primer (GenBank, Accession # L77384).

Probes No 8 and 9 correspond to so called "gene-specific universal mammalian sequence-tagged sites" designed as universal β-adrenergic PCR primers to study mammalian genome (Venta *et al.* 1996). In addition, the original above mentioned mammalian codon usage prim-

ers (No 6 and No7) were synthesized (Venta *et al.* 1996) to serve as a control when a human commercial DNA was a template in PCR amplification (Wyroba *et al.* 1998).

First, we compared the hybridization pattern of isolated genomic DNA digested with different restrictases. At least two DNA species of the same molecular size were detected in hybridization analysis by the probes No 4, 8 and 9 (Surmacz and Wyroba 1997, Surmacz *et al.* 1997, Wiejak *et al.* 1998). Furthermore the probe No 6 has been applied for Southern hybridization. In spite of a different codon usage this probe detected the 6.5 kb DNA species revealed by the probes No 4, 8 and 9 (Surmacz *et al.* 1997).

We next tested two sets of the primers for PCR amplification of isolated *Paramecium* DNA: forward and backward primers respectively - the probes No 4 and 9 (Wiejak *et al.* 1998), and the probes No 8 and 9 (Wyroba *et al.* 1998). In both cases the PCR products of the expected molecular size were detected. A further analysis of these products was undertaken using a strategy similar to that described previously in searching for another *Paramecium* protein (Subramanian *et al.* 1994, Wyroba *et al.* 1995). The PCR products were Southern blotted and hybridized with a new molecular probe which fulfilled the following criteria: (i) - was located within the PCR amplified region; (ii) - was designed to one of the most conservative regions of the β_2-adrenoreceptor, playing an essential role in receptor functioning. This probe (No 1) was constructed to the internal β-adrenergic sequence (Wyroba and Surmacz 1996, Wiejak *et al.* 1998, Wyroba *et al.* 1998) located within the third cytoplasmic loop in the region involved in G-protein interaction, including Ser 262 which is a phosphorylation site in desensitization process (Clark *et al.* 1989, Okamoto *et al.* 1991, Zhao *et al.* 1998).

The PCR generated DNA products of the predicted molecular size hybridized to the probe No 1 thus suggesting that the *Paramecium* DNA species homologous to those encoding for β-adrenergic receptor have been amplified (Wiejak *et al.* 1998, Wyroba *et al.* 1998).

Conclusions

To summarize, it can be said that: (i) - β-adrenergic sites have been mapped at the *Paramecium* cell membrane and within newly formed phagosomes; (ii) - β-adrenergic antagonists inhibit phagocytosis in a dose- and time-dependent manner (the strongest effect observed with the most lipophilic drug - l-propranolol); (iii) - pinocytosis - monitored with fluid phase marker horseradish peroxidase - is increased when phagocytosis is inhibited with l-propranolol; (iv) - catecholamines evoke a stereospecific and dose-dependent enhancement of phagocytosis which is abolished by β-adrenergic antagonists; (v) - phorbol ester and forskolin stimulate phagocytosis and potentiate the effect of catecholamines; and (vi) - at the DNA level - different β-adrenergic-specific molecular probes designed to transmembrane regions of the β_2-adrenergic receptor reveal in genomic *Paramecium* DNA at least two DNA species of the same molecular size in hybridization analysis, while when applied as the PCR primers, generate products of the predicted molecular size.

Contrary to *Tetrahymena* cells, which exhibit unspecific reaction to some antagonists and agonists, *Paramecium* cells respond specifically to β-adrenergic blockers but do not respond to α-adrenergic blockers: prazosine and phentolamine (Wyroba 1989a). *Paramecium* phagocytosis process cannot be stimulated by spiperone (Wyroba 1989c) - the serotonine receptor antagonist, which has been found to be an effective ligand for *Tetrahymena* (Quinones-Maldonado and Renaud 1987). This suggests that *Paramecium* "hormone" receptors, though they appeared very early in evolution, are not different to vertebrates receptors in ability to distinguish agonists from antagonists (Wyroba 1989c).

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Review Article

A Review on the Variability of Testate Amoebae: Methodological Approaches, Environmental Influences and Taxonomical Implications

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Summary. Testate amoebae play an important role in terrestrial and aquatic ecosystems in regards of abundance, biomass, and energy turnover. They respond quickly to alterations in their environment either by changes in community structure or shell morphometry. This paper reviews methodological, ecological, and taxonomical aspects of environmentally influenced shell morphology with emphasis on intraspecific variability. Data from common taxa of testate amoebae, e.g., *Arcella* spp., *Centropyxis* spp., *Cyclopyxis* spp., *Difflugia* spp., *Euglypha* spp., and *Trinema* spp., are presented. It is emphasized that for ecological and taxonomical reasons the range and form ("genetic", "nongenetic") of variability within a given taxon must be known. For this purpose both, morphometrical investigations on field populations and laboratory experiments on clonal cultures will be necessary, to ensure a detailed statistical analysis of reactions, adaptations and interactions to and between several environmental factors (e. g. moisture, food, temperature, fertilizers and pesticides). As an example, it is shown that shell and apertural size of *Cyclopyxis kahli* is mainly affected by food and temperature. Experimentally induced changes in shell morphometry seem to be highly reversible occurring rapidly after a few binary fissions of the amoeba. In spite of the exceptional experimental conditions, neither pronounced alterations in shell morphology nor an exceeding of the shell size range of the respective "wild" taxon could be observed. This applies to all clones and taxa investigated (e.g. *Trinema* spp., *Euglypha* spp., and *Cyclopyxis* spp.). New possibilities in bioindication may be conceivable based on rapid changes in shell morphometry, but taxonomical problems may arise because separation of closely related taxa depends largely on shell size, demanding for additional taxonomic features for separation.

Key words: bioindication, environmental factors, shell variability, testate amoebae.

INTRODUCTION

Testate amoebae are particularly suitable for the fundamental question, whether and how an organism responds to changing environments. They respond to modified conditions by altering abundance or dominance structure (Lousier 1974; Foissner 1987, 1997) and by changing their shell morphometry (e.g. Wanner and Meisterfeld 1994; Wanner 1994b, 1995). The shell architecture of testate amoebae (e.g. shell type and shape, spikes, spines, diaphragms, the aperture) has been commonly used to differentiate between genera or species. Physical limitations, like diffusion-dependent cell size range, surface tension, or biomechanical preconditions of protist skeletons (see Rhumbler 1898, Vogel and Gutmann 1988, Fenchel 1990) and ecological constraints, like habitat adaptation, will also influence the shape of the amoeba shell, resulting in a great variety of different morphs.

For taxonomical and ecological reasons it is important to estimate the range and form ("genetic", "nongenetic", as defined in Mayr 1969) of variability within a given

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taxon. As stressed in Mayr (1969), "the underestimation of individual variation may have caused more than 50% of all synonyms". Furthermore, he stated that "differences between groups of similar specimens ("phena") may reflect either a species difference or intraspecific variation. Therefore a complete understanding of intraspecific variation is necessary before we are able to separate between possible species."

Is for instance the phenomenon of ecomorphosis, the variation caused by the environment (expressed by changes in shell morphology) not only measurable, but also reproducible or even reversible within a few generations? If so, this would give new and fascinating tools for bioindication with testate amoebae, in the laboratory as well as in the field. On the other hand, a strong influence of environmental factors on shell morphometry would lead to serious taxonomic problems, because classification of closely related testate amoebae is primarily based on these characteristics.

This paper reviews some aspects of intraspecific variability in testate amoebae, emphasizing taxonomical and ecological implications. Furthermore, it views on how an asexual organism is to define. Even though meiotic stages had been observed (Mignot and Raikov 1992), testate amoebae reproduce, at least predominantly, by asexual binary fission. In consequence there is the need to reveal the relevant characteristics for species, and the way in which these characteristics are affected by environmental factors.

General features of testate amoebae

The shell encloses the cell plasma and has usually a single aperture for the pseudopodia. A proteinaceous organic matrix is the basic shell component, either solely or functioning as cement, fixing particles in position (Moraczewski 1969, Saucin-Meulenberg *et al.* 1973). There are four main shell types: proteinaceous (species with a flexible or rigid shell), calcareous (only two genera), siliceous (species which secrete their own regular siliceous shell platelets, so-called "idiosomes", Fig. 1), and agglutinate (species which include extraneous mineral particles in their shell structure, so-called "xenosomes", Figs. 2a, b). Details are discussed by Grospietsch (1958), Schönborn (1966), Netzel (1983), Ogden and Hedley (1980), Ogden (1984, 1990, 1991c), Anderson (1987), and Foissner (1987).

Testate amoebae are a polyphyletic, or at least biphyletic, assemblage. The major characteristics, the shell and the pseudopodia, evolved independently representing only

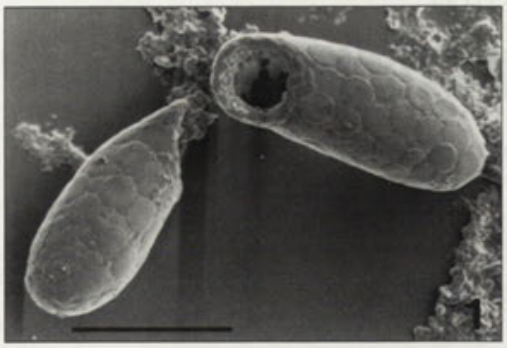

Fig. 1. *Trinema lineare*, EM-micrograph of clone T1, kept at 20°C (±0.5°C) and fed with *Enterobacter aerogenes*. The shell is composed of self-synthesized siliceous platelets, "idiosomes". Scale bar - 20 µm

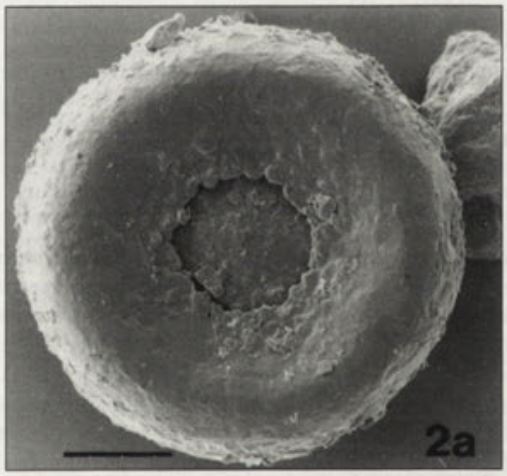

Fig. 2. Morphological variation in *Cyclopyxis kahli*, EM-micrographs of clone G20. a - a single specimen from a subculture of clone G20 (inoculated with 5 individuals of clone G20) kept at 20°C (±0.5°C), fed with *Enterobacter aerogenes*, and provided with sand grains. Ventral view on the aperture (pseudostome). The shell is agglutinated; extraneous mineral particles ("xenosomes") are included in the shell structures. (Scale bar - 20 µm). b - six specimen from a subculture of clone G20 kept at 20°C (±0.5°C), fed with *Saccharomyces cerevisiae*, and provided with sand grains. Note the slight, but recognizable intraclonal variation in shell size. Scale bar - 50 µm

convergent features (Hausmann and Hülsmann 1996). This view is strongly supported by molecular data (Bhattacharya *et al.* 1995, Cavalier-Smith 1997). At present, naked and testate filose amoebae are grouped within the (revised) phylum Rhizopoda, while naked and testate lobose amoebae are grouped into a separate (revised) sarcodine phylum, the Amoebozoa (Cavalier-Smith 1997). This is supported by community structure and biometric data. Foissner (1987) and Wodarz *et al.* (1992) pointed out that taxa of different origin (e.g. lobose and filose testates) may have a different autecology with individual importance for bioindication. Based on a biometrical analysis of twenty-four soil testate amoebae, Lüftenegger *et al.* (1988) observed that Testaceafilosa have wider ranges in morphometric variation as compared with Testacealobosea.

METHODOLOGICAL APPROACHES

The basic methodological tools for collecting, preparation, observation and statistics have been discussed in detail by Bonnet (1964), Schönborn (1966), Laminger (1980), Foissner (1987, 1994), Wanner (1991a, b), Aescht and Foissner (1995), and Dunger and Fiedler (1997). Hence in this chapter some specific methodological problems will be highlighted concerning the analysis and interpretation of variability in testate amoebae.

Field investigations

They provide a "natural environment" for experimental and descriptive analyses. An objective, artifact-free assessment of the environmentally dependend variability will be possible, including all relevant factors and interactions which are affecting the organism of interest. However, this requires a profound and time-consuming quantitative estimation of the spatial and temporal distribution of the entire important (what is important?) environmental factors using e.g. a completely randomized block design (Hurlbert 1984, Köhler *et al.* 1984, Petz and Foissner 1989, Wanner *et al.* 1994a). Furthermore, environmental effects acting indirectly are hardly to assess. Thus it is appropriate to combine field data with those from the laboratory.

Laboratory experiments

They allow an unequivocal assessment of single, selected environmental factors - acting directly and indirectly - under standardized and reproducible conditions. However, only if detailed information about the intrinsic intraclonal variability is available (concerning cultures kept under constant conditions), a laboratory estimation of environmental influences and a delimitation of taxa will be successfull. For this purpose, numerous taxa of testate amoebae had been isolated, cloned (in this context: cells within a culture descending from one isolated specimen), and successfully cultivated in (batch) cultures for even several years (Table 1, Figs. 1, 2a, b, 5).

Quantitative information about the morphological variation in testate amoebae can be attained by measuring different shell parameters (e.g. shell length, - breadth, - depth, apertural size) from about 20-30 specimen (see Hoogenraad and de Groot 1937, Meisterfeld and Schüller 1982, Schönborn *et al.* 1983). With these biometrical data an "Idealindividuum" was constructed, suitable to compare species, populations or clones (Schönborn *et al.* 1983, Fig. 3). Significance tests combined with the analysis of size frequency distribution may help to examine geographic races and ecological variations of testate amoebae (Gillies 1918, Lüftenegger *et al.* 1988, Schönborn and Peschke 1988, Schönborn 1990a, Lüftenegger and Foissner 1991). Precise biometric analyses were also performed using computer image analysis (Wanner 1991a), vector-analysis (Meisterfeld and Schüller 1982) and multivariate statistics allowing examinations of numerous clones or populations of the same taxon as well as the quantification of the shell platelets (Fig. 4; Wanner 1991a, 1995).

Cytochemical and X-ray microanalyses of the amoeba shell seem to be further methods for ecological and taxonomical purposes. Stout and Walker (1976) proposed that tests of different taxa may be distinguished not only by their size and shape, but also by the chemical character of the mineral particles they are composed of. Although important results concerning the amoeba shell has been revealed by this approach - e.g. it was shown that the ionic compositions of the brown and white clones of *Assulina muscorum* are nearly identical (Schönborn and Peschke 1990) - definite and consistent conclusions about local differences within populations or between closely related species could not be drawn (e.g. Awerinzew 1907, Coûteaux and Jeanson 1977, Rauenbusch 1987). However, further research is necessary to clarify if this tool is suitable for research in amoebal variability.

Molecular methods

Taxon-specific DNA probes may be suitable for the identification and characterization of even closely related species excluding modifying environmental influences.

Table 1. Biometrical characterization of clones of testate amoebae. Data in µm and % (CV), partly from Wanner (1995). *Trinema*: 1- length, 2 - breadth, 3 - breadth over pseudostome, 3b - pseudostome diameter; *Euglypha*: 1 - height (longest axis), 2 - breadth, 3 - pseudostome diameter 4 - thickness of shell; *Cyclopyxis*: 1 - shell diameter, 2 - pseudostome diameter; *Centropyxis*: 1 - length (longest axis), 2 - pseudostome length, 3 - breadth, 4 - pseudostome breadth. n - number of shells , $\bar{x}$ - arithm. mean, M - median, CV - coefficient of variation, min, max - minimum, maximum, temp - temperature (°C ± 0,5°C). Each clone was kept under constant culture conditions (mean food density; either *Saccharomyces cerevisiae* (clones D25, D29, F3, G20, additionally supplied with sand grains as shell building material) or *Enterobacter aerogenes* (the other clones). Shell size ranges (longest axis - "1") of "wild" populations are given in parentheses, after Cash *et al.* (1915) and Bonnet and Thomas (1960)*

Clone	n	$\bar{x}$	M	CV	min	max	temp
T4 (*Trinema lineare*, "wild" population: 18 - 35 µm)							
1	456	33.9	34.1	5.6	25.3	39.6	15
2	456	15.4	15.2	8.8	11.0	20.6	15
3	175	12.3	12.2	9.9	9.4	15.0	15
3b	175	8.3	8.2	12.6	6.1	12.2	15
T1 (*T. lineare*)							
1	105	33.2	33.6	5.0	28.5	37.8	15
2	105	15.5	15.2	7.5	12.6	19.6	15
3	105	11.8	11.7	8.1	9.7	14.0	15
3b	105	7.8	7.7	11.4	5.3	10.2	15
T7 (*T. lineare*)							
1	82	33.1	32.7	5.7	28.8	37.5	15
2	82	15.3	15.4	7.1	12.2	18.1	15
3	82	11.5	11.5	7.4	9.2	13.8	15
3b	82	7.4	7.3	9.7	6.0	9.2	15
MT1 (*T. lineare*)							
1	35	30.7	30.9	4.3	28.5	33.7	15
2	35	13.7	13.6	4.6	12.4	15.2	15
3	35	10.3	10.3	7.4	8.7	11.8	15
4	35	6.0	6.1	9.6	5.0	7.2	15
MT2 (*T. lineare*)							
1	35	33.0	33.0	2.5	31.6	34.6	15
2	35	13.8	13.9	4.1	12.9	15.0	15
3	35	11.1	11.2	5.0	9.9	12.2	15
3b	35	7.0	7.0	7.8	5.7	8.3	15
MT11 (*T. lineare*)							
1	33	32.5	32.5	4.2	29.3	35.3	15
2	21	16.0	16.0	5.2	14.5	17.9	15
3	21	12.2	11.9	7.2	10.7	14.7	15
3b	21	6.5	6.4	5.9	5.7	7.4	15
MT12 (*T. lineare*)							
1	35	34.6	34.6	4.1	31.1	38.4	15
2	23	16.2	16.3	4.1	14.6	17.0	15
3	23	12.1	12.2	11.6	8.3	13.8	15
3b	23	6.9	6.8	6.3	6.1	7.9	15
MT3 (*T. enchelys*, "wild" pop.: 32-103 µm)							
1	60	41.5	41.7	3.1	38.2	45.1	15
2	47	15.9	16.0	3.8	14.7	17.1	15
3	47	12.4	12.3	4.9	11.2	13.7	15
3b	47	7.8	7.8	8.7	6.5	9.1	15
a14 (*Euglypha strigosa*, "wild" pop.: 45-100 µm)							
1	26	68.2	68.4	6.3	58.2	78.4	20
2	26	47.5	47.0	6.5	41.9	54.3	20
3	26	19.4	18.9	12.6	14.8	24.7	20
a4 (*E. strigosa*)							
1	801	73.2	73.2	4.8	55.1	83.1	15
1	851	69.7	70.1	6.4	47.8	92.0	20
2	255	45.9	45.8	8.6	27.1	57.2	15
4	255	27.2	27.0	11.3	21.0	59.6	15
x7 (*E. strigosa*)							
1	221	73.9	73.9	4.0	62.2	81.4	15
2	152	41.1	41.0	6.7	34.6	49.8	15
3	152	16.8	16.6	10.2	12.8	24.2	15

Table 1 (con.)

x21 (*E. strigosa*)							
1	320	73.0	73.1	5.0	62.1	80.9	15
2	183	43.1	43.0	7.3	35.8	52.0	15
3	183	17.0	16.8	12.7	11.3	23.6	15
b1 (*E. rotunda*, "wild" pop.: 22-52 µm)							
1	100	38.5	38.1	7.8	32.0	49.6	20
2	100	23.0	23.0	10.2	17.4	32.7	20
3	100	11.3	11.4	16.7	7.7	17.0	20
b5 (*E. rotunda*)							
1	102	41.6	40.9	10.8	33.6	58.0	20
2	102	26.6	26.2	11.5	20.2	39.1	20
3	102	13.6	13.5	13.4	9.9	18.7	20
D25 (*Cyclopyxis eurystoma*, "wild" pop.: 45-65 µm*)							
1	100	41.9	41.1	8.7	36.8	59.2	15+20
2	100	22.6	22.4	14.0	12.4	32.1	15+20
D29 (*C. eurystoma*)							
1	2603	41.2	40.8	8.2	31.0	60.6	20
2	2603	23.1	22.9	13.5	13.9	38.2	20
1	1488	41.9	41.4	8.4	20.9	58.9	15+20
2	1429	23.4	23.0	15.1	12.7	43.7	15+20
1	135	43.0	42.0	10.9	36.3	58.5	15
2	135	26.8	26.3	16.1	16.2	40.3	15
G20 (*C. kahli*, "wild" pop.: 80-98 µm*)							
1	119	72.5	72.4	6.6	62.8	83.2	20
2	119	24.0	24.2	23.8	7.2	34.3	20
F3 (*Centropyxis sylvatica*, "wild" pop.: 65-105 µm*)							
1	149	83.4	82.9	6.2	70.3	97.7	20
2	149	17.2	16.5	36.1	4.7	33.7	20
3	149	83.6	83.3	6.7	71.1	101.4	20
4	149	31.6	32.2	18.1	9.2	48.6	20

Until recently (Bhattacharya *et al.* 1995, Wanner *et al.* 1997), molecular techniques have not been applied on testate amoebae. In most cases neither sufficient amounts of uniform genetical material nor sequence information are available, necessary for RFLP, PCR or sequencing. Additionally, uncommon foreign DNA sources (distinct from the food or the culture medium and located within the amoeba) can inevitably be transferred with the amoebae to each new subculture. This foreign DNA probably belongs to eukaryotic parasites, commensals or symbionts which live inside the amoeba-cell, but are also able to leave the host (Schönborn 1966, Foissner 1987, Anderson and Cowling 1994). As a result the amoeba-free culture medium is contaminated, producing putative "amoebae-clone-specific" DNA patterns (Wanner *et al.* 1997). This severe problem of coamplifying foreign, but culture-specific DNA, can be prevented by using single nuclei of testate amoebae as a reference. Specific primers derived from a sequenced RAPD-DNA fragment demonstrate its amoeba-specific nuclear origin. These RAPD-derived specific primers allow highly specific amplifications of

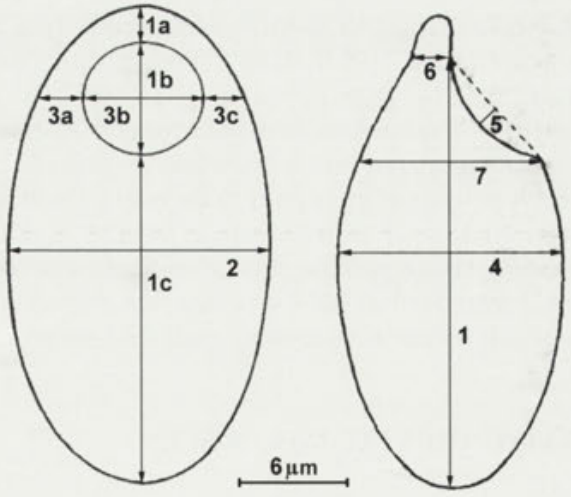

Fig. 3. *Trinema lineare*, "ideal-individual" (from Wanner 1988). Testate amoebae casings had been measured (at least 20-30 shells of each population) and an "ideal-individual" constructed using the mean (median) values as axes (numbers 1-7; "M" in Table 1). Note that this "ideal-individual" is based on numerous, standardized field data, while the EM-micrograph in Fig. 1 is a concrete, cultured specimen

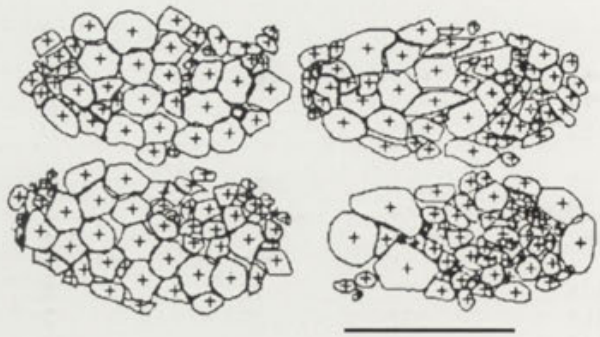

Fig. 4. *Schoenbornia humicola,* shell platelets from two casings, scanned and measured (area, perimeter, centre of gravity) by an image-analysis unit (from Wanner 1991b). Scale bar - 20 µm

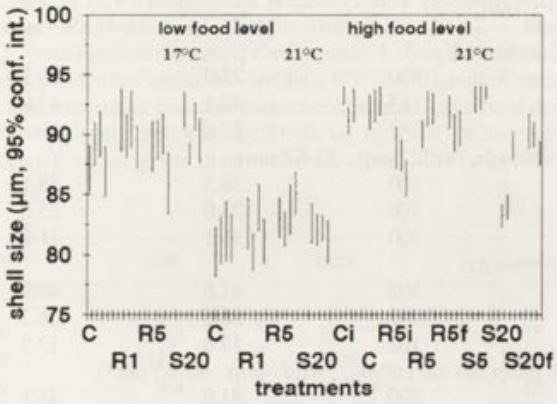

Fig. 5. Shell size of *Cyclopyxis kahli* (clone G20) affected by food, temperature, illumination (i), pretreated inoculi (f), and two insecticides (ripcord ("R" - cypermethrin, a synthetic pyrethroid), spruzit ("S" - an emulsion of 4% pyrethrine and 16% piperonyl butoxide) in different concentrations). A randomized block design and liquid cultures were used; four / three replicates per experiment are shown (from Wanner 1994a, b)

amoebae DNA amplified from a single nucleus, even in the presence of large amounts of contaminating foreign DNA. This approach provides the basic tool for more detailed molecular identification studies in the future (Foissner and Wanner 1995, Wanner *et al.* 1997). Putative contaminated genetic material of testate amoebae can be checked by a taxon-specific probe (generated as described above). If no eukaryotic contamination is present, an estimation of genetic variation among soil isolates of testate amoebae may be possible, using a combination of the polymerase chain reaction and the restriction fragment length polymorphism analysis (PCR, RFLP), as described for the soil ciliate *Colpoda inflata* (Bowers and Pratt 1995). But even in the case of eukaryotic contaminations DNA sequence information derived from a few nuclei may be available, which can be clearly assigned to the respective clone of testate amoebae. Fine tuning these methods as well as generating more specific primer combinations should enable reproducible DNA analysis with respect to intraspecific variation, even from small field samples.

ENVIRONMENTAL INFLUENCES

Morphological adaptations

Numerous authors investigated the effects of natural (e.g. moisture, food, temperature, pH, particle size of the habitat) and anthropogenic (e.g. soil compaction, liming, fertiliziation, pesticides) environmental factors on testate amoebae. Laboratory and field investigations were excellently summarized by Schönborn (1966), and with respect to soil protozoa, by Foissner (1987, 1994, 1997). It was concluded that most morphological adaptations of terrestrial testate amoebae are related to the restricted water resource of the habitat. In general, terrestrial species of the same genus are smaller than the respective freshwater species.

Besides moisture, food is also proposed to be an important factor determining the presence or absence of a given species (Martin 1971). Details about the effects of food on intraspecific variability of testate amoebae were presented by Anderson (1989), who worked on cultures of *Netzelia tuberculata*. The gross morphology of the amoeba shell was not significantly changed by the various food items (either yeast or algae). However, the fine structure of the shell platelets was clearly different exhibiting more regular-shaped, smooth platelets in yeast-fed cells compared to those fed on algae. These differences became visible within one generation after transfer to yeast-containing culture medium. Laminger (1978) reported relationships between soil moisture, shell size and the kind of food ingested by *Trinema enchelys*. An increase of shell size was correlated with an increase of soil moisture. Furthermore, the food consumption of *Trinema enchelys* seemed to vary in relation to soil moisture and shell size. Ogden (1988b) firstly reported a significant change in shell architecture (structure and diameter of the aperture) due to agglutinate material in cultures of *Cyclopyxis kahli*.

To conclude, numerous environmental factors do affect shell morphology of testate amoebae in manifold

ways, impeding taxonomy which is based on shell size and shape. Thus it seemed appropriate to conduct a detailed quantitative and statistically verified experimental evaluation of distinct environmental impacts which may influence the amoeba shell.

Experiments with *Trinema lineare* and *Cyclopyxis kahli*

A two-factorial laboratory experiment with clonal cultures of *Cyclopyxis kahli* and *Trinema lineare* which combined food supply (exact doses of yeast or bacteria) and temperature (constant 15 or 20°C) crosswise revealed numerous effects on shell and aperture size as well as interactions between food and temperature (Table 2; Wanner 1994a, b; Wanner and Meisterfeld 1994; Wanner *et al.* 1994b; Foissner and Wanner 1995). Complex experimental sets demonstrated that even a small increase in temperature causes a significant shell size reduction, which is highly reversible and reproducible (Wanner and Meisterfeld 1994, Wanner 1995). A complex set of consecutive experiments with *Cyclopyxis kahli* (about half a million shells were investigated) allowed a detailed statistical analysis of interactions between and adaptations to several environmental factors. Significant changes in culture growth (lag time, generation time, final culture density) and shell size parameters were primarily dependent on food supply and temperature, whereas insecticides had a minor but still significant influence (Tables 1, 2, Fig. 5). Lime and mineral fertilizer had no direct effects, but were acting indirectly by promoting food density (Fig. 5). Interactions between all tested factors occurred frequently, but no consistent adaptation or selection phenomena could be observed. The shell size effects were reversible within a few days by changing environmental conditions (Wanner 1994a, b; Wanner *et al.* 1994b; Foissner and Korganova 1995; Foissner and Wanner 1995). Because testate amoebae revealed a marked resistance to environmental stress factors, there may be no need to evolve particular adaptation mechanisms. Further investigations are essential to clarify this.

In this context, the influence of illumination on shell size properties was also investigated. Cultures of *Cyclopyxis kahli*, either kept in darkness or illuminated, did not show significant differences in shell size, but cultures additionally treated with a pesticide demonstrated significantly smaller shells than the respective untreated or dark cultures (Wanner 1994b). Because no selection of smaller (here a result of higher temperature) or larger shells was observed in the respective subcultures, shell size modifications are assumed. A possible explanation for the observed smaller amoebae shells with proportionally larger pseudostomes caused by increased temperature may be the temperature-dependent characteristics of diffusion and respiration. An increase of approx. 10°C doubles cellular respiration, while oxygen diffusion within the cell increases only proportionally to the absolute temperature (Fenchel 1990). Testate amoeba may respond to an increase in temperature by extending their active respiratoric surface in proportion to the cell volume, resulting in smaller shells with larger openings (Table 2). To conclude, all the experimentally altered environmental factors did significantly affect shell size (of the respective daughter cells) - but not morphology - in a reversible and definite way, either directly or indirectly.

Variation in cell size

Variation in mean cell size in protist populations was examined as early as 1908 by Popoff, who noted an inverse relationship between temperature and cell/macronucleus size in the ciliate *Frontonia leucas*, and Harding (1937), who studied food-dependent cell size alterations in the ciliate *Glaucoma* sp. Environmentally induced cell size alterations had been also observed by Choi and Peters (1992). Cold-water isolates of the heterotrophic nanoflagellate *Paraphysomonas imperforata* had the largest volumes at the coldest growth temperature. Because of the characteristics of flow cytometry used in this study, it was possible to detect even small (but reproducible and statistically significant) changes in cell size (e.g. 8.2 µm at -1.5°C, 8.07 µm at 6°C, and 7.89 µm at 15°C).

In contrast, Baldock *et al.* (1980), who kept six species of naked amoebae at four different temperatures, found no consistent relationship between cell volume and temperature. This applies also to the planktonic foraminifer *Orbulina universa*, which was cultured at four different temperatures between 19.5 to 28°C, the range at which this species is abundant in the ocean. Although average final shell length (594-669 µm) and shell morphology (increased frequency of individuals forming a second spherical chamber) were significantly affected, largest shell sizes were observed for the two intermediate temperatures. This may be due to other factors more important than temperature (Caron *et al.* 1987). Furthermore, species-dependent optimum growth temperature and culture conditions have to be taken into consideration (e.g. James and Read 1957).

The reversible nature of variability

The reversible nature of variability in protists was demonstrated by Heal (1963), who induced an increase in

Table 2. Characterization of shell parameters of *Cyclopyxis kahli* in relation to food and temperature. All measurements in µm. Food: Ea - *Enterobacter aerogenes*; Sc - *Saccharomyces cerevisiae*; temperature: 15°C; 20°C. All mean shell and pseudostome diameters differ significantly (ANOVA, $P<0.05$). Further explanations see Table 1. From Wanner and Meisterfeld (1994)

Treatment	n	$\bar{x}$	M	CV	min	max
Shell diameter						
Ea, 15°C	140	86.9	87.0	5.8	75.0	98.2
Sc, 15°C	140	88.1	88.1	5.7	73.5	100.1
Ea, 20°C	140	84.0	83.4	5.8	73.7	104.0
Sc, 20°C	140	88.2	87.8	7.0	71.4	104.3
Pseudostome diameter						
Ea, 15°C	111	24.3	24.0	13.4	12.1	32.3
Sc, 15°C	125	26.3	26.6	10.6	15.8	33.3
Ea, 20°C	125	27.3	27.5	12.0	17.1	38.4
Sc, 20°C	125	27.7	27.4	11.6	19.2	39.9

shell size in the testate amoeba *Nebela tincta* after rewetting its habitat (*Sphagnum* mosses). Although he did not speak of different generations, he must have sampled consecutive generations of testate amoebae, because his experiment went on for 91 days. Thus he observed shell size alterations that referred to the respective daughter cells. Schönborn and Peschke (1988) and Schönborn (1990b) showed that the short-spined, soil dwelling testate amoebae *Euglypha ciliata* is able to redevelop long spines when kept in aequeous soil cultures. Within 48 days, the mean spine length of the daughter cells increased from 5.12 to 12.8 µm. In the ciliate *Tetrahymena pyriformis*, Saini and Saxena (1986) and Agarwal and Saxena (1990) noted that the ciliates recovered from treatment with organophosphorus insecticides (e.g. reduction in cell size) by being transferred to toxicant-free medium. Within 6 h, they returned to normal shape, size and locomotion. Soose *et al.* (1994) reported that inhibition of cell density and cell volume in *Tetrahymena thermophila* was completely reversible upon removal of the antibiotic adriamycin. Environmental-dependent reversible cell size alterations are also observed in heterotrophic microflagellates. Irrespective of the duration of starvation, cells of *Ochromonas* sp. immediately increased in volume when exposed to food bacteria (Fenchel 1982). However, after cell divisions started, cell volume increased only slowly. After long starvation periods, balanced growth was not achieved for several generations. The lag time was also dependent of the duration of starvation.

Further environmental influences

Besides food, temperature and various drugs or pesticides, particle size in lake sediments (Schönborn 1968) affects size distribution of the amoebae. Berger *et al.* (1985) demonstrated in a field experiment that *Trinema complanatum* was significant smaller in size in compacted soil. Finally, the deficiency of silicate (Hegner 1919a, Netzel and Grunewald 1977, Anderson 1990), calcite (Casper and Schönborn 1985), or the quality/lack of shell building material for agglutinated forms (e.g. Ogden and Meisterfeld 1989) in the surrounding medium causes striking alterations or deformations in the shell structure.

To summarize, interacting environmental factors either natural or "anthropogenic", affect especially shell size of (the daughter cells of) testate amoebae, acting directly or indirectly in a reversible mode.

TAXONOMIC IMPLICATIONS

There are qualitative shell characters useful for distinguishing taxa, as discussed in Ogden and Meisterfeld (1989). Besides biometrical data, surface composition (in combination of knowledge of the general biology and ecology), shell matrix properties, apertural structure, and cytoplasmic features as size and structure of the nucleus, number and location of the nucleolus, are suitable for species differentiation.

As shown above, environmental factors can especially affect shell size and aperture, which are relevant for an exact definition of closely related taxa, e.g. ecologic and geographic races (see Wanner 1994a, b; Bobrov *et al.* 1995, Foissner and Korganova 1995). Thus, identification and assessment of specific environmental factors responsible for the shell variability of distinct species or races are necessary prerequisites for further taxonomical and ecological work on testate amoebae.

Examples for variation in testate amoebae

Already in 1864, Wallich observed that shell size in *Difflugia* spp. is "modified to an extraordinary degree by the nature of the localities in which they happen to be found" with respect to direct influences via food supply, and climatic conditions which may act indirectly by changing sustenance. Penard (1902) mentioned polymorphism in *Centropyxis constricta* and *Trinema* spp. Leidy's minute field investigations in 1879 caused him to critisize his famous colleague Ehrenberg mentioning (p. 227) that "*Trinema enchelys* is very variable in form and size, and thus helped Ehrenberg to swell the long list of his *Difflugias* and *Arcellas*". Leidy himself accepted only one species, *Trinema enchelys*, within the whole genus *Trinema* because he observed numerous transitions between different morphs. Finally, Penard (1890) clearly defined *Trinema enchelys*, *T. lineare*, and *T. complanatum* (discussed in Penard 1902 and Cash *et al.* 1915).

Up to now, numerous authors draw attention to the fact that many "species" of testate amoebae may not represent "true" species but different morphs or variations of the same taxon. Bobrov *et al.* (1995) conducted a detailed morphological comparison of shell size characteristics of two geographically isolated populations of *Nebela militaris*, *Hyalosphenia papilio*, and *Trigonopyxis arcula sensu lato*. Only the first two taxa revealed statistically significant differences between the Siberian and Ontario populations, while *T. arcula sensu lato* (including *T. arcula major* and *T. minuta*) may be better considered as a single polymorphic complex rather than different species. Generally, the authors stressed that shell morphology measurements alone are not sufficient for delineating species. Additional morphological, ecological and genetic studies should be undertaken on the same material to improve taxon-specific differentiating.

Variation and synonymy

Cowling (1994) reviewed the relationship between body size /shape of soil protozoa and environmental influences, and presented data about intraspecies variation and adaptations. Decloître (1964) presented an impressingly long list of aberrant and variable forms within numerous genera and species (e.g. *Nebela*, *Difflugia*, *Centropyxis*, *Cyclopyxis*, *Cyphoderia*, *Arcella*, *Assulina*, *Trinema*, and *Euglypha*). Consequently he recommended to be careful with hasty descriptions of new taxa based on aberrant forms. Using morphometry, light microscopy and SEM, Foissner and Korganova (1995) redescribed the shell morphologies of terrestrial testate amoebae. They concluded that morphometric criteria are of very limited taxonomic value, and only applicable for species separation if they are very distinct. Generally, to avoid an oversplitting into apparently closely related species, they suggested that individuals of field populations very likely belong to the same species if the coefficient of variation of the main metric shell characters (e.g. shell and pseudostome / aperture size) does not exceed 30 - 50%. Additionally, they recommended at least one extra reliable morphological character for separation. Thus *Cyclopyxis kahli cyclostoma, C. kahli obliqua, C. dispar, C. dulcis, C. insolitus*, and *C. profundistoma* is synonymized with *C. kahli* and *Cyclopyxis gigantea, C. bathystoma* and *C. kahli grandis* synonymized with *C. intermedia*. Supplementary discussions are given in Lüftenegger *et al.* (1988), Wanner (1988), and Lüftenegger and Foissner (1991), who characterized numerous soil testate amoebae morphometrically.

Heal (1961) mentioned difficulties in separation of closely related species, e.g. in *Centropyxis* spp., *Nebela* spp., *Heleopera* spp., and *Phryganella* spp., and recommended additional culture experiments to distinguish species. In 1963 he stressed that, although morphometric studies have been made to assess the validity of various species, it is necessary to obtain more information about the reasons of variation. In this work, he measured about 1060 tests of the *Nebela tincta-bohemica-collaris* group from *Sphagnum* and found that *Nebela tincta* and *N. flabellulum* are distinct species, but *N. collaris* sensu lato includes *N. bohemica*. Remarkably, he demonstrated that the size of *Nebela* increased when wet *Sphagnum* was flooded experimentally. Thus it was concluded that *Nebela tincta* in the range of 75-95 µm is distinct from larger forms, but specimens ranging from 95-155 µm in length form a cline due to the variation in water content of the habitat. Lüftenegger *et al.* (1988) also studied morphometric variation in the *Nebela tincta-parvula-bohemica-collaris* group and separated this species complex on the basis of shell size into *N. tincta* (with lateral pores) /*N. parvula* (no lateral pores, both taxa about 80-110 µm) and *N. bohemica/N. collaris* (about 100-200µm). However, some intermediate forms occurred in the populations, too. The presence or absence of lateral pores as a valid taxonomical criterion and further taxonomical problems in *Nebela* are (controversially) discussed in e.g. Leidy (1879), Cash and Hopkinson (1909), Deflandre (1936), MacKinlay (1936), Hoogenraad and de Groot

(1937), and Heal (1963). But - as stated in Lüftenegger *et al.* (1988) - as long as no better criteria are known, it may be useful for separation.

With regard to the change in size within a species, Jung (1936) stated after measuring 21 shells of *Assulina muscorum*, that this species becomes smaller with decreasing water content of the habitat. A quite controversially discussed case study of intraclonal variability in testate amoebae was presented by Medioli *et al.* (1987). They claimed that intraclonal variability represents the "minimum" variability that can be expected in a given (uniparental) species. Only morphotypes falling outside the interval of variability will belong to other species. Based on this hypothesis, they synonymized about 100 taxa to the single species *Difflugia tricuspis*. Unfortunately, as discussed in Ogden and Ellison (1988) and Ogden and Meisterfeld (1989), they did not consider additional data, as those belonging to the idiosome bearing genus *Netzelia*.

Extensive information about morphometry, shape and structure of testacean shells is given by Ogden (1980, 1981, 1983, 1984, 1991a, b, c), Ogden and Hedley (1980), Ogden and Coûteaux (1987), Ogden and Meisterfeld (1989), and Schönborn (1992). Generally, it was underlined that the combination of detailed species descriptions and information deriving from clonal cultures concerning the natural variability of shell formation may be helpful in finding reliable specific features. In turn, the number of new species based on poor diagnoses should be restricted.

Variation in different taxa and shell characteristics

To sum up, description (or redescription) of species as well as species-based ecological work is only useful if reliable biometric data are provided. In this context, the analysis of the coefficients of variation is of special interest, because it allows direct comparisons and evaluations of differently sized shell characteristics. However, it must be taken into consideration that different taxa as well as different shell characteristics within a given taxon do vary divergently. For example, the shell morphology in *Centropyxis* spp. is more variable than in *Arcella* spp., and in both genera the aperture ("pseudostome") is more variable than the shell size (Heal 1963; Meisterfeld 1978; Lüftenegger *et al.* 1988; Wanner 1988, 1991a, 1995; Wanner and Meisterfeld 1994). Univariate as well as multivariate statistics of main shell characteristics of terrestrial testate amoebae showed that the smallest cubic axis (the thickness or height) of the shell varies independently as compared to the other axes (Wanner 1988, 1991a). This indicates an adaptation of the amoeba shell to the thin water-film covering the soil substrate (e.g., Heal 1963, Bonnet 1964, Schönborn 1966, Foissner 1987, Wanner 1988). The feature complex "pseudostome" also shows a strong variability. The respective diameters and the invagination are not coherent because they vary independently (Wanner 1988, 1991a). Furthermore, a computerized image-analysis of the shells of six populations of *Schoenbornia humicola* revealed no significant differences in shell size, while shell platelets showed highly significant differences in form and size (6061 platelets of 110 shells had been measured), indicating habitat specific influences (Wanner 1991a). Moreover, the platelets may also be derived from other species (Schönborn *et al.* 1987). Finally, it had been proved quantitatively using image-analyzed clonal cultures of testate amoebae (Wanner 1994b, 1995, Wanner and Meisterfeld 1994, Wanner *et al.* 1994b), that all tested taxa, clones and cultures showed a taxon-specific, highly reproducible and reversible variability influenced by interacting culture conditions. However, it is important to know that, in spite of the "extreme" experimental conditions in culturing *Cyclopyxis kahli*, neither pronounced alterations in shell morphology nor an exceeding of the shell size range of the respective "wild" taxon could be observed. The latter applies to all clones and taxa investigated (Table 1).

Species delimitation and reasons for variation in testate amoebae

Based on the above collected data, the following questions arise: Which are the features characterizing a species, and in which way are these features affected by environmental factors?

On one hand, habitat structures may stabilize the morphological characteristics of a taxon, on the other hand micro-scaled variable environmental factors may influence the morphological characteristics in various ways (Schönborn 1989, 1992). The current biological species-concept may be insufficient for organisms with a predominant asexual reproduction (Meisterfeld 1979, Tibayrenc *et al.* 1991, Poljansky 1992). Alternatives, like the phylogenetic species concept (Cracraft 1983), or the evolutionary species concept (Simpson 1961), have some practical disadvantages for parthenogenetic species and asexual clones, as discussed in Quicke (1993). Additionally, with the evolutionary species concept, it is not possible at any one time to know the historical fate of an extant lineage, and the phylogenetic species concept may result in an extreme oversplitting of species, as compared to the more traditional biological species concept. Working

with an "ecological species" - a morphological divergence is interpreted as an expression of a reproduction community - this problem may be circumvented to a certain degree (Willmann 1985). To conclude, only a more or less temporary solution based on a combined approach, including criteria for monophyletism, ecological niches and morphological and genetical similiarities, will be successful (Zakryś 1997).

Observations on clonal cultures of testate amoebae indicate high variations in shell characteristics which are used for species determination (discussed in e.g., Heal 1963, Meisterfeld 1979, Schönborn and Peschke 1990, Wanner 1994a, Foissner and Korganova 1995), problems which also occur in other protist groups (e.g., Zakryś 1997). However, there are existing stable characteristics useful for species discrimination, but these features have to be estimated in each individual case (Meisterfeld 1984, Ogden and Meisterfeld 1989). Yet, due to missing culture and molecular data, the actual reasons for variation in testate amoebae can be proved in a few cases only.

To summarize, variable shell features of testate amoebae are commonly used for taxonomic purposes. As discussed above it is likely, that a considerable part of the present known taxa (about 1600 species) may be only variations of "true" species (controversially discussed in Medioli *et al.* (1987), Ogden and Ellison (1988), Ogden and Meisterfeld (1989), Foissner and Korganova (1995). Assuming this hypothesis, there is need to know in which way species may split in different morphs or variations.

(a) The "classic" studies on clonal cultures postulated either persistent modifications ("dauermodifications"- Jollos 1917, 1924a, b) or mutations (Jennings 1916; Root 1918; Hegner 1919a, b, 1920; Reynolds 1923) as a basis for variation.

(b) Chardez and Leclercq (1963) separated *Euglypha strigosa* into four habitat-specific ecotypes with different spines: *Euglypha strigosa heterospina* (aquatic), *Euglypha strigosa strigosa (Sphagnum)*, *Euglypha strigosa muscorum* (mosses), and *Euglypha strigosa glabra* (terrestrial). Schönborn (1983) interpreted these morphs as "Dauermodifikationen", because in his aqueous cultures, spineless terrestrial shells transformed into spiny morphs. Putative extra-chromosomal factors firstly substantiated the concept of "dauer-modification", but Danielli (1959) showed that mainly chromosomal genes are responsible for most features of amoebae. Thus persistent modifications occur, but are likely to play only a minor role (Schönborn 1983, 1992).

(c) Subsequently Schönborn developed the concept of an "adaptive polymorphism" as explanation for culture experiments on stability and selection in varieties and morphs of testate amoebae, namely *Trinema complanatum* and *Euglypha laevis*. Four different morphs of *Trinema complanatum* isolated from spruce forest humus and soil samples (small-rounded, large-rounded, waisted, and small morph), indicated a discontinuous size polymorphism. After Schönborn (1992) adaptive forms are hidden behind this discontinuity. In clonal cultures of *Trinema* and *Euglypha*, the features of the morphs remained relatively constant, but over the course of several months other morphs occurred. These morphs could be transformed into each other by selection, while "pure" shell size variability, as shown for *Trinema complanatum*, may have a modificatoric basis. Furthermore, in clonal cultures of *Euglypha* with round pseudostomes ("*E. rotunda*") individuals also occurred with oval pseudostomes ("*E. laevis*"). Thus Schönborn (1992) synonymized the later described *E. rotunda* (for practical reasons, *E. rotunda* was maintained in Table 1).

However, only an estimation of the genotypical conditions will allow further knowledge on variation, as discussed for *Euglena* spp. (Zakryś 1997) and *Amoeba proteus* (Poljansky 1992). Besides molecular approaches, which are essential for the future, clonal cultures of testate amoebae (informing about intraclonal variability) are important for taxonomy too (e.g. Ogden and Coûteaux 1988). Furthermore, ultrastructural research on shell structure, cytoplasm (pseudopodia, nucleus), and organic matrix are necessary (e.g. Stout and Walker 1976; Netzel 1977, 1983; Ogden 1979, 1981, 1983, 1988a, 1991a, b, c; Ogden and Coûteaux 1987; Ogden and Meisterfeld 1989, 1991; Ogden and Pitta 1989).

CONCLUSIONS

"Conventional" bioindication with testate amoebae - using community structure parameters like abundance, dominance, species spectra, and biomass - is successfully established since a long period. As compared to naked amoebae, flagellates, and ciliates, no costly or time-consuming methodology is necessary, since direct observation of an aequeous suspension is sufficient for qualitative and quantitative analysis. With respect to forest soils or agroecosystems, their relatively small species richness, as compared to other protist groups, faciliates practical work on bioindication.

However, field investigations based on shell size alterations may not be practicable at the moment, since even few environmental factors produce complex morphomet-

ric alterations. In contrast, laboratory tests using clonal cultures of testate amoebae seem to be more promising. Experimentally changed environmental conditions resulted in distinct alterations in aperture and shell size, which were shown to be highly reproducible and reversible within a few generations. If reliable qualitative shell characteristics are available, shell size variability may cause no severe taxonomic problems, but regarding closely related taxa, where separation occurs mainly by size characteristics, definition of species and bioindication may be impeded.

To sum up, intraclonal variability plays an pivotal role in basic and applied research. On one hand, it implies some current taxonomic and ecologic problems, but on the other hand, it offers new and promising possibilities for the future. Based on the above referred data, the following research objectives are proposed:

- the search for additional qualitative species characteristics, morphological as well as molecular features, as a basis for species delimitation;

- the comprehensive evaluation of clonal variability in numerous taxa of testate amoebae to provide basic information for taxonomic and ecologic evaluations;

- the development of routine laboratory (toxicity) tests by means of shell morphometry alterations as a new tool for bioindication - e.g. to replace /supplement animal experiments;

- the establishment of molecular methods for the characterization and delineation of (contaminated) field populations by amplifying and sequencing DNA deriving from single amoeba nuclei;

- the establishment of contamination-free cultures to detect sexual processes in testate amoebae using RAPD-derived DNA-fragment patterns. Such cultures may be good candidates for a subsequent ultrastructural search for meiosis, and may help to clarify the above discussed problem of species definition;

- finally, basic research on so-called "dauermodifications", seemingly forgotten by most scientists, may be renewed using testate amoebae as convenient model organisms.

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How are Plastid Proteins of the Apicomplexan Parasites Imported? A Hypothesis*

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Summary. Apicomplexa include parasitic protozoans that are the causative agents of diseases such as malaria, toxoplasmosis and coccidiosis. Interestingly, their cells contain not only 35-kb plastid DNA, but also a modified plastid. Recently it has been demonstrated that the plastids (termed "apicoplasts") are surrounded by four membranes, which points to a eukaryotic alga as their ancestor. Considering the probable absence of ribosomes on the outermost membrane of the apicoplasts, I propose that their nuclear-encoded proteins are first transported into the ER, then to the Golgi apparatus, and finally to the plastids. Thus the presequences of these proteins should have both a signal peptide and a transit peptide. If this hypothesis is confirmed, the complex targeting mechanism of the apicomplexan plastid proteins may become an excellent target for chemotherapeutic agents in the future.

Key words: Apicomplexa, apicoplast, Chlorarachniophyta, plastid, protein targeting, signal peptide.

Abbreviations: ER - endoplasmic reticulum, PER - plastid ER, RER - rough ER

INTRODUCTION

According to the common view, the main function of plastids is photosynthesis. However, these organelles also participate in other processes, such as the biosynthesis of amino acids, fatty acids and haem, the reduction of nitrites and sulphates and the accumulation of starch (Howe and Smith 1991, Wallsgrove 1991, Emes and Tobin 1993). As the result, in many organisms, even after the loss of photosynthesis, the plastids are not eliminated. Among the protists such organisms are represented, for example, by a euglenoid *Astasia longa* (Bodył 1996) and a percolozoan *Psalteriomonas lanterna* (Hackstein *et al.* 1997). Interestingly, modified plastids (termed "apicoplasts") are also present in a large group of parasitic protozoans of the phylum Apicomplexa (for a review see McFadden *et al.* 1997). The apicoplasts contain 35-kb plastid (pt) DNA, almost all of whose genes encode the components of a transcription and translation apparatus (Wilson *et al.* 1996). The apparatus probably enables expression of several open reading frames (ORF) of unknown function (Wilson *et al.* 1996). At present it can be only supposed that these proteins are engaged in some non-photosynthetic functions of the apicoplasts and/or of regulation processes which make their loss impossible.

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The low number of genes in the 35-kb ptDNA suggests that most plastid proteins in apicomplexans are nuclear-encoded. But how are these proteins targeted to the apicoplasts?

Diversity of mechanisms of the plastid protein import

The extant algae display several different mechanisms of protein import to their plastids (Fig. 1). In glaucocystophytes, red algae and green plants whose plastids are surrounded by only two membranes, the process has a post-translational character (Fig. 1c) (Robinson *et al.* 1998, Schwartzbach *et al.* 1998, Soll and Tien 1998). The first stage of the targeting is the synthesis of the precursor proteins on cytosolic ribosomes. The precursors contain amino-terminal presequences that consist mainly of the transit peptide. There is, however, a group of proteins which also have a signal peptide-like domain. But the domain is located behind the transit peptide and participates in the intraorganellar sorting of the proteins into the thylakoid lumen. In addition, the segments differ in their origin. The signal peptide-like domain was probably present already in cyanobacterial export proteins, while the transit peptides were acquired by plastid proteins as late as during the primary endosymbiosis. It is suggested that the interaction between the transit peptides and lipids in the outer plastid membrane induces specific secondary structures in them which are then recognised by the receptor proteins. At a subsequent stage of import, the protein precursors are transported through the envelope membranes into the stroma. The transport takes place in the so-called contact sites which are junctions between the translocation complexes of the outer and inner plastid membranes. Recently several components of each of these translocons have been identified (e.g. Toc 75, Toc 86, Tic 55, Tic 110). Following their arrival at the stroma, the precursors of stromal proteins are processed to mature forms by the stromal processing peptidase (SPP) which removes transit peptides from them. Further sorting and processing events are required for proteins of the thylakoid membrane and the thylakoid lumen. It appears that the post-translational mechanism of protein targeting to the plastids of glaucocystophytes, red algae and green plants remains in close association with the character of their outer membrane. If it was derived from the host's phagosomal membrane, as suggested by Schnepf (1964), the vesicular pathway should occur in such algae (see the further discussion). For this reason it should be supposed that the

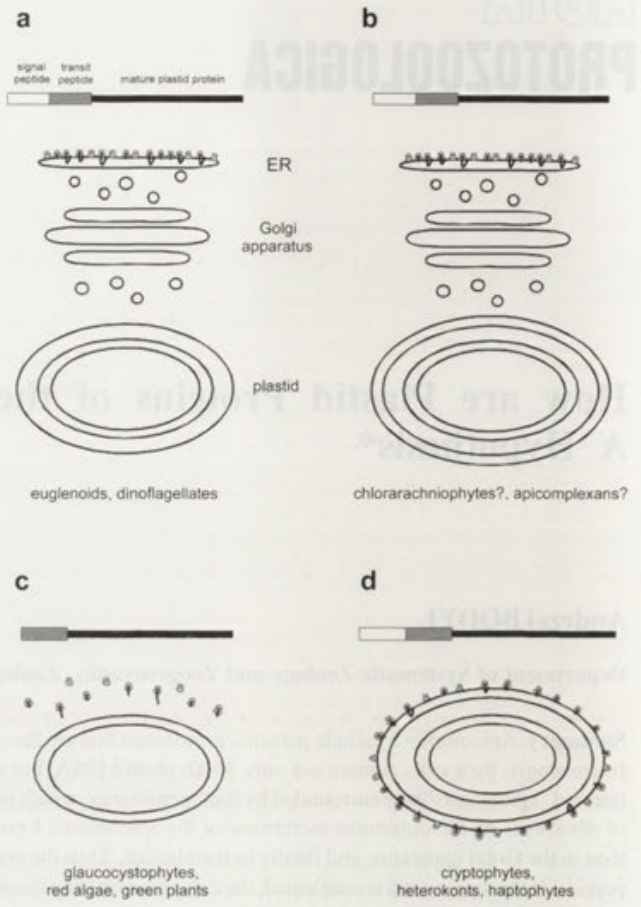

Fig. 1. Various pathways of plastid protein transport from the cytoplasm to the plastids. The presequence structure of the nuclear-encoded plastid proteins characteristic of each pathway is presented as well. A signal peptide-like domain (not shown) is located behind the transit peptide and participates in the intraorganellar sorting of the thylakoid proteins into the thylakoid lumen

membrane is homologous with the outer membrane of cyanobacterial envelope (Cavalier-Smith 1982). The hypothesis is very strongly supported by the discovery of the homologues of the outer membrane proteins of two-membrane plastids in cyanobacteria (Soll and Tien 1998). Other arguments in favour of the prokaryotic origin of the outer plastid membrane of glaucocystophytes, red algae and green plants can be found in the papers of Cavalier-Smith (1993, 1995).

A completely different import pathway is found in euglenoids and many dinoflagellates whose plastids have an envelope built of three membranes (Fig. 1a). Sulli and Schwartzbach (1995, 1996) demonstrated that the *Euglena* plastid proteins were first transported co-translationally into the endoplasmic reticulum (ER), then

to the Golgi apparatus, and only later - with the use of pre-plastid vesicles - they reached the plastids (see also Schwartzbach *et al.* 1998). These data agree with the presequence structure of the nuclear-encoded euglenoid plastid proteins. The presequences are very long (*ca* 140 amino acids) and contain a signal peptide at their amino-termini (e.g. Kishore *et al.* 1993, Lin *et al.* 1994, Henze *et al.* 1995). The signal peptides differ from the above-mentioned signal peptide-like domain, not only in their physico-chemical properties (von Heijne *et al.* 1989) but also in their origin. Like the transit peptides, they were probably acquired only during endosymbiotic events: primary endosymbiosis (if three-membrane plastids have a prokaryotic origin, as suggested by Cavalier-Smith 1982), or secondary endosymbiosis (if three membrane plastids are of a eukaryotic origin, as proposed by Gibbs 1978) (for discussion see Cavalier-Smith 1993). It is very likely that also in dinoflagellates the vesicular pathway is engaged in the targeting of plastid proteins. Such an assumption is supported by the studies of Norris and Miller (1994). These authors found that the amino-terminal extension of peridinin-chlorophyll *a*-binding protein (PCP) from *Symbiodinium* sp. resembled signal peptides (see also Schwartzbach *et al.* 1998). The presence of the vesicular pathway not only in euglenoids but probably also in dinoflagellates suggests that the outermost membrane of their plastids is derived from the host's phagosomal membrane (for a variety of arguments see Cavalier-Smith 1993). Thus, if three-membrane plastids originated from eukaryotic algae, it should be supposed that during secondary endosymbioses it was not the phagosomal membrane that was lost, as proposed by Gibbs (1978), but the endosymbiont's plasmalemma. The scenario presented by Cavalier-Smith (1982) is much more parsimonious; it does not require any loss of any membrane. Besides, in this scenario the outermost plastid membrane may be derived only from the host's phagosomal membrane, since the cyanobacterial envelope is formed of only two membranes. Irrespective of whether we assume that the ancestors of three-membrane plastids were prokaryotic or eukaryotic endosymbionts, their two inner membranes would be homologous with the plasmalemma and the outer membrane of cyanobacteria.

The algae provided with four-membrane plastids probably have two different import pathways (Figs. 1b, d). Each of them is accompanied by a specific structure of the outermost plastid membrane. The four-membrane plastids are present in a large group of algae grouped in four phyla: Cryptophyta, Heterokonta, Haptophyta and Chlorarachniophyta. An inner and outer pair of membranes can be distinguished in the envelope of such plastids. It is assumed that the outer pair originates from the host's phagosomal membrane and the endosymbiont's plasmalemma, whereas the inner pair (the so-called plastid envelope) is derived from the envelope membranes of the endosymbiont's plastid (Cavalier-Smith 1986). In cryptophytes, heterokonts and haptophytes the outermost membrane bears ribosomes which suggests that in these groups the phagosomal membrane has fused with the rough ER (RER) membrane (Cavalier-Smith 1986). For this reason the outer pair of membranes of these plastids was termed the plastid ER (PER). In 1979 Gibbs proposed a model of protein import to the four-membrane plastids with PER (Fig. 1d). According to her model the plastid proteins are first transported co-translationally into the lumen of plastid ER. Then they are re-packed to transport vesicles budding off from the inner membrane of PER which - following the fusion with the outer membrane of the plastid - liberate them into the lumen of the plastid envelope. At the final stage the proteins pass through the innermost membrane into the stroma. The model has been recently confirmed by molecular data. All the cytoplasmically-synthesized plastid proteins in cryptophytes and heterokonts have complex presequences which are provided with both the signal peptide (probably responsible for the transport through the outer PER membrane) and the transit peptide (probably participating in the transport through the inner membrane of plastid envelope) (e.g. Apt *et al.* 1995, Kroth-Pancic 1995, Liaud at al. 1997, McFadden and Gilson 1997). Additionally, Bhaya and Grossman (1991) showed that the precursor of fucoxanthin-chlorophyll *a/c*-binding protein (FCP) from the diatom *Phaeodactylum tricornutum* was transported and processed by pancreatic microsomal vesicles (see also Schwartzbach *et al.* 1998).

In chlorarachniophytes the outermost plastid membrane is ribosome-free which indicates that it has been derived solely from the phagosomal membrane (Hibberd and Norris 1984). Based on these data I have recently proposed that the nuclear-encoded chlorarachniophyte plastid proteins are first targeted into the ER, then to the Golgi apparatus and ultimately to the plastids (Fig. 1b) (Bodył 1997). The possibility of this pathway in chlorarachniophytes is indicated by the existence of the identical import mechanism in euglenoids (and probably in dinoflagellates) in which the outermost plastid membrane is probably homologous with the host's phagosomal membrane (see the earlier discussion). Besides, in the chlorarachniophyte cells the Golgi apparatus is located in the close vicinity of each plastid (Hibberd and Norris

1984). An additional support for this transport mechanism is provided by the evolutionary pathway of four-membrane plastids. The conversion of a eukaryotic alga into a plastid encounters an array of limitations of which the most serious is the origin of a new targeting mechanism of plastid proteins (Cavalier-Smith 1986). However, all these difficulties can be avoided by assuming that the ancestors of chlorarachniophytes, cryptophytes, heterokonts and haptophytes had three-membrane plastids which were later substituted by four-membrane plastids (Bodył 1997). If this scenario is correct, eukaryotic endosymbionts may use the co-translational mechanism of protein import to the three-membrane plastids. Thus the protein targeting to the four-membrane plastids should take place through the Golgi apparatus. This complex import mechanism has been probably preserved in chlorarachniophytes whose the outermost plastid membrane is devoid of ribosomes, while in cryptophytes, heterokonts and haptophytes it has been modified as a result of fusion of their outermost plastid membranes with RER membranes (for details see Bodył 1997). If chlorarachniophyte plastid proteins are actually transported through the endomembrane system, their presequences should contain signal peptides at their amino-termini.

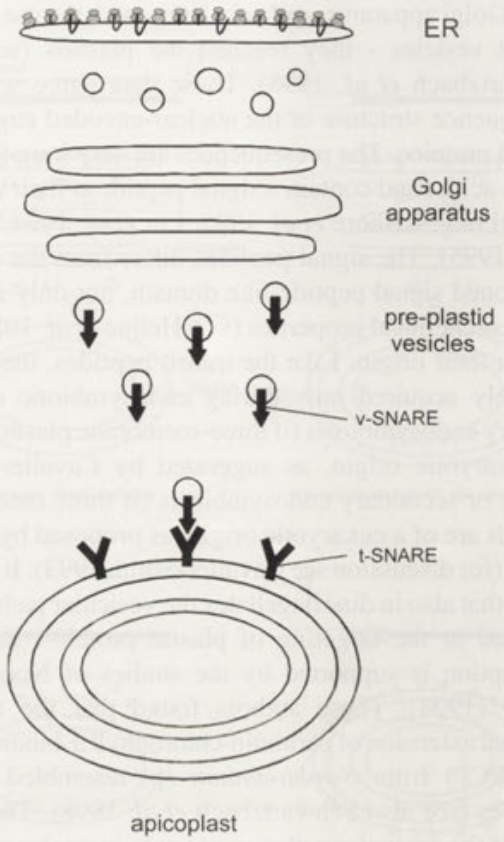

Fig. 2. The complex import mechanism of apicoplast proteins as apotential target for chemotherapeutic agents. Only SNARE proteins of pre-plastid vesicles (v-SNAREs) and of the outermost apicoplast membrane (t-SNAREs) have been indicated in the diagram. It can be supposed that a modification of either the v-SNAREs or the t-SNAREs could preclude the fusion of pre-plastid vesicles with the outermost apicoplast membrane

Pathway of protein import to the apicomplexan plastids

The above considerations indicate that not only the number of envelope membranes but also the nature of the outermost membrane determine the mechanism of plastid protein targeting. Thus, using only the data on the plastid ultrastructure, an attempt can be made at predicting the import mechanism of the cytoplasmically-synthesized plastid proteins. The earlier electron microscope studies on the apicoplasts suggested the presence of two or three envelope membranes (McFadden *et al.* 1996). If these plastids are surrounded by two membranes, a post-translational pathway should be suspected (Fig. 1c). Thus the presequences of apicoplast proteins would be probably provided only with a transit peptide. If apicoplasts have a three-membrane envelope, their nuclear-encoded proteins could be transported via the vesicular pathway (Fig. 1a). At the same time their presequences should have a signal peptide at their amino-termini. However, Köhler *et al.* (1997) has recently shown that four membranes surround apicoplasts, which clearly indicates that their ancestor was a eukaryotic alga. If the outermost membrane of these plastids bears ribosomes, the apicomplexan plastid proteins may be transported directly into the lumen of the plastid ER (Fig. 1d). Since PER forms connections with the rough ER (Gibbs 1981), they may be also targeted into the lumen of RER, and from there they could migrate to the lumen of PER, omitting the Golgi apparatus. This mechanism of transport would require the presence of both a signal peptide and a transit peptide. Additionally, the proteins should be provided with a signal for their retention in the ER (Bar-Peled *et al.* 1996). But electron micrographs published by Köhler *et al.* (1997) suggest that the outermost membrane of the apicoplasts is ribosome-free. The presence of four envelope membranes and the probable absence of ribosomes on the outermost membrane resembles the situation found in chlorarachniophyte plastids (see the earlier section). Considering the similarities, I propose that the nuclear-encoded apicoplast pro-

teins are first transported co-translationally into the ER, then transport vesicles carry them to the Golgi apparatus, and only later - with the help of pre-plastid vesicles - they reach the plastids (Fig. 1b). If so, the presequences of such proteins should be bipartite and consist not only of a signal peptide, but also of a transit peptide. Two other arguments lend support to this hypothesis. Firstly, apicomplexan cells, like those of chlorarachniophytes, display Golgi apparatus located in proximity to the apicoplasts (McFadden *et al.* 1997). Secondly, the new model of the evolution of four-membrane plastids (see the earlier section) applies especially well to the apicoplasts. Molecular phylogenetic analyses indicate that Dinoflagellata are a sister-group of Apicomplexa (e.g. Kumar and Rzhetsky 1996, Silberman *et al.* 1996, Van de Peer *et al.* 1996). Although these protists have very diverse kinds of plastids, their ancestral type are three-membrane plastids containing peridinin as the main carotenoid (the so-called typical plastids) (Schnepf 1993). The remaining plastids are probably of a recent and independent eukaryotic origin (for a review see Schnepf 1993). It can be supposed that all these "unusual" plastids have evolved via the substitution of the typical plastids. From the view-point of the apicoplast origin, the most interesting plastids are those of *Lepidodinium viride* with four envelope membranes the outermost of which is ribosome-free (Watanabe *et al.* 1987, 1990). Recently, I have proposed that such a *L. viride*-like protist, possessing both three- and four-membrane plastids, was the ancestor of Apicomplexa (Bodył 1998a).

PERSPECTIVES

The apicomplexan plastids are a welcome new target for chemotherapeutic agents. Recently both antibiotics and herbicides inhibiting plastid function have been shown to stop growth of apicomplexans *in vitro* (Fichera and Roos 1997, McConkey *et al.* 1997, Roberts *et al.* 1998). However, the proverbial Achilles' heel of the apicomplexan cells may be the targeting mechanism of their nuclear-encoded plastid proteins. The presence of vestigial plastids in these obligatory parasites suggests that they constitute a compartment where one or several vital metabolic pathways/cycles take place (e.g. biosynthesis of amino acids or fatty acids, see McFadden *et al.* 1997, Roberts *et al.* 1998). Thus it should be supposed that efficient blocking of one of these functions might be lethal to the apicomplexan cells. How could this be done? If apicomplexan plastid proteins were transported via the vesicular pathway, one of the ways would be to prevent the fusion of pre-plastid vesicles with the outermost plastid membrane. Consequently, the proteins would be mistargeted to the endomembrane system and would never reach their final destination i.e. plastid. Since SNARE proteins, present both on the transport vesicles (v-SNAREs) and on the target membranes (t-SNAREs), are probably responsible for the specificity of vesicular transport (for reviews see Edwardson 1998, Weis and Scheller 1998), it can be speculated that a modification of either v-SNAREs located on pre-plastid vesicles or t-SNAREs located on the outermost apicoplast membrane could preclude the fusion of pre-plastid vesicles with this membrane (Fig. 2). Detection and/or construction of chemical factors which are able to modify SNARE proteins could be helped by an examination of endosymbiotic associations with such pathogenic bacteria as *Mycobacterium*, *Chlamydia* and *Legionella*. Interestingly, all these microorganisms are capable of inhibiting phagosome-lysosome fusion (Russell 1998). Twenty three *icm/dot* genes, recently detected in *Legionella pneumophila*, encode components of transport machinery which is probably located in the bacterial inner and outer membranes (Kirby and Isberg 1998, Segal and Shuman 1998). It appears that the function of this machinery is transferring effector proteins to the host's cell, which inhibit or modify the endocytic pathway. Segal and Shuman (1998) suggested that one of these proteins (the IcmG/DotF protein) might interact with the SNARE system.

The subject of future studies should be ascertaining not only the number but also the homology of the membranes surrounding apicoplasts. A direct test for the three possible pathways of protein import to the plastids may be provided by: (1) isolating and sequencing nuclear-encoded apicoplast proteins, and (2) detailed tracing of their transport route from the cytoplasm to the plastids. If the apicoplast envelope consists of four membranes, further experiments should be also aiming at recognising mechanisms which are involved in the transport of apicoplast proteins through the remaining three envelope membranes.

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Recently Waller et al. (1998) have demonstrated that nuclear-encoded apicoplast proteins contain signal peptides, which suggests that they are imported via the endomembrane system. However, at present it is still unknown if these proteins pass only through the ER, or perhaps also through the Golgi apparatus.

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ACTA
PROTOZOOLOGICA

Nuclear Phenomena During Autogamy in the Marine Ciliate *Euplotes crassus*: a Tangled Cytogenetic Process Fostering Evolutionary Conservatism

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Summary. Nuclear phenomena have been studied during autogamy in single cells of the ciliate *Euplotes crassus* comprising the group of closely related, marine, single dargyrome, cirrotype 10 forms *Euplotes vannus-crassus-minuta*. Evidence for sexuality has been soundly derived at two different levels of methodology: (i) the overall cytological description by light microscopy, and (ii) the existence of differences in the DNA content measurable by Feulgen cytophotometry among differently staged micronuclei. The micronuclear events include four prezygotic and two postzygotic divisions. The first and the fourth prezygotic divisions are mitotic, while the second and third divisions are meiotic. Both the mitotic products of the first prezygotic, mitotic division undergo a classic two-step meiosis, which reduces levels of micronuclear DNA contents according to the ratio 4c : 2c : 1c. Only six of the resulting eight haploid nuclei are destroyed, leaving two survivors to enter the fourth prezygotic, mitotic division. Of the four fission products two swell and differentiate into gamete nuclei while the other two degenerates. Thus, contrary to some descriptions of both conjugation and autogamy in marine *Euplotes*, stocks comprising the population "G" of *E. crassus* have the fourth prezygotic, mitotic division leading to gamete nuclei differentiation. The fusion of these last yields a zygotic nucleus that undergoes two postzygotic, mitotic divisions; two of the resulting four products typically degenerate, one differentiates into the single macronuclear anlage and one into the micronucleus. Meanwhile, the old (prezygotic) macronucleus is completely destroyed after breaking into various fragments. The integrated cytological and cytophotometric approaches reliably establish the sexual nature of the autogamic reorganization of *E. crassus*. Nevertheless, notwithstanding the occurrence of typical two-step meiosis, the autogamous cytogenetic system comprises consistent and real deviating elements that counter a random genetic recombination; an idiosyncratic feature of evolutionary value addressed in the discussion section.

Key words: autogamy, cytogenetic events, ecogenetic strategies, *Euplotes*.

INTRODUCTION

The nuclear phenomena during conjugation of *Euplotes* species are rather well known. There exist more or less complete descriptions of the process in *Euplotes eurystomus*, *Euplotes patella*, *Euplotes harpa*, *Euplotes charon*, *Euplotes woodruffi* (see references in Raikov 1972, pp. 184-185), *Euplotes vannus*, *Euplotes crassus*, *Euplotes minuta*, *Euplotes cristatus* (see Nobili *et al.* 1978, for a review), and *Euplotes raikovi* (Miceli *et al.* 1981). Since then, conjugation processes have been described in detail in *Euplotes octocarinatus* (Kuhlmann and Heckmann 1991) and briefly in *Euplotes plicatum*

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(Valbonesi *et al.* 1997). In all these cases, the micronuclear activity typically includes four prezygotic divisions, that is, divisions leading to the formation of gamete nuclei that fuse to produce the zygotic or fertilization nucleus (synkaryon): a premeiotic (first prezygotic) diploid mitosis, two meiotic divisions, and a postmeiotic (fourth prezygotic) haploid mitosis.

In the last two decades, however, some data appeared stating that stocks comprising the *E. vannus-crassus-minuta* group of closely related species may lack the postmeiotic division, two of the usually eight products of meiosis differentiate directly into gamete nuclei. Such a variation in the micronuclear behavior has been described during both conjugation (Lueken 1973, Machelon 1983) and autogamy in single cells (Fleury and Fryd-Versavel 1981). Yet, even the most recent reports maintain that the postmeiotic micronuclear division exists at least during conjugation in *E. crassus* (Baird and Klobutcher 1988, Price *et al.* 1994). The question arises as to whether this nuclear division may be also considered a typical event of autogamy in marine *Euplotes*.

A postmeiotic division has been maintained to occur in autogamous stocks of *E. minuta* (Siegel and Heckmann 1966, Nobili and Luporini 1967) but no detailed account of the sequence of nuclear developmental features accompanying the reorganizing process has yet been published. Difficulties in producing populations progressing synchronously throughout the autogamous process likely underlie the evident literature shortcoming dealing with this issue. In the present paper, the time frame of nuclear events during autogamy in marine *Euplotes* is reported and illustrated by timely genuine photographs which, to date, have been at most replaced in the literature with diagrammatic nuclear views or schematic representations of specimens undergoing sexual reorganization.

The ability to undergo autogamy is a strain-specific character in members of the *E. vannus-crassus-minuta* group where (i) autogamous stocks naturally occur at very low frequency with respect to the far more common nonautogamous ones (Luporini and Nobili 1967, Luporini and Dini 1977, Dini 1984, Dini and Giannì 1985), (ii) sex in autogamous stocks takes the form of both autogamy and conjugation, and (iii) the autogamy trait is genetically determined by allelic differences at a single locus (Dini and Luporini 1980). Autogamy has been described also in the freshwater strains and one exceptional marine strain of the euryhaline *E. woodruffi* (Kosaka 1970, 1982a, b, 1992; Ito 1971; Murakami 1986). In these cases, the two spindles of the postmeiotic division are present.

The present report gives accounts of the cascade of nuclear events characterizing development of autogamy in *E. crassus* at two levels of methodology: (i) the overall cytological description by light microscopy, and (ii) the difference between haploid and diploid nuclei expressed as DNA content, measurable by Feulgen cytophotometry. Both levels concur in qualifying autogamy in *E. crassus* as a true sexual process involving two-step meiosis. The pattern of nuclear events consists of four prezygotic divisions, the last one (postmeiotic) leading to the pronuclear formation of mitotic nature. This is a matter of theoretical as well as practical importance in view of its genetic consequences that, in turn, establish the genetic system of an evolutionary line, that is, the amount of diversity and evolutionary progress of which it is capable.

MATERIALS AND METHODS

The five stocks GI, GV-VIII analyzed in the present study represent a population of *E. crassus* collected from the Somalian coasts near Mogadishu and previously employed in extensive breeding (Luporini and Dini 1977) and evolutionary (Gates 1990) studies. This is the largest autogamous population comprising the cluster of the marine, single dargyrome, cirrotype 10 forms, *E. vannus-crassus-minuta*, which has been to day recorded in the literature. The classical taxonomic practice allowed stocks of the population "G" to be assigned to *E. crassus* (Luporini and Dini 1977); an identity recently confirmed by Dini *et al.* (1993) following a new reliable approach for identifying taxa within the above-mentioned group of closely related species. All "G" stocks were analyzed for the cascade of nuclear events during autogamy, even if stock GVIII was the mostly used for producing satisfactory numbers of staged cells since its ability to respond better to procedure employed for staging.

Lines established from stocks were cultured in Pyrex triple depression slides and fed the green microalga *Dunaliella salina* grown in Medium 1 (Committee on Cultures, Society of Protozoologists), except that an artificial seawater was used (see Dini and Luporini 1985). Experimental cultures were maintained at about two fissions/day by feeding excessive food and kept at 23±1 °C while exposed to alternate light and darkness with a rhythm of 12 h.

In order to induce autogamy, the culture medium was withdrawn from the log cultures 12 h after the last feeding using a micropipette under the low powers of a dissecting microscope. The concentrated cells were washed with starvation medium (pure artificial seawater filtered through a sheet of filter paper, heated twice at 85 °C, and stored for 1 day at room temperature before use), gently transferred from the triple depression slides to Syracuse dishes using a coarse pipette, and finally resuspended in starvation medium to a cell density adjusted to about 1.5-2.0 x 10^3 cells/ml. Such a method produced cell lines showing the highest degree of synchronization in progressing throughout the autogamy process. The washing and resuspending in starvation medium (shiftdown) was scored as 0 time. Forth the next 12 h, cell samples were taken every 0.5 h and then fixed and stained.

Cells of *E. crassus* undergoing autogamy (autogamonts) precociously assume an outward appearance defined as "comma shape" (Luporini and Dini 1977) which has its nuclear counterpart in the initial macronuclear fragmentation and triggering of the micronuclear meiosis. A large number of comma-shaped cells usually occurred in the analytical lines about 3 h after the shiftdown. Within 0.5 h they were removed and transferred to Syracuse dishes in starvation medium. A population of autogamonts with ±0.5 h synchrony was thus established. This provided the further samples of autogamonts at 0.5 h intervals from 3 to 12 h. The experiment was repeated delaying by 15 min the first sampling of starving cells with respect to the shiftdown (= 0 time), but maintaining the interval of 0.5 h between the successive samples up to 12 h. Combining the two experiments, the development of nuclear changes up to the formation of the new macronuclear anlage could be thus dissected into intervals of about 15 min. For each interval, the most frequent staged cells typified the nuclear change of the autogamous process. Samples were also taken after 12 h, but at 2-h intervals, in order to follow the developmental events of the old (prezygotic) and new (postzygotic) macronuclei, which are spread out over almost 70 h since the shiftdown.

Cytological analysis of nuclear phenomena were performed on cells pipetted with some microliters of culture fluid over grease-free slides (cleaned with ethanol and flame). The Sanfelice fixative (chromic acid 1% aqueous solution, 16 parts; formalin, 8 parts; glacial acetic acid, 1 part) was dropped onto the cell culture sample in a 2:1 ratio. After 5 min, the mixture of the culture fluid and fixative was withdrawn with a micropipette under a dissecting microscope (leaving a little fixative around each cell), and 95% ethanol was immediately dropped over the fixed cells which stuck to the slides. After air drying, slides were dipped in 1N HCl overnight at laboratory temperature of 23±1 °C, then rinsed with distilled water and Feulgen stained. They were placed in Schiff's reagent for 30 min, passed throughout three rinses of 2 min each in 0.1 M $Na_2S_2O_3$, washed in tap water for 10 min, dehydrated through ethanol and toluene, and finally mounted in synthetic neutral mounting medium. Observations were carried out by brightfield-, phase contrast, and differential interference contrast (DIC) microscopy. Photographs were taken with a Leitz Orthoplan photomicroscope.

A quick, temporary acetic-orcein staining procedure was utilized to monitor the nuclear state of the experimental autogamous population. This made it possible to take samples of autogamonts for measurements by Feulgen cytophotometry at given points in the autogamous process. In these cases, before Feulgen staining, a coverslip was placed on the fixed autogamonts that were pressed with the fingers. This helped to flatten the cells facilitating measurements of the micronuclei.

Feulgen/DNA absorption of differently staged micronuclei was measured at 550 nm using the Barr & Stroud integrating microdensitometer, type GN5. Micronuclear DNA content was expressed as absorption units.

RESULTS

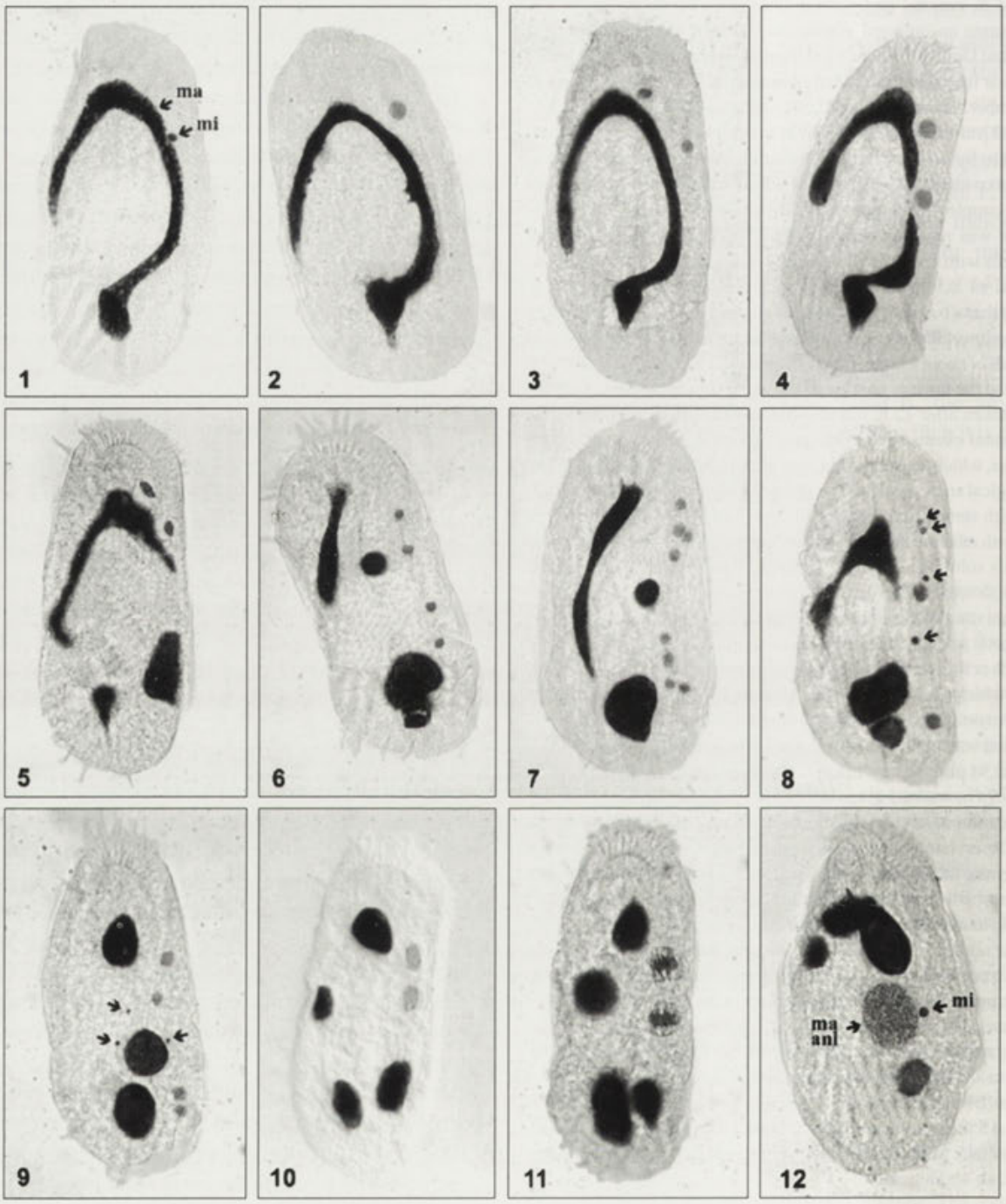
Nuclear processes during autogamy

In the dorsally-ventrally flattened *Euplotes crassus*, the micronucleus and its fission products occurring during the autogamic process line the left cell's border. In Figs. 1-12, the micronuclear elements of the reorganizing nuclear apparatus appear on the right cell's border because photographs are specimen's ventral views. Timing of the nuclear events at autogamy is reported in Fig. 13.

The nuclear apparatus of a vegetative cell (preautogamont) of the population "G" of *E. crassus* comprises a question-mark-shaped macronucleus and one micronucleus adjacent to it (Fig. 1). This latter undergoes four prezygotic divisions while proceeding through the autogamous process. The specimens starting autogamy show a swollen micronucleus detached from the macronucleus (Fig. 2). Then, the micronucleus undergoes the first prezygotic division which gives rise to two micronuclei in the cell (Fig. 3). These last enlarge (Fig. 4) while entering the prophase of the second prezygotic division. The chromatin now becomes tightly aggregated and polarized (Fig. 5). The phenomenon concurrently involves both prophasic nuclei that acquire a structure resembling the "parachute" stage typical of the Euplotidae's meiotic I prophase (for more about this stage, see Raikov 1972, 1982). Meanwhile micronuclei progress through prophase of the second prezygotic division, the macronucleus starts fragmenting (Figs. 4, 5). It constricts in several places and forms up to four large fragments, most frequently three (Fig. 5); few additional small spherical fragments may occur.

The second prezygotic division produces four nuclei linearly arranged, two located in the anterior and two in the posterior half of the cell (Fig. 6). They derive from products of the first prezygotic division that, just before dividing, locate at the equatorial plane of the cell along its left border. The bipolar telophasic spindles of the first prezygotic division products overlap meanwhile they elongate along the long axis of the cell, thus producing the defined, longitudinal nuclear arrangement shown in Fig. 6. Among the fragments of the broken prezygotic macronucleus, most round up whereas one keeps a rod-shaped structure. This organizational patter of the fragmented macronucleus typically associates with the presence of the second prezygotic division products.

Progressing through the autogamic process, it is relatively frequent the occurrence of staged autogamonts with an anterior and a posterior group of nuclei lined up along the cell's border and comprising each even a different number of nuclei ranging from two to four. Figure 7 shows a micronuclear arrangement in two groups each of four nuclei. They are the final products of the third prezygotic division that occurs between the seventh and eighth hour (cf. Fig. 13) since the shiftdown. The last quarter of this one-hour interval is characterized by a steady increase in the frequency of autogamonts endowed with two groups



Figs. 1-12. Microphotographs of Feulgen-stained autogamonts of *Euplotes crassus* during the first 12 h of the nuclear reorganization process. All photographs are specimens' ventral views; hence micronuclei appear on the right that actually represents the left cell's border (x 3,600). 1 - typical nuclear apparatus of pre-autogamonts, 2 - detachment from the macronucleus and enlargement of the micronucleus in prophase of the first prezygotic (mitotic) division, 3 - first prezygotic division derivatives, 4 - micronuclei in prophase of the second prezygotic (meiotic I) division and beginning of macronuclear degeneration, 5 - parachute-like stage characterizing the prophasic meiotic I micronuclei and further macronuclear fragmentation, 6 - end of meiosis I characterized by four micronuclei and by the typical presence of a rod-shaped macronuclear fragment, 7 - end of the third prezygotic (meiotic II) division producing eight micronuclei arranged in two groups each of four nuclei, 8 - two surviving meiotic II products in prophase of the fourth prezygotic (postmeiotic) division along with identical staged micronuclei becoming relics (arrows), 9 - end of the postmeiotic division resulting in four derivatives arranged in two groups each of two nuclei associated with exclusively rounded up macronuclear fragments and micronuclear relics (arrows), 10 - gamete nuclei just before to form the synkaryon, 11 - products of the first postzygotic division in metaphase of the second postzygotic division, 12 - early stage in the development of the macronuclear anlage associated with the surviving micronucleus. ma - macronucleus, ma anl - macronuclear anlage, mi - micronucleus

each of four nuclei vs. autogamonts with arrangements varying in the number of grouped nuclei. It therefore seems safe to assume an asynchrony of micronuclei in progressing from the stage represented by the Fig. 6 to that shown in Fig. 7. Yet, the possibility of a discriminating commitment of the four second prezygotic division products to proceed through meiosis, thus producing even final numbers of meiotic products less than eight, appears far less tenable. Spindles of nuclei engaged in the third prezygotic division stretch along the long axis of the cell and overlap in such way that the resulting four micronuclei aligned in each quartet do not share descent from the same pair of products of the second prezygotic division. Substantial differences do not appear to occur in the organization of the macronuclear fragments with respect to the pattern shown in Fig. 6.

Only two of the eight products of the third prezygotic division enlarge and enter the fourth prezygotic division (Fig. 8) while the others remain inactive and condense (Fig. 8, labeled with arrows). The two nuclei progressing through the autogamic process derive each from a different quartet. The fourth prezygotic division gives rise to four nuclei lying in a row along the cell's border, two located in the anterior and two in the posterior half of the autogamont (Fig. 9). All of this is the consequence of a stretching followed by an overlapping of spindles of dividing nuclei. Meanwhile, relic nuclei of the third prezygotic division move toward the center of the cell where they are completely resorbed (Fig. 9, labeled with arrows). All the macronuclear fragments assume the form of spherical fragments, thus permitting this stage to be distinguished clearly from the earlier stage typified by a closely similar micronuclear arrangement (two pairs of micronuclear products of the second prezygotic division), yet showing an anterior, rod-shaped macronuclear fragment (cf. Fig. 6).

Among the four derivatives of the fourth prezygotic division, one nucleus of the anterior and one of the posterior pair of nuclei give rise to the gamete nuclei (Fig. 10); the remaining two nuclei condense and are resorbed. The analysis of the overlapping spindle positions in 32 staged autogamonts did not provided helpful clues about the kinship of nuclei comprising the two groups produced by the fourth prezygotic division. Hence, whether the gamete nuclei are sister nuclei or not, or else whether they are the result of a random choice, is not evident during autogamy of *E. crassus* "G" stocks. The fusing gamete nuclei are two rather large nuclei (Fig. 10). They stain very lightly and can be well observed only with the differential interference contrast microscopy. On the basis of the ability to fulfil karyogamy, a haploid state of these nuclei following a meiotic process may be thus assumed. Note should be made of maneuvers performed by gamete nuclei to form the synkaryon which occurs in a region of the autogamont more apical than the synkaryon localization in the partners of a conjugating pair of *E. crassus*. While the anterior gamete nucleus in the autogamont actually stands still, the posterior one migrates about half the length of the cell. Such a behavior mimics that of the "stationary," posterior gamete nucleus in each co-conjugant *Euplotes* where it performs a relatively long migration to meet the anterior "migratory" gamete nucleus of the mating partner after this latter is pushed only a very short distance across the conjugation junction. Hence, the behavior of gamete nuclei in autogamy emphasizes again the literal incorrectness of the "stationary-migratory" terminology usually referred to gamete nuclei in conjugation. The "resident" and "transfer" terms suggested by Nelsen *et al.* (1994) and referred to the posterior "stationary" and the anterior "migratory" gamete nuclei, respectively, appear more appropriate. Nevertheless, a further variation in the terminology could be suggested considering the gamete nuclei behavior at autogamy: the inversion of the conjugational "stationary-migratory" terminology in referring to the anterior and posterior autogamous gamete nuclei, respectively, reflects properly the actual maneuvers performed by these last nuclei.

The synkaryon divides twice in succession. Fig. 11 shows two metaphases of the second postzygotic division characterized by chromosomes occupying the equatorial plane of the nucleus. The postzygotic nuclei divide simultaneously and divisions proceed rapidly (cf. Fig. 13). From the stage characterized by the four products of the fourth prezygotic division (Fig. 9) to that representing the end of the micronuclear divisions (Fig. 11), the pattern of the macronuclear fragments does not apparently change, the above-mentioned variation in the number of the spherical fragments notwithstanding.

Out of the four products of the postzygotic divisions, two usually degenerate, one remains condensed and becomes the new micronucleus, and one enlarges differentiating into the single postzygotic macronuclear anlage of each exautogamont (Fig. 12). The two surviving nuclei are those located in the posterior half of the cell. Since only one postzygotic product is induced to become the macronuclear primordium, in *E. crassus*, the exautogamous clone is composed of a single karyonide. The fragments of the old (prezygotic) macronucleus gather at the two extremities of the individual (Fig. 12) and start autolysis during the development of the new (postzygotic) macro-

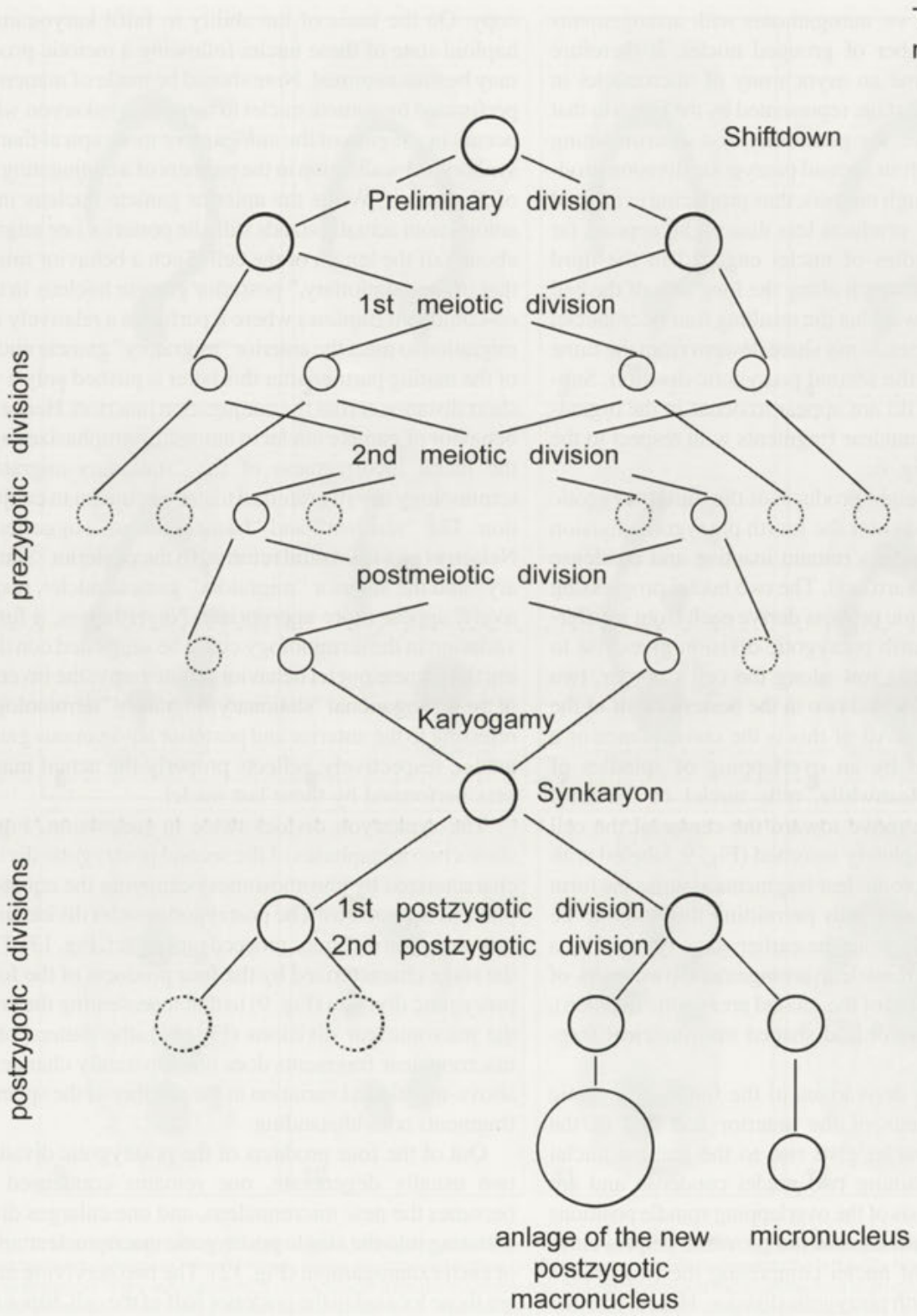


Fig.13. A diagrammatic view of the complex series of micronuclear divisions characterizing typically the autogamic reorganization process occurring in the "G" stocks of *Euplotes crassus*. Large and small circles refer to diploid and haploid nuclei, respectively. Resorbed nuclei present a dashed contour. The largest circle at the bottom of the diagram refers to the new developing postzygotic macronucleus, which, together with the diploid micronucleus on the right side, will reconstitute the typical nuclear framework in the offspring. Lines that do not directly connect nuclei denote uncertainty dealing with positional relationships of fission products in the arrangement following a round of nuclear divisions whose spindles overlap. On the right are reported data dealing with timing of the micronuclear divisions

nuclear anlage. There is asynchrony in the autolytic process. The posterior fragment(s) is (are) resorbed later than the anterior ones. The resorption ends up when the new macronucleus starts to elongate. However, prior to the first post-autogamic cell division no more macronuclear fragments are present. Even if the posterior fragment(s) of the old macronucleus (Fig. 12) sometimes occur(s) very close to the macronuclear anlage, we have neither observed fusion between these two structures nor we have got evidence for regeneration of fragments of the parental macronucleus.

Cytophotometric data

The amount of Feulgen-stained DNA has been measured in the micronuclei or its derivatives at various stages of autogamy. The results, expressed in absorption units (a.u.), are presented in Table 1 (cf. also Fig.13).

Euplotes crassus is known to enter conjugation with micronuclei in the G_2 phase (Luporini and Dini 1975). Vegetative micronuclei of this species are always in G_2 since DNA is replicated as early as in ana-telophase of vegetative mitosis. This is also true when shifted down individuals (pre-autogamonts, cf. Fig. 1) are considered, which show a 4c level of DNA content equaling a mean of 71 a.u.

The DNA content (57 a.u.) shown by the products of the first prezygotic division (cf. Fig. 3) ranges between 2c and 4c levels. The nuclei in prophase of the second prezygotic division (cf. Fig. 4) seem to have completed the DNA replication, since their DNA content (75 a.u.) is that expected for diploid G_2 (4c), suggesting a mitotic nature for the first prezygotic (premeiotic) division. Such a situation has its counterpart in the first prezygotic division products of conjugating *E. crassus* where an autoradiographic analysis has shown that (i) DNA synthesis takes place shortly before the onset of the second prezygotic (meiotic I) division, rather than at the end of the first prezygotic (mitotic) division of the micronucleus (Luporini and Dini 1975), and (ii) this synthesis is related to the meiotic process. Accordingly, the analyzed products of the first autogamous prezygotic division are assumed to represent a heterogeneous population comprising some representatives just engaged in diploid S phase forecasting their entry into prophase of the second prezygotic (meiotic) division.

The DNA contents corresponding to a mean of 35 a.u. (2c level) and 22 a.u. (1c level) were recorded in the micronuclear derivatives of the second (cf. Fig. 6) and third (cf. Fig. 7) prezygotic division, respectively. The 4:2:1 ratio of the DNA contents of micronuclei in the stages shown in Figs. 4, 6, and 7 strongly points to the occurrence of a classical meiosis with two nuclear divisions. An observation, however, is relevant to this point. The DNA content (22 a.u.) of the meiotic II (third prezygotic division) products is somewhat higher than the 1c level which would equal here 17-18 a.u. Probably, among the measured meiotic II derivatives, some were already in haploid S phase preparing for the fourth (postmeiotic) prezygotic division.

The clearest haploid presynthetic (G_1) condition seems to occur in the four products of the postmeiotic division (cf. Fig 9, nuclei giving rise to the gamete nuclei). Their DNA content is about 1/4 that of micronuclei in pre-autogamonts and must be held for 1c (17-18 a.u.). It is unknown whether differentiation of the gamete nuclei involves any DNA replication, since the gamete nuclei stain too faintly to be measured.

As could be expected, the synkaryon derivatives (cf. Fig.11) are diploid postsynthetic (4c DNA).

DISCUSSION

Our results clearly show that stocks comprising population "G" of *Euplotes crassus* possess the fourth prezygotic (postmeiotic) division of the micronucleus (cf. Fig. 13). Two meiotic II products out of eight participate in this division. There is the stage of four (morphologically) identical nuclei (Fig. 9), derived from the postmeiotic mitotic division, which cannot be confused with an earlier similar stage (Fig. 6) because of the different shape of fragments of the parental (prezygotic) macronucleus. The lack of staging methodologies in analyzing the sequence of nuclear events may foster bias in discriminating between the foregoing two stages both showing four micronuclei. If this is not the case, hence, the situation briefly described during autogamy by Fleury and Fryd-Versavel (1981), characterized by the lack of the postmeiotic division, represents only one of the possibilities that may be encountered among autogamous populations of marine *Euplotes* comprising the *E. vannus-crassus-minuta* group. A situation that has its counterpart among conjugating populations of this group of closely related marine species. The pattern of four prezygotic micronuclear divisions occurring in the autogamous "G" stocks parallels that observed during conjugation in the *E. vannus* strains of Heckmann (1963), in the *E. crassus* strains of Heckmann (1964), Salvano (1974), Luporini and Dini (1977), Baird and Klobutcher (1988), and Price *et al.* (1994), as well as in all strains *of E. minuta* studied up to now (see Nobili

Table 1. DNA content of micronuclei at various stages of autogamy of *Euplotes crassus*

State of the micronucleus	DNA content (absorption units) (Mean ± S.E.)	No. of nuclei measured	Ploidy and cell cycle phase
In interphasic vegetative cells (pre-autogamonts)	71 ± 0.68	19	Diploid G_2
After the end of the 1st prezygotic (mitotic) division	57 ± 1.35	26	Diploid G_1+S
At prophase of the 2nd prezygotic (meiotic I) division	75 ± 0.95	22	Diploid G_2
After the end of the 2nd prezygotic (meiotic I) division	35 ± 0.89	7	Haploid G_2
After the end of the 3th prezygotic (meiotic II) division	22 ± 1.98	16	Haploid G_1 + S
After the end of the 4th (postmeiotic) prezygotic division	17 ± 1.02	12	Haploid G_1
After the end of the 1st postzygotic division	68 ± 2.02	4	Diploid G_2

et al. 1978, for a review). Yet, conjugating *E. crassus* strains of Lueken (1973), as those of *E. vannus* (looking much like *E. crassus*) studied by Machelon (1983) differ for the lack of the fourth prezygotic division, that is, the postmeiotic one. When the different behavior of the meiotic II products is considered at the generic level, then it clearly appears that most *Euplotes* species perform the postmeiotic division, whatever their habitat, marine, brackish, and freshwater, may result to be (see Introduction for references). A similar extent of intra-generic variation characterizes also the behavior of the synkaryon which regularly undergoes two divisions, intra-specific cases of a single synkaryon division notwithstanding (see Wichterman 1967, Dini and Giannì 1985).

Among other hypotrichs, a postmeiotic division is typically absent during conjugation in the freshwater *Aspidisca costata* (Diller 1975) as well as in the marine *Aspidisca* sp. (closely resembling *A. leptaspis*, Dini *et al.* 1987): meiotic products appear to differentiate directly into gamete nuclei. The lack of a premeiotic, mitotic division represents a further difference between *Aspidisca* and *Euplotes* which, on the other hand, share a quite similar pattern of variation when the number of fissions of the synkaryon is concerned (Diller 1975, Dini *et al.* 1987).

Cytophotometric measurements clearly show that there is a four-fold reduction of the DNA content from the vegetative and the premeiotic micronuclei to the postmeiotic ones. The first are in a diploid G_2 phase of the cell cycle, while the last are in a haploid G_1 phase. It follows that the second and the third out of the four prezygotic micronuclear divisions are meiotic. The first prezygotic (premeiotic) division is a diploid mitosis characterized by a delayed S phase, as in conjugation (Luporini and Dini 1975), and the fourth prezygotic (postmeiotic) division is a haploid mitosis since it starts and ends with a haploid DNA amount. The peculiarity of this mitosis must be however the rather late DNA replication, because measured meiotic products are still mainly in G_1 (Table 1).

Evidence from two levels of methodology, (i) the overall cytological description, and (ii) the Feulgen cytophotometry, thus warrants a reliable acceptance of sexuality in autogamy of *E. crassus*, that is, the occurrence of a nuclear reorganization involving both a typical two-step meiosis and fertilization; events so far assumed on the basis of argumentation of comparative biology. This addresses the matter of the genetic consequences of such a cytogenetically complex, uniparental, sexual process. The occurrence of a postmeiotic (mitotic) division of the two surviving meiotic II products (cf. Fig. 13) accounts for the possibility that the two gamete nuclei may arise from one or two different products of meiosis. It follows that autogamy in *Euplotes* is not a promising system for causing a transition of all genes from the heterozygous to the homozygous condition in a single generation, as it occurs in other ciliate cytogenetic systems related to the autogamic reorganization (see Dini and Nyberg 1993). A positional analysis of the postmeiotic fission products in representatives of the studied population "G" of *E. crassus* did not allow to establish reliably the kinship of the two surviving gamete nuclei. Such a kind of analysis has been carried out successfully in conjugating *E. patella* (Katashima 1960) and *E. octocarinatus* (Kuhlmann and

Heckmann 1991). In both species, gamete nuclei have been recognized as sisters in about 90% of cases. The occurrence of cytologically non-sister gamete nuclei in 78% of cases has been reported in autogamonts of *E. woodruffi* (Murakami 1986). No genetic support has been provided to all these cytological observations. When genetic markers have been used, evidence has been adduced consistent with randomness in the persistence of sister or non-sister gamete nuclei in conjugating Heckman's stocks of *E. vannus* (Heckmann 1963) and *E. crassus* (Heckmann 1964). A trend toward a significant higher frequency in favor of non-sisters has been reported in conjugating *E. crassus* stocks of Luporini and Dini (1977) and Baird and Klobutcher (1988). Whatever the matter stands, this variation does not challenge the lack of uniformity in whether the two gamete nuclei arise from one or two different meiotic products. In this latter case, the parental genotype in a heterozygous stock can be maintained. However, to the extent that the possibility for the sister meiotic products to persist is preserved, the transition of all genes from a heterozygous to a homozygous condition continues to be a real event. Hence, notwithstanding the constrain represented by the occurrence of non-sister, genetically different, gamete nuclei, also autogamy in *Euplotes* should be finally doomed to homozygosity. Passing through successive autogamies, a heterozygous autogamous stock should enhance more and more the frequency of homozygous individuals following the persistence of sister gamete nuclei. Yet, literature records the appearance of a nonparental genotype as an extremely rare or nonexistent event in heterozygous, wild, autogamous stocks of *E. minuta* (Nobili and Luporini 1967) and *E. crassus* (Luporini and Dini 1977, Dini 1984). It becomes clear that peculiar cytogenetic events must occur during the autogamic nuclear process leading to the maintenance of the parental genotype, and that these events are closely coupled with the capacity to undergo autogamy. Dini and Giannì (1985) have provided a speculative but testable cytogenetic model for the maintenance of the parental genic state in autogamous *Euplotes* stocks. However, the statement that the perceived end result is supported by cytogenetic events alone can hardly be tenable. Regeneration of the fragments of the old (prezygotic) macronucleus or their fusion with the newly developing (postzygotic) macronuclear anlage could contribute to the production of "heterokarya" thus perpetuating the parental genic state. Nevertheless, no evidence for the occurrence of such phenomena has been obtained. Finally, the occurrence of a continued selection in favor of parental genotypes should not be overlooked. The extent of the mortality rate at reorganization after autogamy recorded in at least some marine *Euplotes* (Luporini and Dini 1977) supports a role of selective events countering nonparental genotypes.

Adequate reasons exist for drawing an analogy between *Euplotes* autogamy and the automictic parthenogenesis of certain groups of higher organisms, that is, between two genetic systems promoting maximum genetic stability at the expense of an evolutionary flexibility.

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ACTA
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Ultrastructural Details of the Plasmodial Rhizopod *Synamoeba arenaria* Grell

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Summary. During the last years, several plasmodial protists of the littoral zone have been discovered, cultivated, and characterized. Here, we present and discuss results on the ultrastructure of *Synamoeba arenaria* Grell. Especially characteristic in S. *arenaria* ultrastructure is the microtubule-organizing centre (MTOC). This plaque-like structure is positioned in proximity to the nucleus and comprises two electron-dense plaques separated by a transparent, partly granularly structured, cleft. During nuclear division MTOCs divide via typical V-shaped intermediary structures. The ultrastructure details of the MTOC suggest a phylogenetic relationship between *Synamoeba arenaria* and *Corallomyxa chattoni* and *Stereomyxa ramosa.*

Key words: MTOC, plasmodial rhizopod, *Synamoeba arenaria,* ultrastructure.

INTRODUCTION

Synamoeba arenaria is a marine plasmodial rhizopod that was first described by Professor Karl G. Grell (Tübingen), who was an enthusiastic pioneer in research of plasmodial protists. He isolated it from samples of sand, collected in the littoral zone of the coast North-East from Santa Cruz de Tenerife, Canary Islands (Grell 1994b).

Reticulate amoebae, among them *S. arenaria,* offer some interesting features: They are important grazers of bacteria in marine and freshwater sediments and play a role in cycling benthic carbon and nutrients (Grell 1989; Gothe *et al.* 1993; Gothe 1994; Grell 1994a,b; Rogerson *et al.* 1996). Ultrastructural organization, function, and biochemistry of this phylogenetically old group of organisms are of special interest for the establishment of phylogenetical relationships among protozoa (Benwitz and Grell 1971; Grell and Benwitz 1978; Page and Siemensma 1991; Grell *et al.* 1990; Grell 1991, 1994c; Cavalier-Smith *et al.* 1996; Linder *et al.* 1997).

Besides further physiological observations, the present study describes for the first time ultrastructural details of *S. arenaria,* including the microtubule-organizing centre (MTOC), which initiates microtubule growth and organizes microtubule array.

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MATERIALS AND METHODS

Culture of *Synamoeba arenaria*

Synamoeba arenaria was cultured in plastic Petri dishes of 3 cm in diameter containing seawater, heated to 90°C and filtered before use.

This medium was replenished every 8-10 days. Cultures were maintained in daylight at 22-24 °C and fed with the pennate diatom *Amphiprora* sp. (Grell 1994b).

Light Microscopy

Light microscopic observations were done using a Zeiss Axioplan microscope, in phase contrast mode using a water immersion 40x objective.

Scanning Electron Microscopy

For scanning electron microscopy, cultures settled on a coverslip were fixed by the method of Wollweber *et al.* (1981) for 1 h at room temperature in 1% glutaraldehyde, postfixed in 1% osmium tetroxide for 1 h and 1% tannin for 1 h, stained with 0.5% uranyl acetate for 1 h, dehydrated in acetone, and critical point dried. The samples were examined in a Zeiss-DSM 962.

Transmission Electron Microscopy

For transmission electron microscopy, settled plasmodia or pellets of plasmodia (1000 g for 5 min) were fixed for 1 h at room temperature in a solution of 1% formaldehyde and 0.5% glutaraldehyde in 10 mM Pipes (pH 7.2), 1 mM EGTA and 1 mM $MgSO_4$, postfixed in 0.2% osmium tetroxide for 1 h, 0.05% tannin for 45 min, and stained with 0.5% uranyl acetate for 12 h, dehydrated in acetone, and embedded in Vestopal. Observation was performed in a Tesla BS 540 or a Philips 400 T transmission electron microscope.

RESULTS

Light microscopic observations

Under the conditions realized in our laboratory, *Synamoeba arenaria* forms a meroplasmodium, i.e., a permanent association of cells connected by a reticulopodial network. The diameter of the network increases with the age of the organism (Figs. 1-3). The cell volume of *S. arenaria* was calculated to range between 4,000 and 8,000 μm^3 (see also Rogerson *et al.* 1996). Within the network, there is an incessant streaming of ground cytoplasm and particles. While the particles in the larger reticulopodia appear to move in well-defined cytoplasmic streams, the movement of single smaller particles is saltatory with mean velocities of about 15 µm/s.

Electron microscopic observations

Scanning electron microscopy (Figs. 4, 5) certifies the light microscopic observations that the plasmodial rhizopod is capturing and digesting protistan prey. Not only the whole body can catch and phagocytize the prey diatoms but also the tips of free-ending reticulopodia are used for grasping prey (Fig. 4). As already demonstrated in experiments on consumption rates of *S. arenaria* (Rogerson *et al.* 1996), intracellular bacteria were detectable also in electron microscopic images (Figs. 8, 21).

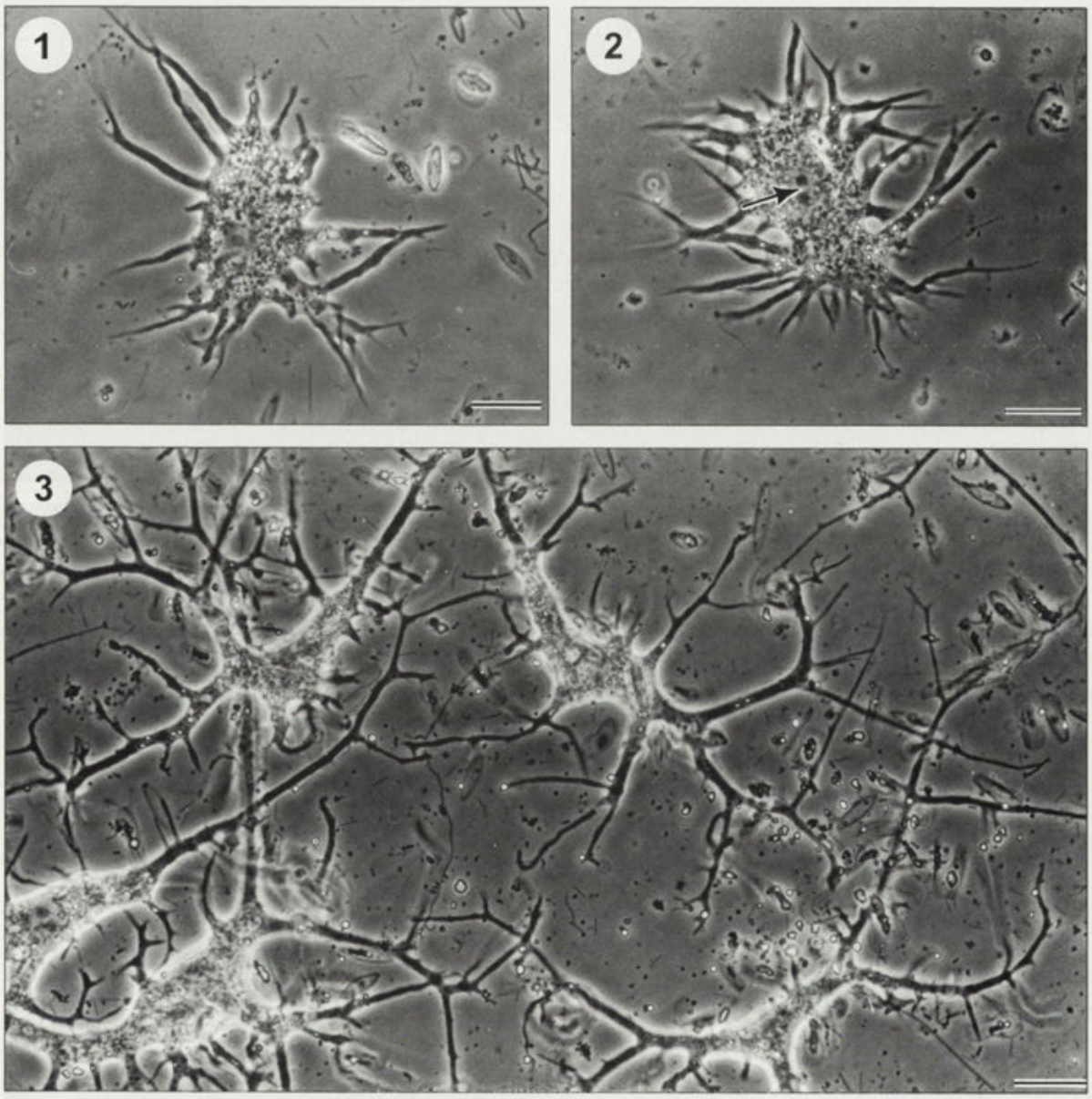
The cytoplasm of *S. arenaria* is not subdivided into ecto- and endoplasm. An elaborated network of vacuoles (sometimes more than 10 µm in diameter) is a dominating constituent of especially the central cytoplasm, while the cortical plasma shows smaller vacuoles. Dependent on the nutritional state of the cell, the vacuoles contain several stages of digested diatoms, empty shells, and other prey remnants (Figs. 6-9, 17).

The cell membrane is more or less covered by a surface coat sometimes with extracellular deposits (Figs. 13-17). As in other plasmodial rhizopods, membranosomes communicate with the cell membrane (Figs. 13-15). Smooth endoplasmic reticulum, membranous cisternae, and more or less dense membranosomes as constitutive parts of other membrane systems occur (Figs. 13-17). Mitochondria with vesicular cristae (Figs. 12, 16) are distributed throughout the cell. Golgi dictyosomes appear arranged mainly around the MTOC (Figs. 19, 22).

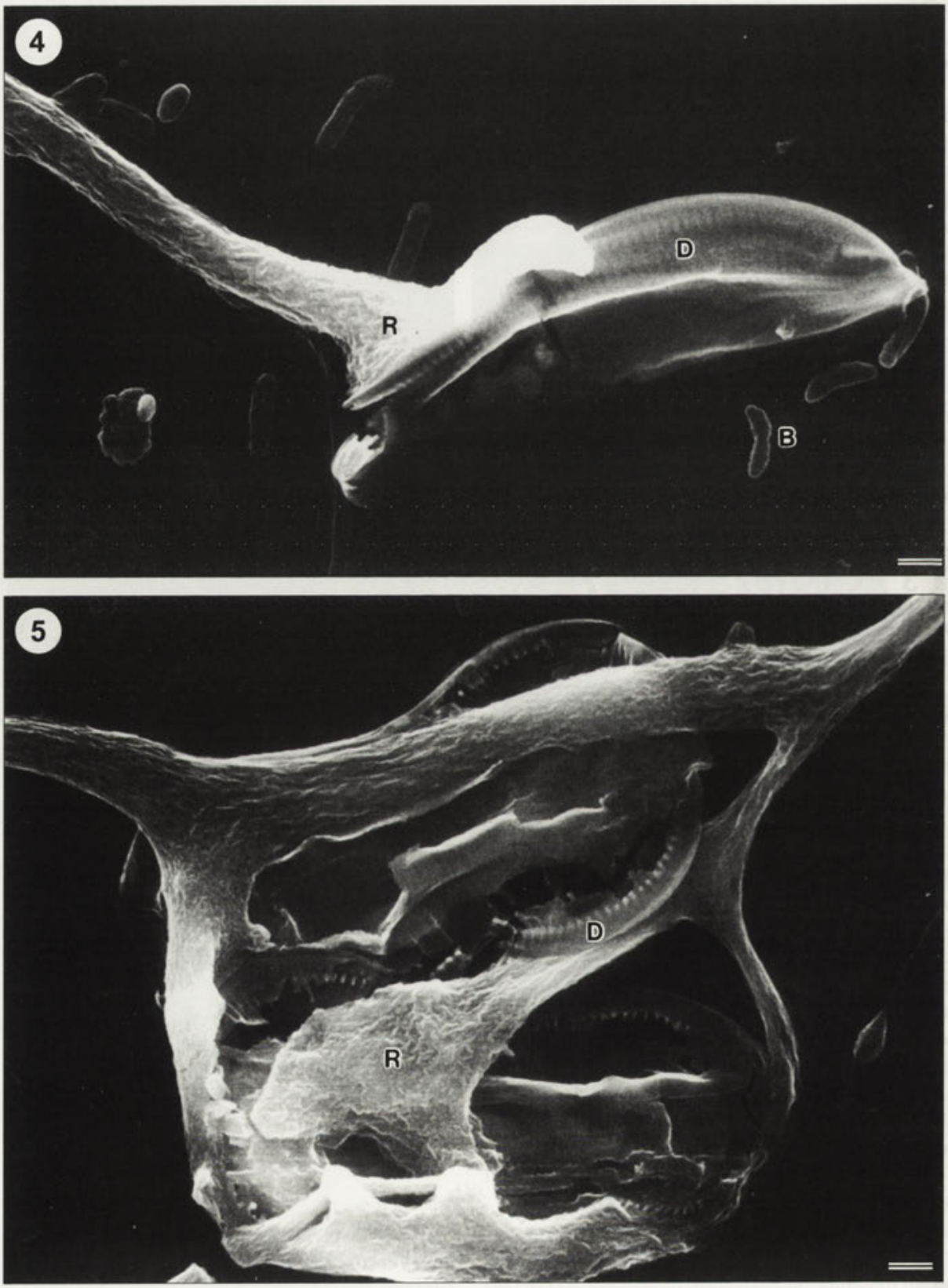
Synamoeba arenaria is an uninucleated protist, whose nucleus usually has an eccentric position in the cell. Nuclei are rounded or ovally shaped with irregular surfaces (Figs. 7-9, 23, 24). Their diameters vary from 4 to 7 µm. The nuclei are surrounded by nuclear envelopes which are interrupted by regularly arranged nuclear pore complexes. Tangential sections of the pores show the typical arrangement of cytoplasmic particles around a central channel with a central body (Figs. 10-12, 19, 20).

The karyoplasm contains chromatin, which appears as fine fibrils, and one electron-dense central nucleolus (Figs. 7, 8, 23). A characteristic appearance shows the MTOC of *S. arenaria* which is situated in direct vicinity to the nucleus (Figs. 9, 19, 20-24). In the centre of the MTOC, a structure of two electron-dense outer zones and a flaked inner zone can be seen (Figs. 18-22). Numerous microtubules escape from amorphous material on the outer zone. They radiate between the radially arranged endoplasmic reticulum and Golgi cisternae into the cytoplasm. The microtubules are typically associated with granular or filamentous material, possibly representing the microtubule-associated proteins (Figs. 18-22).

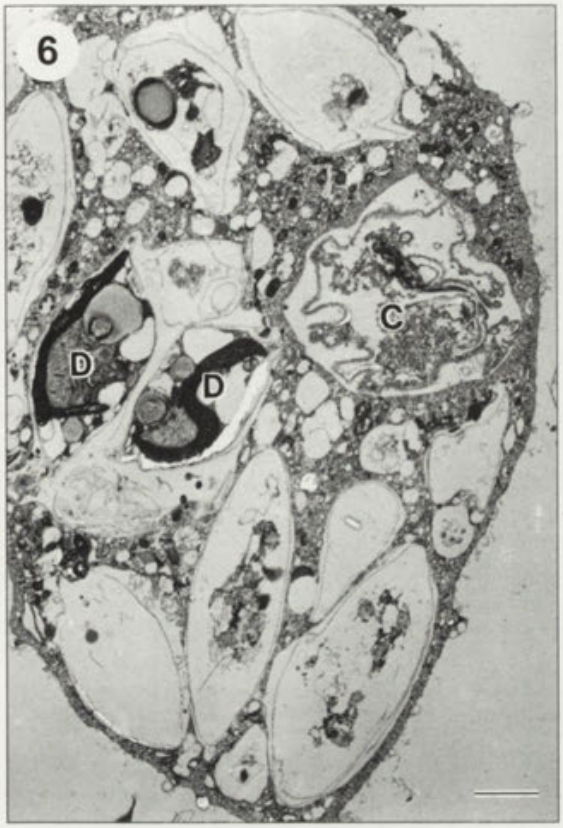
During nuclear division, the MTOC divides *via* typical V-shaped intermediary structures (Fig. 18). Immediately after division of the nucleus, before starting cytokinesis, two MTOCs appear in immediate proximity of the daughter nuclei (Figs. 23, 24).

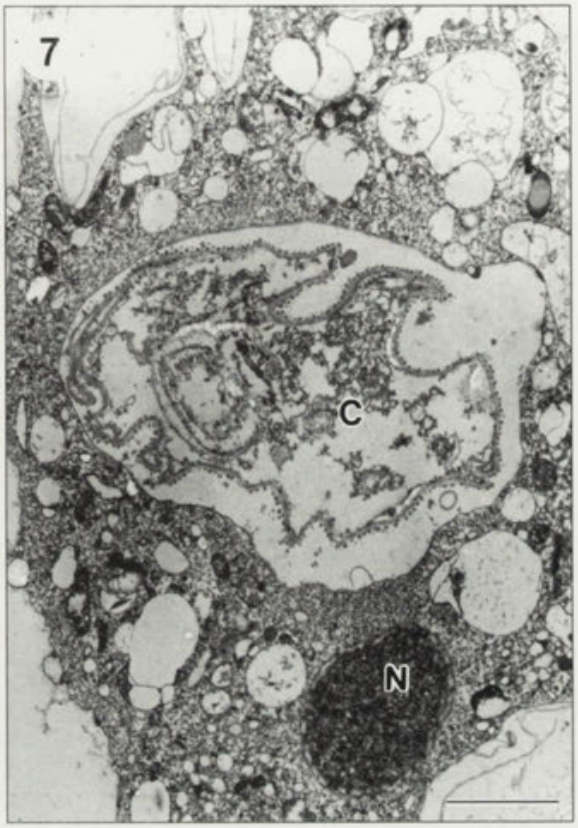


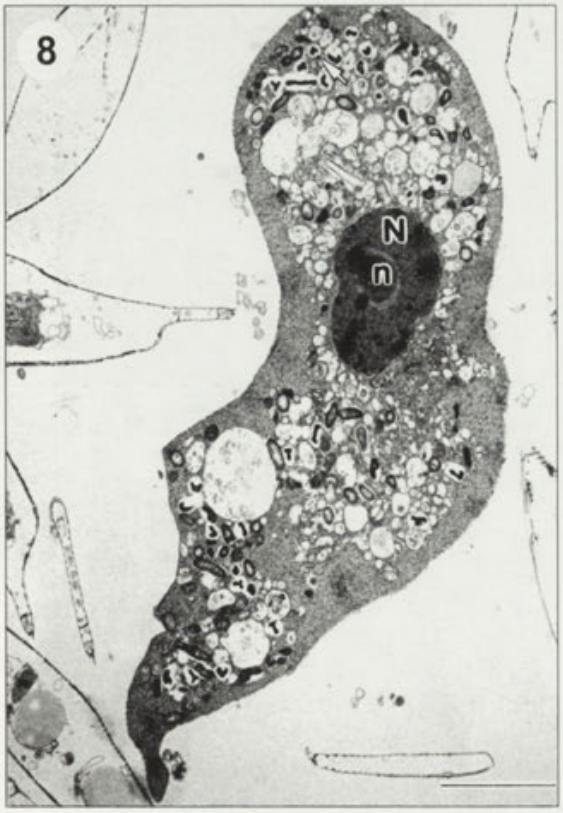
Figs. 1-3. Living *Synamoeba arenaria* (phase contrast images).1 - single cell; 2 - cell with two nucleoli (arrow), indicating a late state of mitosis; 3 - five cells as part of a meroplasmodium. Scale bars - 25 µm



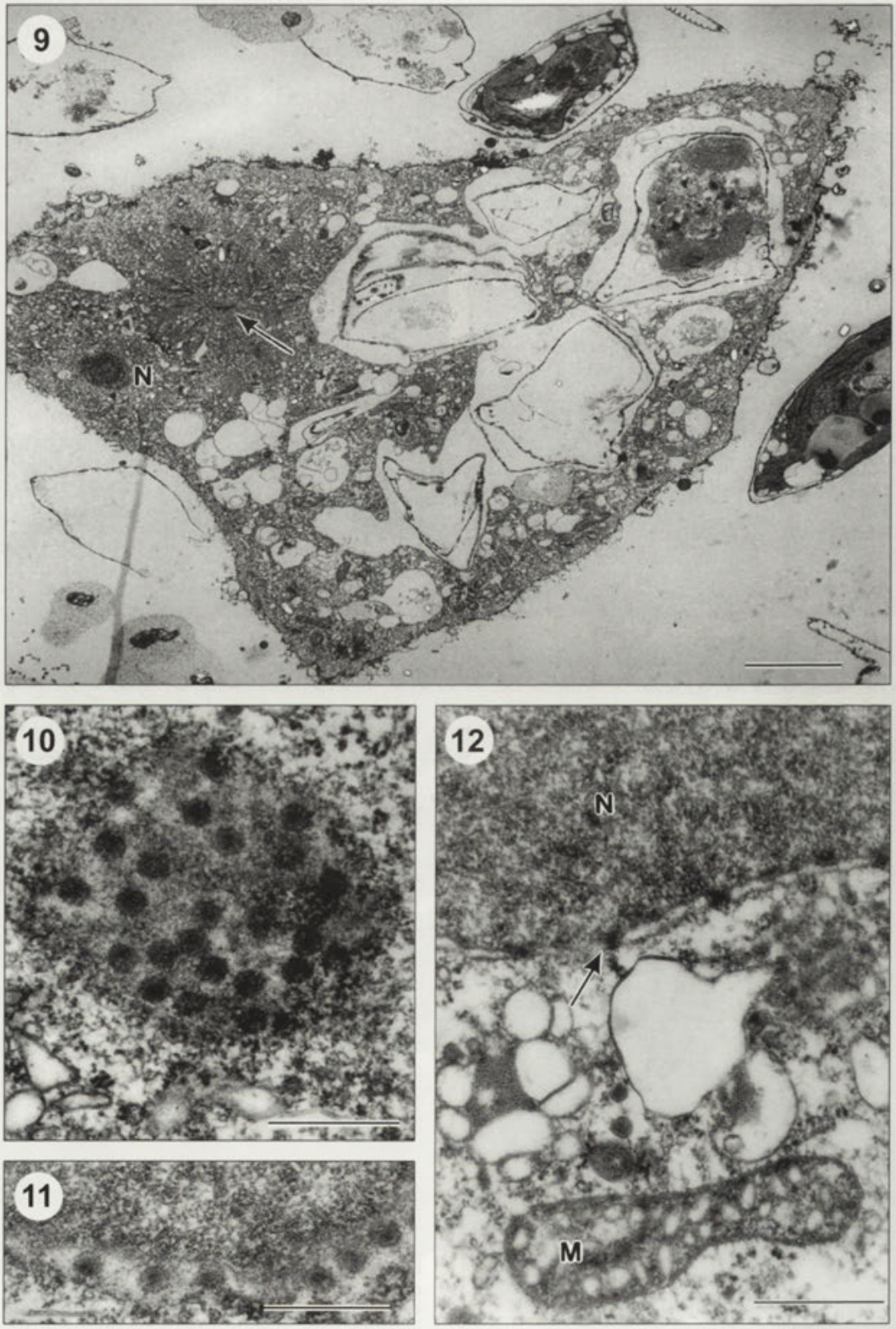
Figs. 4, 5. Nutrition of *Synamoeba arenaria* (scanning electron microscopy). 4 - tip of a reticulopodium catching a diatom as prey, bacteria in the surrounding medium; 5 - remaining shells of diatoms after digestion. Scale bars -1μm. B - bacteria, D - diatom, R - reticulopodium



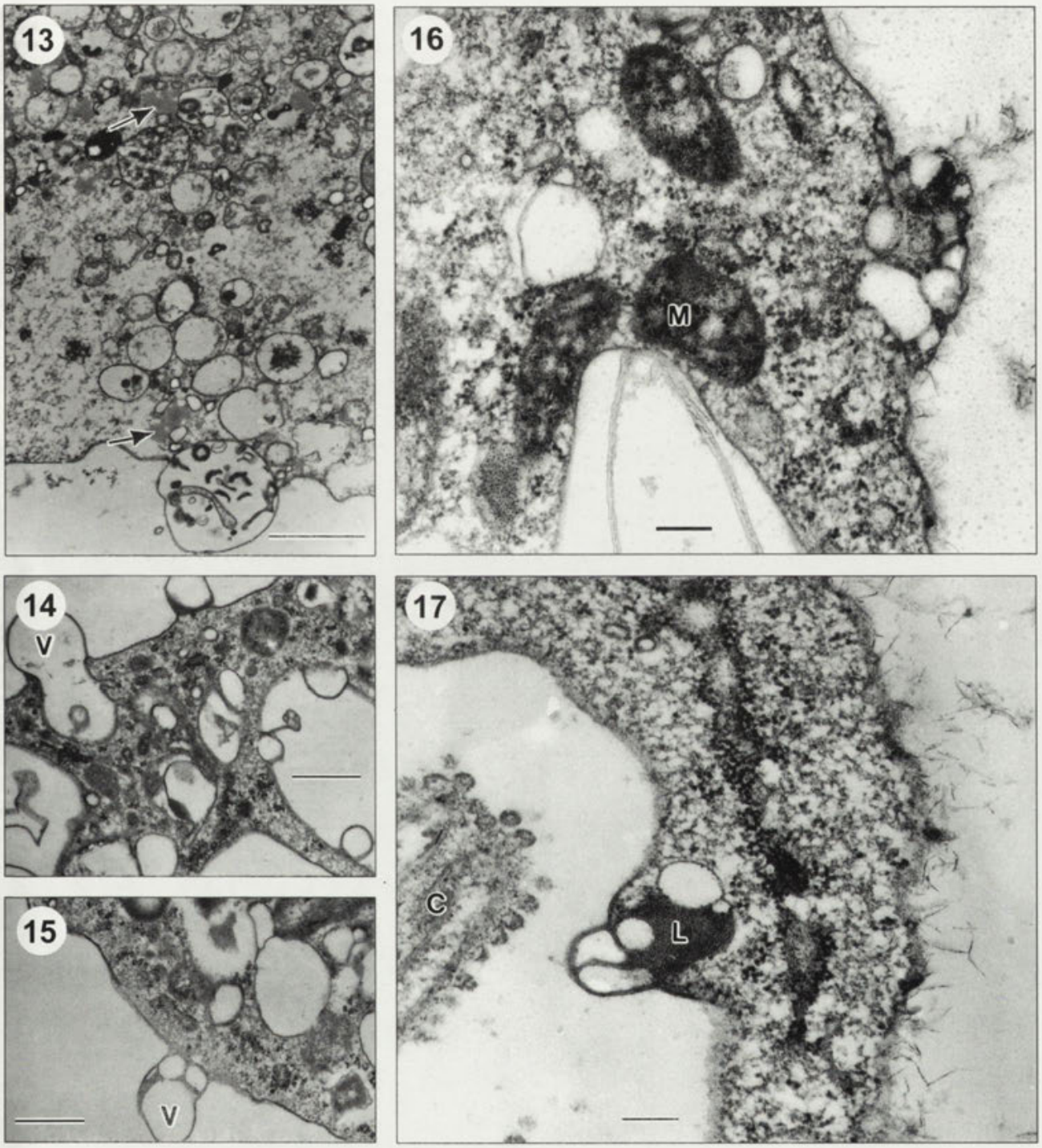




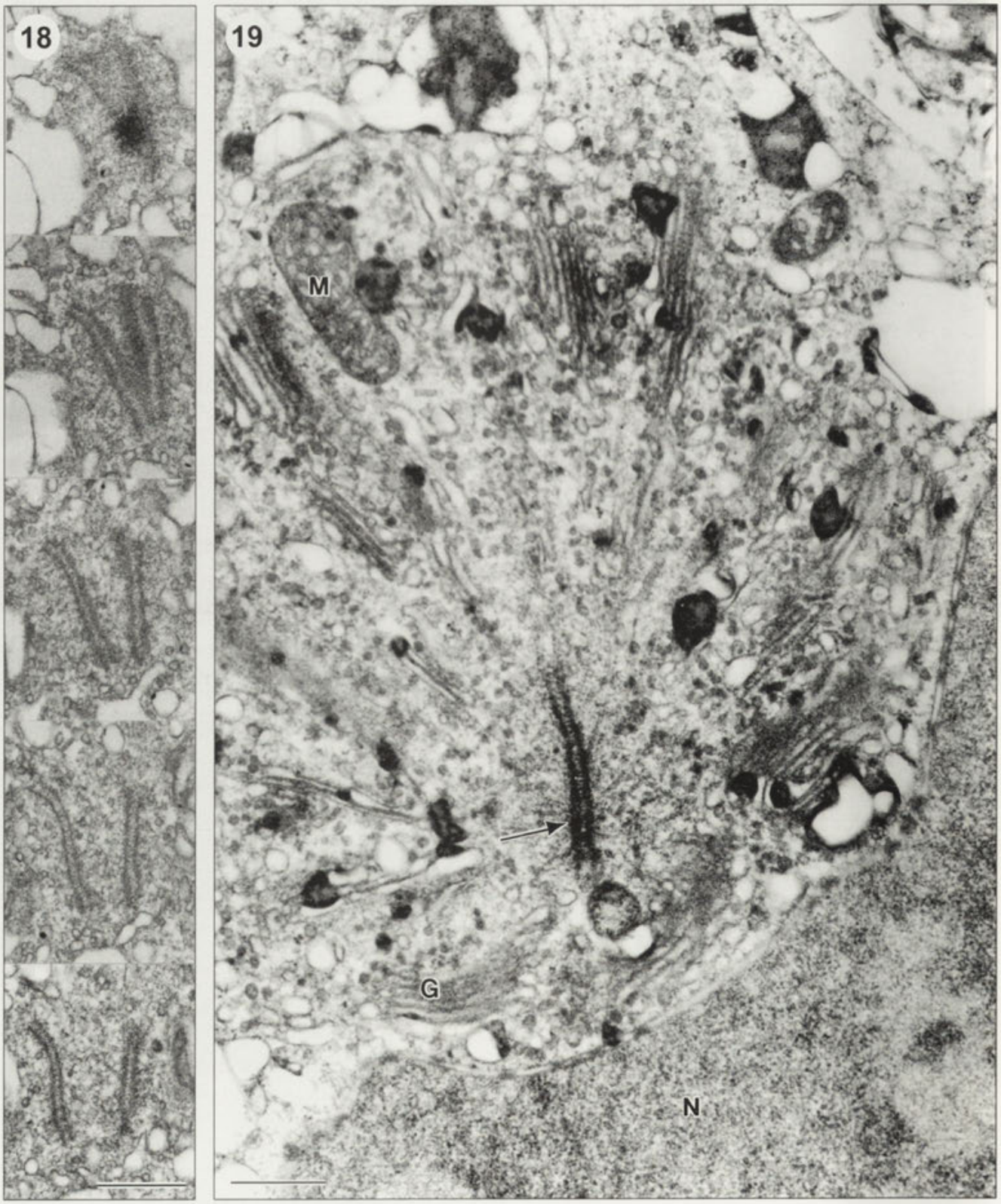
Figs. 6-8. Nutrition of *Synamoeba arenaria* (transmission electron microscopy).6 - vacuoles with diatoms and a ciliate as prey; 7- central vacuole with a ciliate as prey. Scale bars - 2 µm. 8 - starving *Synamoeba arenaria*, vacuoles are much smaller than in Figs. 6,7 and 9 and often contain bacteria (arrow); around the amoeba empty diatom shells can be seen. Scale bars - 5 µm. C - ciliate, D - diatoms, n - nucleolus, N - nucleus



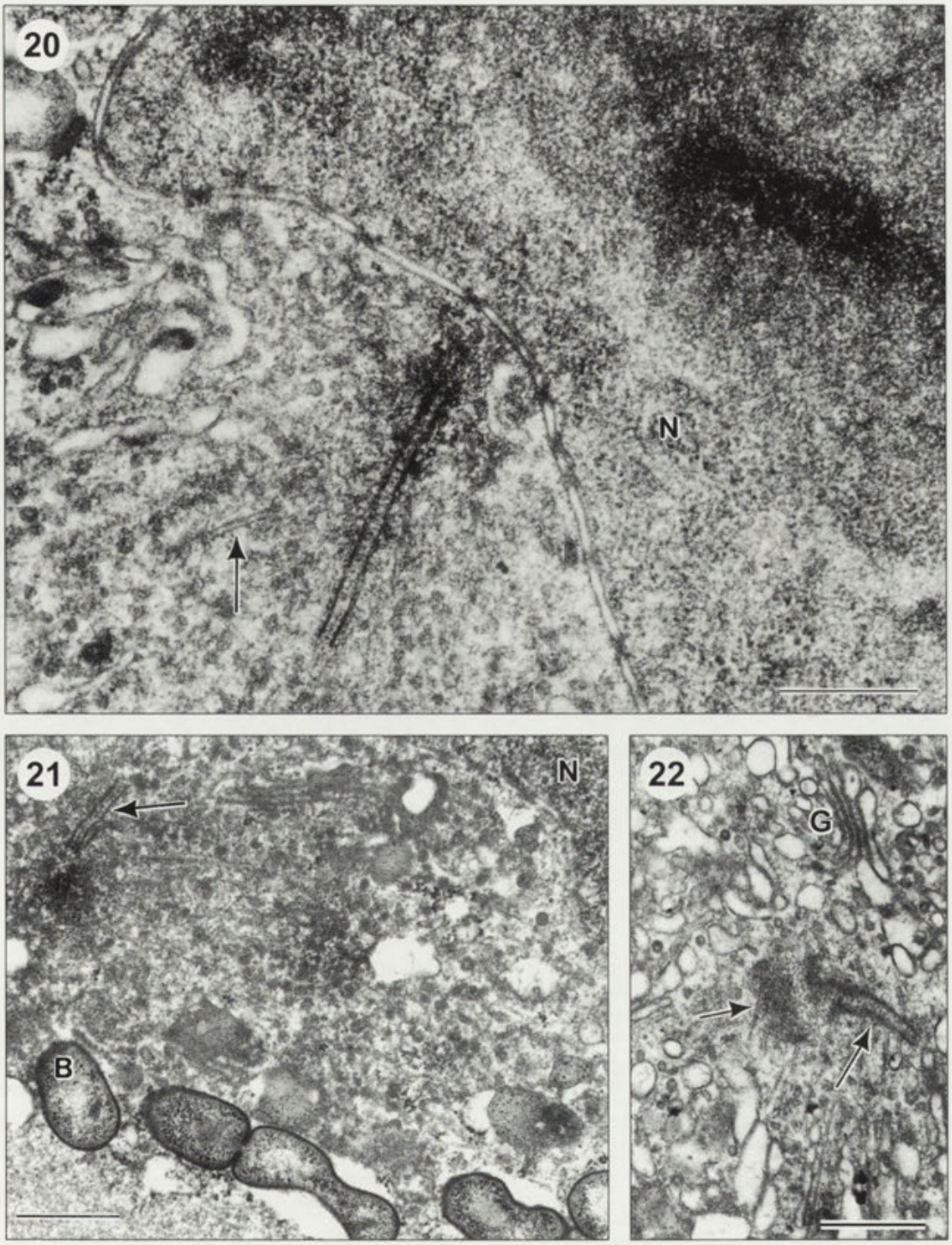
Figs. 9-12. Ultrastructural details of *Synamoeba arenaria*. 9 - interphase; overview, demonstrating the non-central position of the nucleus and the MTOC (arrow); 10 - nucleus, tangentially sectioned with numerous pores; 11 - nuclear membrane, tangentially sectioned with pores; 12 - section through a mitochondrion, nucleus and nuclear pores (arrow). Scale bars - 0,5 µm. M - mitochondrion, N - nucleus



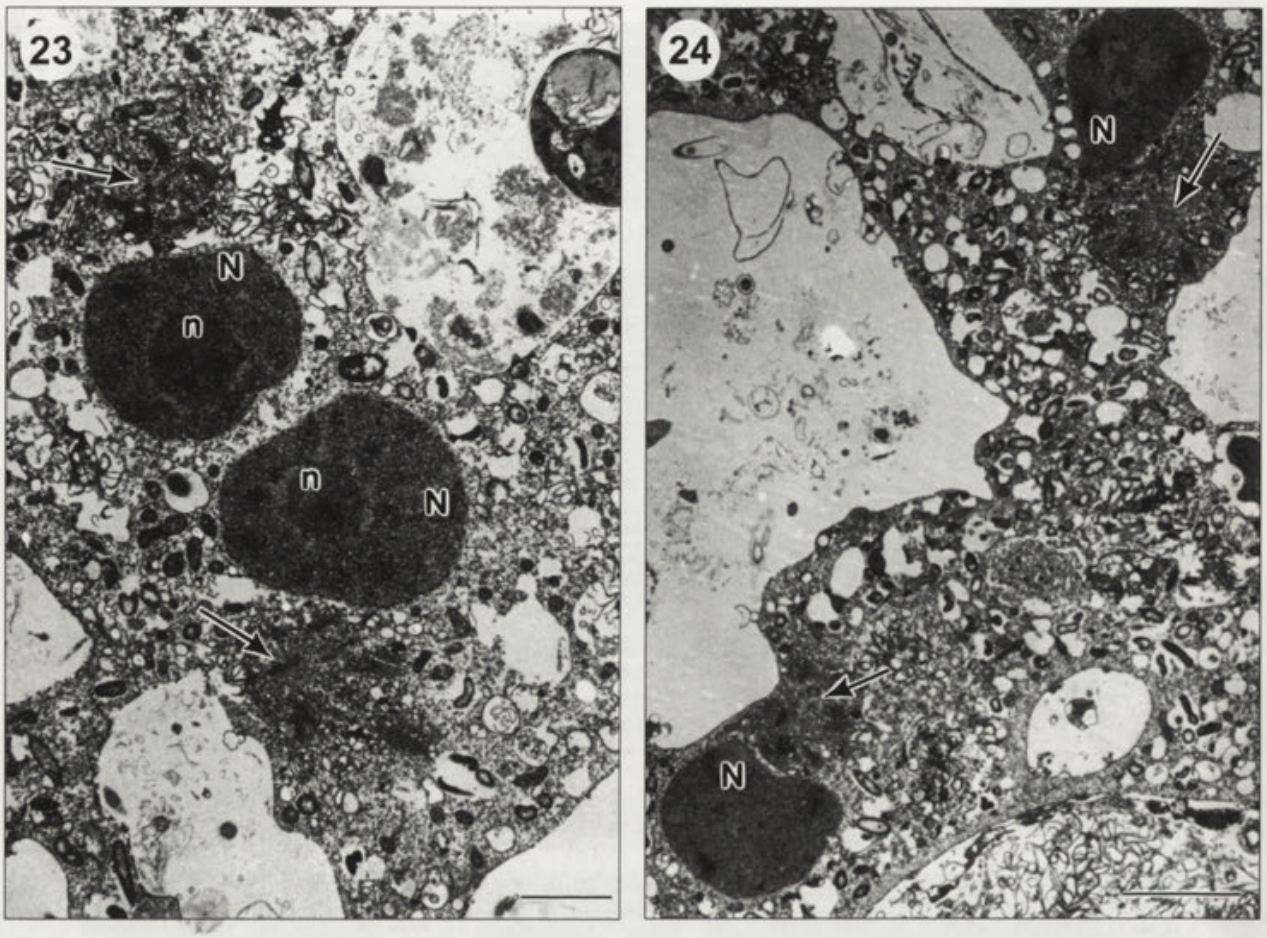
Figs. 13-17. Plasma membrane of *Synamoeba arenaria*. 13 - plasma membrane with a large vesicle fused, containing cytoplasmic material; numerous lipid bodies (arrows) can be seen in the cytoplasm. 14, 15 - smooth plasma membrane; vesicles fused with the plasma membrane. Scale bars - 2 µm. 16 - plasma membrane revealing extracellular deposits. 17 - inner membrane from a vacuole with a ciliate as prey. Scale bars - 100 nm. C - ciliate, L - liposome, M - mitochondria, V - vesicles



Figs. 18,19. MTOC of *Synamoeba arenaria.* 18 - serial section of dividing MTOC; 19 - location of the MTOC (arrow) nearby Golgi dictyosomes and nucleus. Scale bars - 0.5 µm. G - Golgi, M - mitochondrion, N -nucleus



Figs. 20-22. MTOC and nucleus of *Synamoeba arenaria*. 20 - MTOC in direct vicinity of the nucleus, microtubules (arrow) arising from the MTOC, typically associated with granular or filamentous material; 21 - MTOC (arrow) in the vicinity of the nucleus, inclusions of bacteria; 22 - centriole pair (arrows). Scale bars - 0,5 μm. B – bacteria, G - Golgi, N – nucleus



Figs. 23, 24. Dividing cells of *Synamoeba arenaria* with two nuclei at different distances; the MTOCs are marked by arrows. Scale bars - 23 - 2 µm, 24 - 5 µm. N - nucleus, n - nucleolus

DISCUSSION

Protozoa show very different types of mitosis, a fact that causes some difficulties in their phylogenetic classification (Heath 1986, Raikov 1994, Hausmann and Hülsmann 1996, Roos and Guhl 1996, Linder *et al.* 1997).

In the plasmodial rhizopod *S. arenaria,* the MTOCs for the spindle microtubules are situated in the cytoplasm and gain direct access to the chromosomes through the nuclear pores of the nuclear envelope, which does not disappear before starting mitosis (Fig. 20). The MTOCs of *S. arenaria* are always tightly associated with the nuclei. Rather than being a cylinder or a balk as observed in other plasmodial species (Grell and Benwitz 1978, Unger *et al.* 1992, Guhl and Roos 1994), the MTOC of *S. arenaria* is a plaque-like structure, which can be seen from serial sections (Fig.18).

In ultrastructural studies of marine amoebae, Grell and Benwitz (1978) described similar „centrosome complexes" in connection with nuclei in the meroplasmodia *Corallomyxa chattoni* and *Stereomyxa ramosa.* In opposite, *Corallomyxa mutabilis* has no centrosome and *Stereomyxa angulosa* has this structure at any place of the cell (Grell and Benwitz 1978). The centrosome ultrastructure of *Corallomyxa chattoni* and *Synamoeba arenaria* is very similar. Our results on ultrastructure of *S. arenaria* MTOC confirm the phylogenetic relationship to *Corallomyxa chattoni* and *Stereomyxa ramosa,* suggested by Grell (1994, oral information). In addition, there is a certain similarity between the MTOC of *S. arenaria* and centroplast-like MTOCs, which were described for various species of centrohelidian heliozoans (Bardele 1975, 1977). Also the localization of the dictyosomes mainly around the MTOC in *S. arenaria* is similar to that found

in heliozoa (Bardele 1975, 1977). These facts support discussions on phylogenetic relationships between the plasmodial rhizopods and heliozoa.

Acknowledgement. We thank Prof. Dr. Karl G. Grell (Tübingen) for generous gifts of *Synamoeba arenaria*, Ch. Herrmann and S. Hein for valuable technical assistance in electron microscopy.

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ACTA
PROTOZOOLOGICA

Ultrastructural Observations on *Loma acerinae* (Jírovec, 1930) comb. nov. (Phylum Microsporidia)

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Summary. *Glugea acerinae* Jírovec, 1930 is assigned to the genus *Loma* Morrison and Sprague, 1981 as *L. acerinae* (Jírovec, 1930) comb. nov. *L. acerinae* induces xenomas which when mature, have a centrally located hypertrophic nucleus with deeply invaginated nuclear envelope; their plasmalemma produces a finely granular xenoma wall. Parasite nuclei are unpaired throughout the cycle. Elongated or rounded merogonial plasmodia are coated with flat cisternae of host ER. Sporogonial plasmodia cleave within a parasitophorous vacuole (PV) into uninucleate sporoblast mother cells which produce sporoblasts by binary fission. The PV wall does not arise as a product of the sporont surface. Small vesicles containing dense substance move from the xenoma cytoplasm to discharge their contents into the PV space. Thus a granular matrix is produced in which tubular formations appear, unconnected with sporoblast surface. The dense substance and tubules ultimately separate the PV space into separate compartments for individual spores. In meront cytoplasm, microtubules were observed. In sporonts, virus-like particles were detected. The transfer of *Glugea acerinae* into the genus *Loma* is based on features identical with those of the type species, *L. branchialis,* like sporogony taking place in a PV, separation of spores within the PV by dense, tubule-containing matrix and the structure of the xenoma. The position of several other *Loma* species warrants further study.

Key words: *Glugea*, *Gymnocephalus cernuus*, *Loma*, microsporidian ultrastructure, virus-like particles.

Abbreviations: ER - endoplasmic reticulum, G - Golgi aparatus; Gr - granular mass; H - host; Nu - nucleus; Hnu - host cell nucleus; P - parasite; Pg - posterior granule; Pt - polar tube; PV - parasitophorous vacuole, RER - rough endoplasmic reticulum, SP - mitotic spindle plaque, SPV - sporophorous vesicle, VLP - virus-like particles, W - xenoma wall

INTRODUCTION

Several species of microsporidia infecting fish were earlier assigned to the genus *Glugea* Thélohan, 1891 simply because they produced xenomas in host tissues (Canning and Lom, 1986). The use of electron microscope changed the taxonomic position of some of them. Microsporidia inducing formation of small xenomas in the gills of cods and salmonids were transferred to a separate genus *Loma* Morrison and Sprague, 1981 (Morrison and Sprague 1981a, b, c, 1983, Canning and Lom 1986). Morrison and Sprague (1981a) defined *Loma* as xenoma forming, "apansporoblastic, unikaryotic disporoblastic",

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unlike *Glugea* lacking plasmodial stages, with partial development in parasitophorous vacuoles in which they produced 1 or 2 spores only and a certain amount of tubules. Sporogony occurred everywhere in the xenoma. This definition was amended in Canning and Lom (1986) and Larsson (1988) to incorporate findings of plasmodial stages in merogony and sporogony in other *Loma* species; this essential difference was explained (Loubès *et al.* 1984) by incomplete original observations of Morrison and Sprague. The need of an exact definition of *Loma* persists, the more so, that species of this genus were proven to be important pathogens of cultured salmonid fishes (e.g., Kent *et al.* 1989).

One of the former "glugeas" is *Glugea acerinae* Jírovec, 1930 described from a single specimen of ruff, *Gymnocephalus cernuus*, collected in the Elbe River near Mělník in the Czech Republic. The parasite formed up to 350 μm large xenomas located in tunica propria, muscularis and submucosa of the intestinal wall. Jírovec (1930) presented description of developmental stages of the parasite and of the xenoma it produced. Since his original finding, *G. acerinae* has not been found again, until recently, one of us (M.P.) found it quite commonly in ruffs in Finland. This gave us an opportunity to reexamine the generic assignment of *G. acerinae*, to study its ultrastructure and revise the status of *Loma*.

MATERIALS AND METHODS

The fish investigated were caught from small lakes and a river (Kirmustenjärvi, Vähäjarvi and Toivanjoki) in southern Finland. The internal organs were first examined under a binocular microscope to detect the microsporidian xenomas. The organs were then dissected and contents of cysts were spread in a drop of 0.9 % NaCl between a slide and coverslip for identification. Spores in distilled water were closed in glass capillaries and transported to České Budějovice for photography and measurements. Fresh spores were photographed at an exactly determined magnification and measured in enlarged prints (n = 30).

For light microscope preparations, samples fixed in Bouin's fixative were processed with routine paraffin techniques and the sections were stained with Mayer's haematoxylin, chromotrope and fast green.

For transmission electron microscopy, opened intestines were immersed immediately after the fish were caught in 2.5 % glutaraldehyde in phosphate buffer (0.1 M, pH 7.2). During a few hours' fixation smaller pieces were dissected from the intestines. The pieces were washed in the same buffer and brought to the Electron Microscope Unit of the Institute of Biotechnology, University of Helsinki, where they were post-fixed in 1 % OsO_4 and embedded in epoxy resin. The blocks were transported to České Budějovice for sectioning. The sections were double stained in uranyl acetate and lead citrate and observed in the JEM 1010 electron microscope at 60 kV accelerating voltage.

RESULTS

Light microscopy

In addition to their presence in the intestinal wall, xenomas were sometimes also found in the walls of the oesophagus and stomach, in the mesentery, anus, gall bladder, liver and even in the ovary. The spores were elongate ellipsoidal, slightly narrower at the anterior end and their size was 2.19 (1.9-2.9) x 4.64 (4.0-5.1) μm (Fig. 1). The large posterior vacuole, 2.45 (2.0-2.9) μm long, was flanked at both sides by the coils of the polar tube and its vaulted anterior border extended beyond the mid-spore length.

Electron microscopy

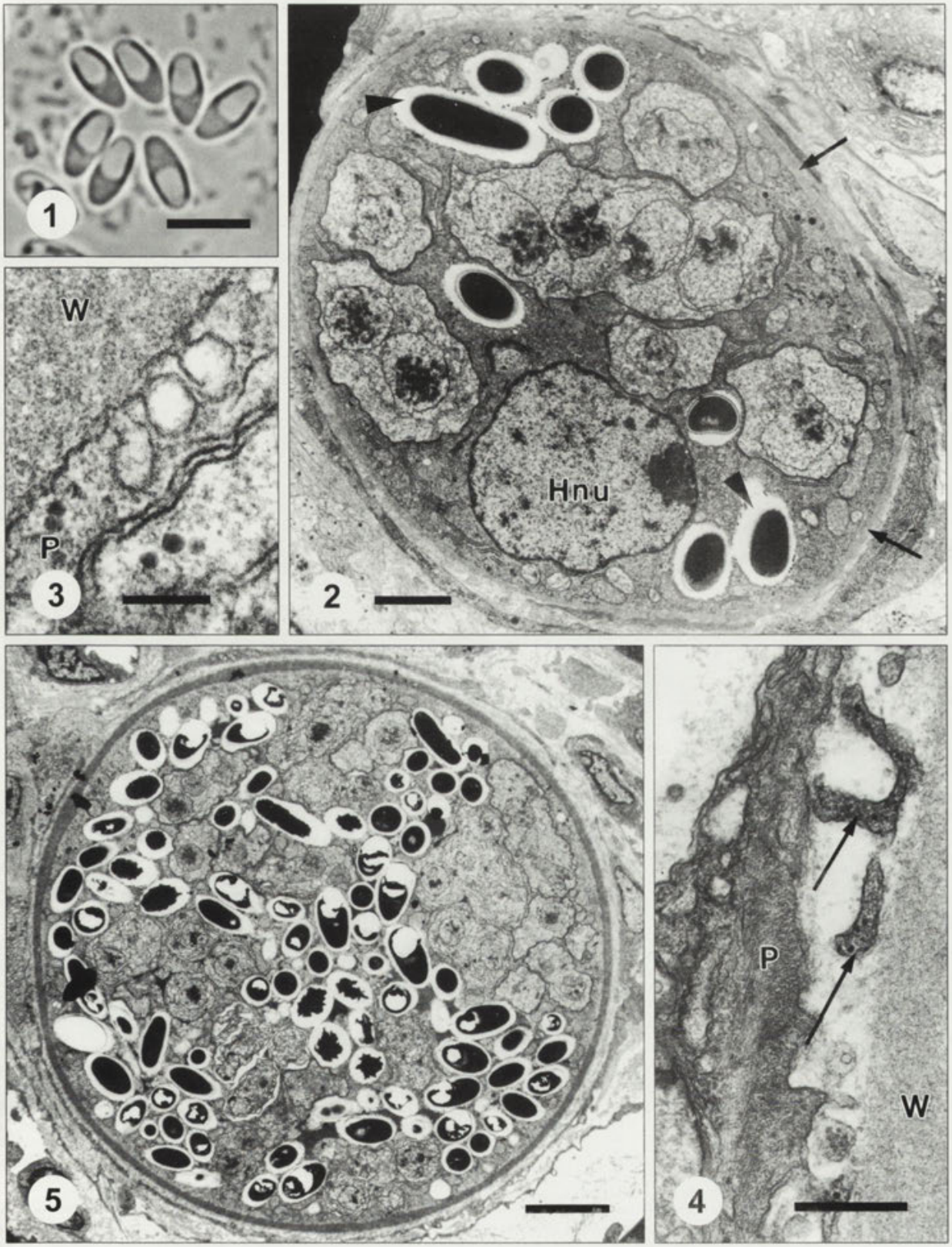
Xenomas

In cross section xenomas were section almost regularly circular. The wall consisted of an amorphous, finely granular layer, up to 300 nm thick in small, initial xenomas (Fig. 2), and slightly thicker in grown xenomas. The substance of the wall was obviously secreted by the plasmalemma of the cell. On the outside, the wall was not distinctly delimited and there were adhering extracellular elements of the host, including collagen fibres and/or extremely flattened fibroblasts. In young xenomas the host cell plasmalemma immediately beneath the wall appeared more or less even while in more advanced xenomas there were numerous pinocytotic-like vesicles which opened into the wall (Fig. 3). These were subtended by a vesicular layer in the cytoplasm. In some of the grown xenomas, plasmalemma extended into numerous irregular processes and the cytoplasm beneath them was full of interwoven microfibrils (Fig. 4).

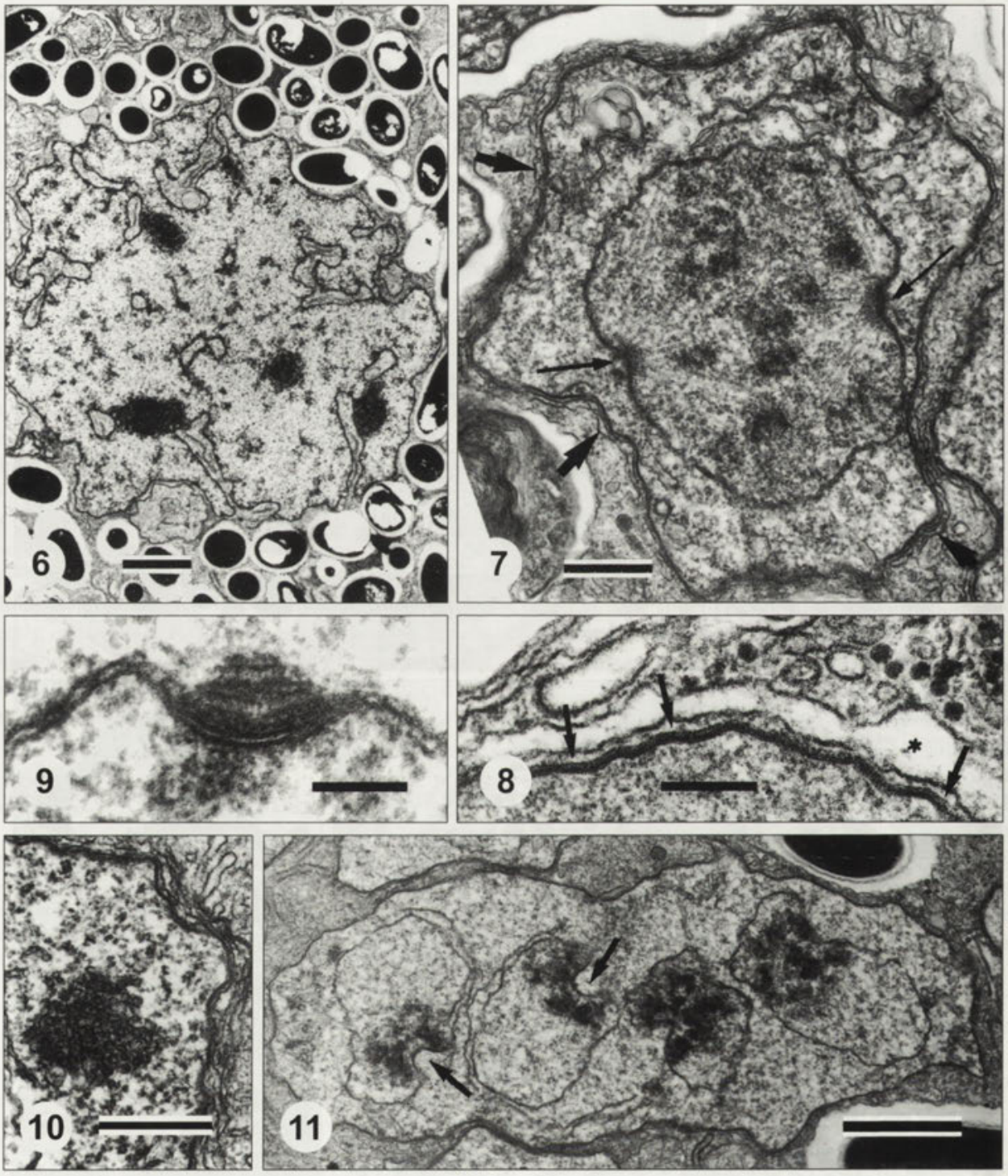
Constituents of the xenoma cytoplasm include a variety of vesicles, RER cisternae often with a rather dense contents, numerous mitochondria, scarce microfibrils and few rather simple Golgi bodies. No annulate membranes were detected.

In early xenomas, the host cell nucleus lay at the side, had compact outline and a persisting nucleolus shifted to one side (Fig. 2). Later on, the highly hypertrophic nucleus assumed a central position, with a fragmented nucleolus and a highly sinuous surface with many invaginations (Fig. 6). There seemed to be very few nuclear envelope pores.

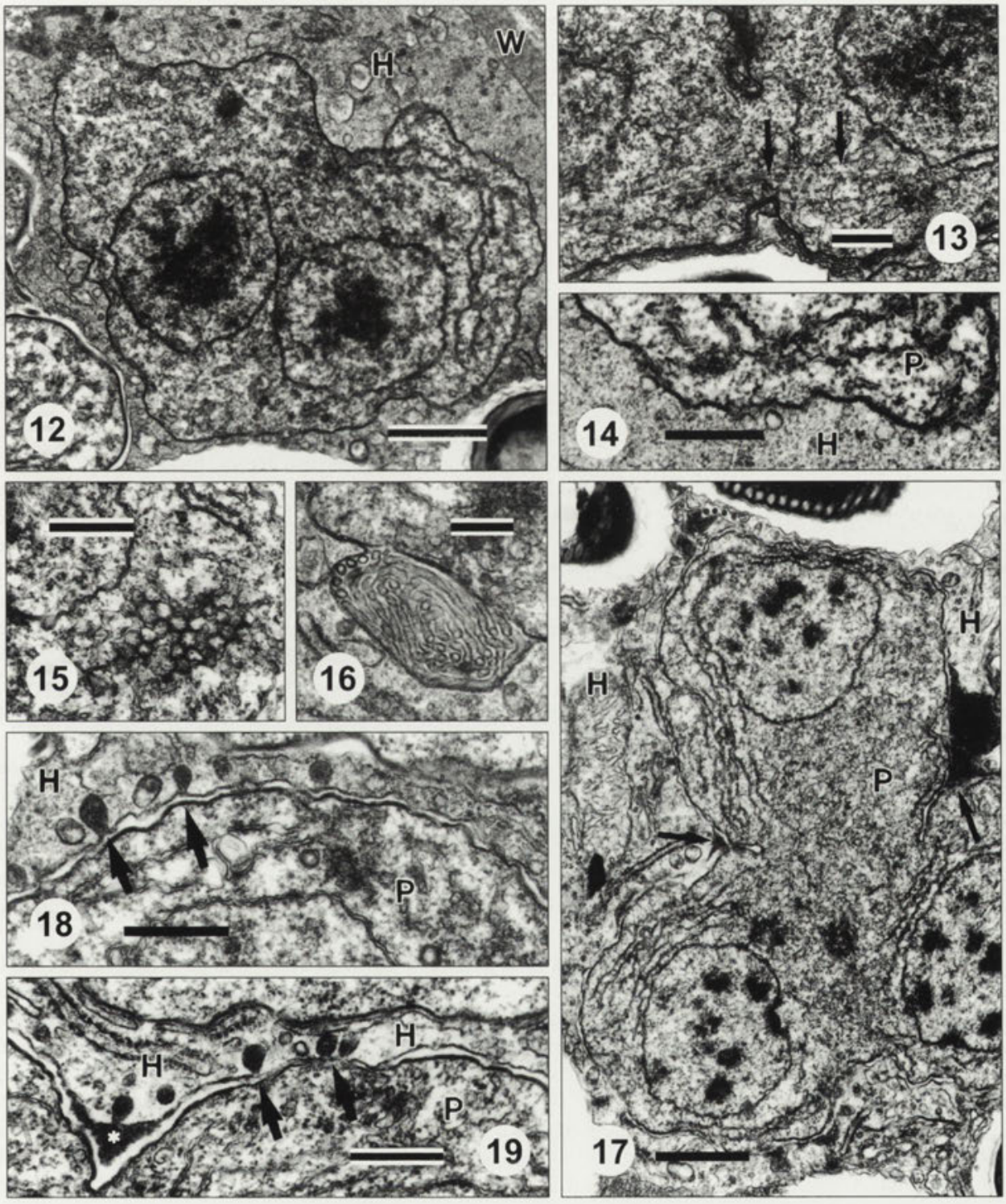
Even in early xenomas, mature spores could be observed (Fig. 2) along with developing meronts, providing



Figs. 1-5. *Loma acerinae*: 1 - fresh spores, scale bar - 5 µm; 2 - section through an early xenoma with its nucleus and spores (arrowheads) in separate compartments, arrows point at the xenoma wall, scale bar - 2 µm; 3 - vesicles opening in the xenoma cell membrane and discharging into the xenoma wall, scale bar - 150 nm; 4 - periphery of a grown xenoma with cytoplasmic projections (arrows), scale bar - 0.5 µm; 5 - grown xenoma, with host cell nucleus beyond the plane of section, scale bar - 5 µm. Hnu - host cell nucleus, P - parasite, W - xenoma wall



Figs. 6-11. *Loma acerinae*: 6 - hypertrophic nucleus of the host cell, scale bar - 3 µm; 7 - meront; in the plane of section one dividing nucleus. Big arrows point at the ER cisternae cover, arrows at the mitotic centers, scale bar - 0.5 µm; 8 - meront cell wall with glycocalyx coat; arrows point at the thin host cytoplasm layer separating the host ER cisterna (*), scale bar - 150 nm; 9 - the mitotic spindle plaque, scale bar - 150 nm; 10 - agglomeration of small vesicles in meront cytoplasm, scale bar - 0.5 µm; 11 - elongate, multinucleate meront, arrows point at depressions in nuclear envelopes harbouring spindle plaques, scale bar - 250 nm



Figs. 12-19. *Loma acerinae*: 12 - early sporont in direct contact with host cell cytoplasm, scale bar - 1 μm; 13 - part of meront cytoplasm with cytoplasmic microtubules (arrrows), scale bar - 0.5 μm; 14 - part of early sporont surface devoid of ER cover, scale bar - 0.5 μm; 15 - group of small vesicles, early form of the Golgi system (?), scale bar - 0.5 μm; 16 - paramural body wedged into the sporont cell wall, scale bar - 250 nm; 17 - sporont cleaving into sporoblast mother cells with granular material (arrows) wedged into the separation furrows, scale bar - 1 μm; 18, 19 - vesicles with dense contents (arrows) discharging into the SPV space and building a mass of granular substance (*), scale bar - 0.5 μm. H - host, P - parasite, W - xenoma wall

evidence for the absence of synchrony in parasite development. In grown xenomas, mature spores and developmental stages were found throughout the cytoplasm (Fig. 5). In their final stage, xenomas only contained mature spores.

Merogony

Merogony stages were always in direct contact with host cell cytoplasm. At a distance of about 10-15 nm from the meront surface extended a discontinuous layer of extremely flat cisternae of endoplasmic reticulum (Fig. 7). Meront plasmalemma was covered by a 9-10 nm thin (Fig. 8) dense layer. Meronts ranged from uninucleate ones, which were rather compact or stellate (Fig. 7), to multinucleate ones of elongate (Fig. 11) or rounded shape with up to 5 nuclei seen in one plane of section. Signs of nuclear division were common (Fig. 7).

The spindle microtubules were as a rule attached to chromosome kinetochores (Fig. 7) while some of them extended free across the nuclear space to the opposite side of the nuclear envelope. The mitotic spindle plaque (SP) lay on the outside of the nuclear envelope in a small depression, which became quite deep in the last stage of mitosis; it consisted of three double discs interconnected by microtubules (Fig. 9). There were no polar vesicles associated with the SP. The same pattern of mitotic division occurred during sporogony.

In the cytoplasm were numerous free ribosomes, various vesicles, cisternae of endoplasmic reticulum, mostly small, occasionally forming loose stacks of up to 3 flat cisternae and rarely a small whorl (Fig. 11), microtubules situated free in the cytoplasm, at a distance from the nucleus (Fig. 13) and, rarely, tightly packed groups of minute vesicles (Fig. 10).

Sporogony

Sporonts were represented by multinucleate sporogonial plasmodia which obviously originated by transformation of multinucleate meronts. They were differentiated from meronts by the absence of flat cisternae of ER on their surface and by the gradually increasing thickness of the dense layer coating the plasmalemma. There were some stages which might be interpreted as early sporonts which had lost the host ER coat, yet still lay in direct contact with host cytoplasm (Figs.12, 14). Later, a gap appeared between the sporont and the host cytoplasm to expand further and become the sporogony vacuole. The membrane lining this vacuole - in spite of meticulous scrutiny - was not observed to arise from the sporont surface by blister formation or by delamination and thus seemed to be completely of host cell origin. Consequently, the sporogony vacuole is not a sporophorous vesicle (SPV) but a parasitophorous vacuole (PV).

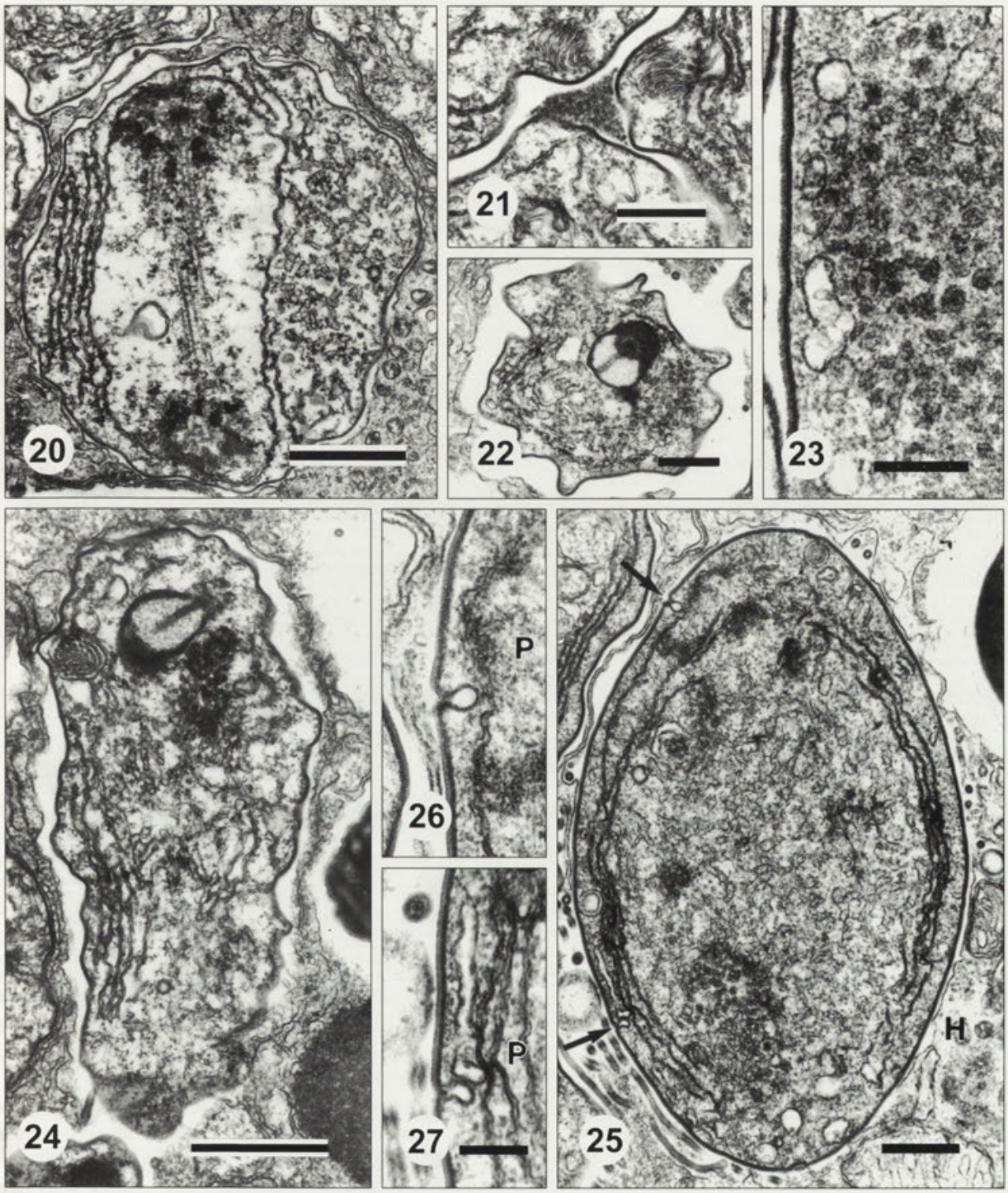
The flat ER cisternae became more numerous in the sporont cytoplasm and slightly more inflated. Groups of vesicles appeared - possibly early stages of the Golgi system ? - and these persisted in the later stages of sporoblast mother cells (Fig. 15) and meshworks of profiles with dense contents. While the sporont was still undivided, structures resembling paramural bodies appeared. In addition to their prevailing position beneath the cell membrane, some lay within the cavity of the split sporont wall (Fig. 16) while others appeared to lie externally to the sporont, as balls of twirling tubules.

Sporonts divided by segmentation (Fig. 17), with nuclear mitoses commonly seen. At the cleavage furrows appeared wedges of a dense, finely granular substance. There were tiny vesicles with more or less dense contents in the host cytoplasm (Fig. 18) . The vesicles appeared to approach the PV membrane and to fuse with the it and to discharge their contents into the PV space (Fig. 19). The dense contents seemed to be the source of the granular matrix which gradually developed into massive septa separating products of sporont division within the PV.

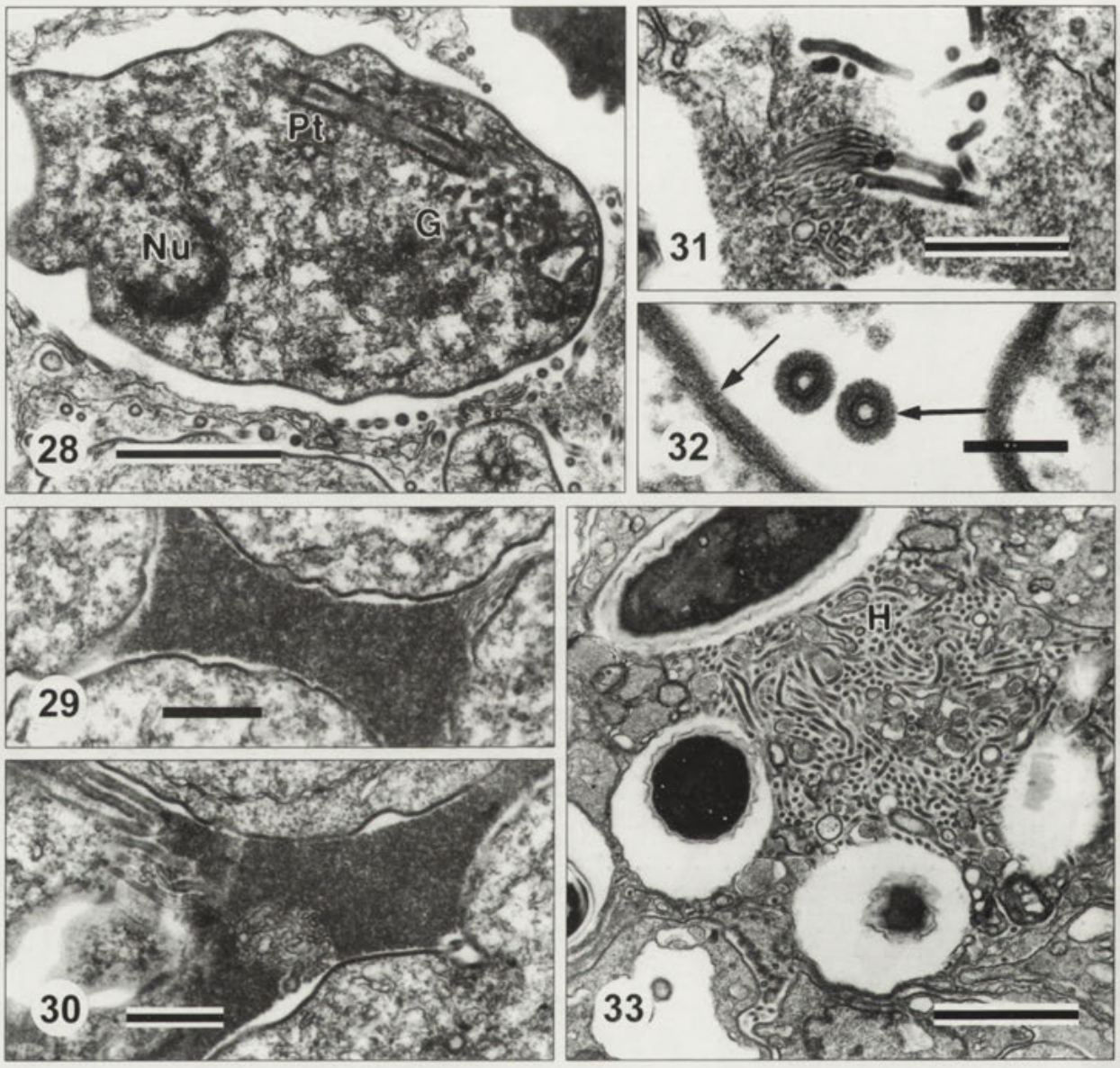
In some of the sporonts, virus-like particles (Fig. 23) of the size of about 50 nm were irregularly distributed, forming no paracrystalline patterns.

The more or less rounded products of the sporont segmentation engaged in binary fission (mitosis might already have been started during segmentation). We consider these cells to be sporoblast mother cells. The last stages of their division showed an elongate nucleus extending all across the cell (Fig. 20). No indication of a meiotic process was observed.

The newly formed sporoblasts still harboured paramural bodies beneath their cell membrane (Fig. 21). The bodies persisted at least until the start of polar tube formation (Fig. 24). The sporoblast wall, mostly crumpled due to fixation, grew thicker up to about 30 nm across. The gap between sporoblast wall and the wedged granular material grew gradually wider. In the sporoblast cytoplasm, a meshwork of tubule-like cisternae with either lucent or dense contents, interpreted generally as primitive Golgi apparatus, could be seen close to structures signalling the start of polar tube formation (Figs. 22, 24). Quite rarely, early sporoblasts kept a smooth ellipsoidal shape (Fig. 25), while the wall showed invaginations suggestive of uptake of external material (Figs. 26, 27). In both early and advanced sporoblasts, nuclei displayed an eccentric condensation of chromatin (Fig. 28) reminiscent of the



Figs. 20-27. *Loma acerinae*: 20 - dividing nucleus of a sporoblast mother cell, scale bar - 1 μm; 21 - early sporoblasts with interposed granular substance inbetween and with paramural bodies at their periphery, scale bar - 0.5 μm; 22 - apex of a sporoblast with early stage of polar sac and polar tube, scale bar - 0.5 μm; 23 - virus-like particles in sporont cytoplasm, scale bar - 200 nm; 24 - sporoblast, scale bar - 1 μm; 25 - anomalous sporoblast with micropore-like openings in its cell wall (arows), scale bar - 0.5 μm; 26, 27 - enlarged cell wall openings of the former, scale bar - 200 nm. H - host, P - parasite

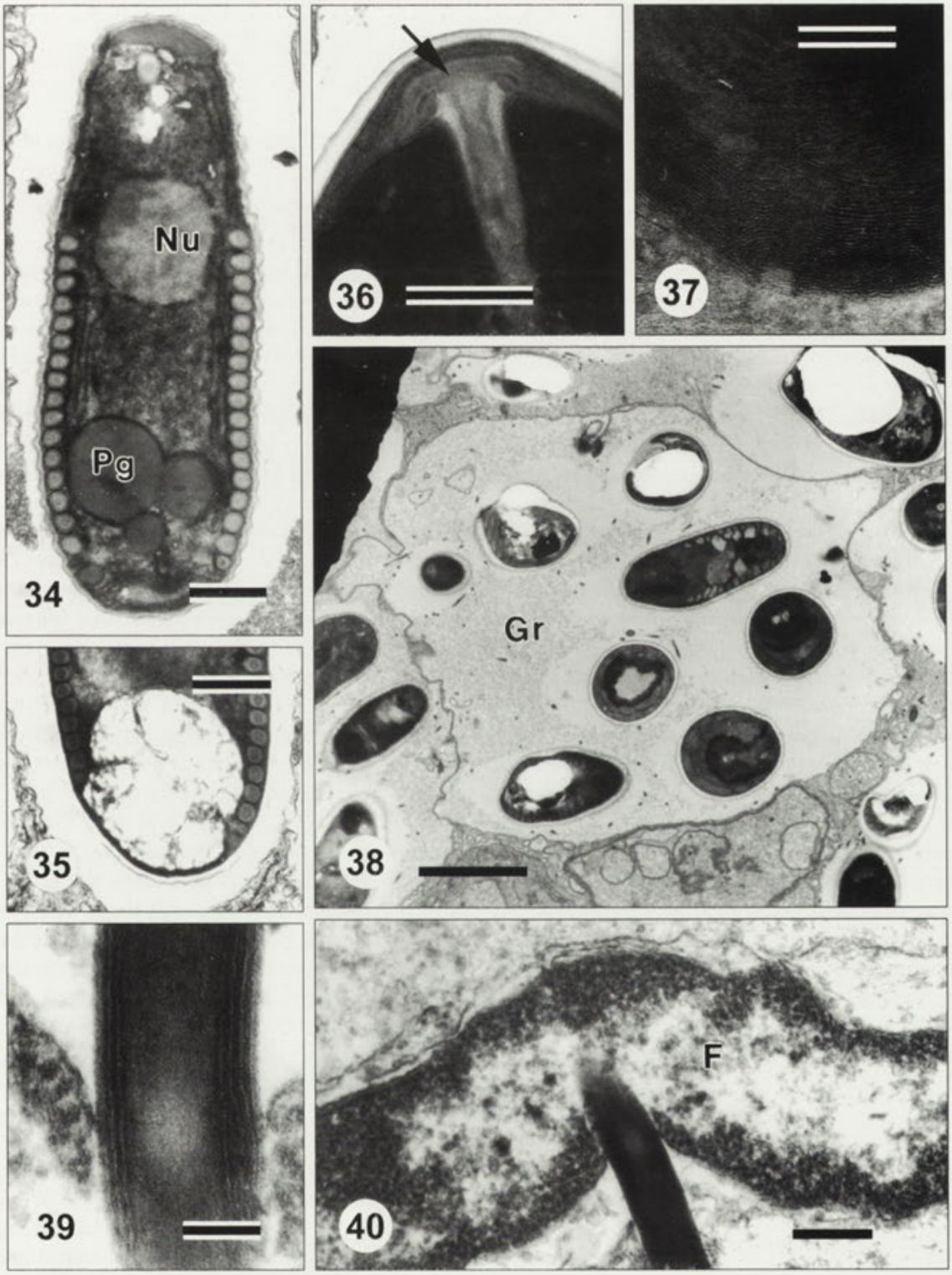


Figs. 28-33. *Loma acerinae*: 28 - oblique section of a sporoblast, scale bar - 1 µm; 29, 30 - granular substance interposed between early sporoblasts from which tubules appear to originate, scale bars - 0.5 µm; 31 - tubules of different diameter, some with clubbed ends, scale bar - 0.5 µm; 32 - transverse sections of two tubules; arrows point at the glycocalyx at their and the sporoblast's surface, scale bar - 100 nm; 33 - mass of tubules in the substance separating the spore compartments, scale bar - 1 µm. G - Golgi, H - host, Nu - nucleus, Pt - polar tube

telophase stage of the sporoblast mother cell mitosis. The polar tube formation followed the pattern known in other microsporidia, i.e., from a bulbous primordium (Figs. 22, 24) with rudiments of the future polar sac grew the primordial polar tube (Fig. 28).

During the separation of early sporoblasts by the accruing interposed granular substance there appeared tubular structures which gradually increased in number in the septa between the sporoblasts. They did not seem to be produced by the parasite cell directly but rather originated in association with the granular substance (Figs. 29, 30). They were of different diameter (Fig. 31), the narrower ones perhaps developing into the typical wider ones terminated mostly by a bulbous end. They had a diameter of 60-65 nm (Fig. 32) and on transverse section showed a tube of unit membrane coated with dense material. The tubules, straight or very tortuous, sometimes occupied considerable space between the sporoblasts (Fig. 33).

Developing spores resided already inside distinct separate compartments built of membranes and granular substance. Inside their single nucleus of rounded or



Figs. 34-40. *Loma acerinae*: 34 - almost mature spore within its own compartment and with dense posterior globules, scale bar - 0.5 µm; 35 - vacuole in the posterior end of the spore, scale bar - 0.5 µm; 36 - apex of the spore with polaroplast, polar sac with anchoring disc (arrow), scale bar - 200 nm; 37 - lamellar part of the polaroplast, scale bar - 250 nm; 38 - sporophorous vesicle space with granular mass- separating the spores - turned pale, scale bar - 2 µm; 39 - incompletely extruded polar tube with multiple walls, scale bar - 100 nm; 40 - polar tube passing through the nucleus of a fibroblast (F) adjacent to the xenoma, scale bar - 250 nm. Gr - granular mass, Nu - nucleus, Pg - posterior granule

irregular shape the chromatin appeared deposited in hazy flakes. In the posterior part of the spore appeared one to three dense homogeneous vacuoles (Fig. 34) to be replaced eventually in the mature spore by the empty space of the posterior vacuole (Fig. 35).

In mature spores, the anchoring disc with basal part of the tube lay slightly laterally. The polar sac embraced the cup-shaped mass of the anterior lamellar part of the polaroplast (Fig. 36). Its lamellae, about 18-20 nm thick (Fig. 37) abutted with their thickened ends on the basal part of the tube. The posterior part of the polaroplast was composed of rather indistinct vesicles. The dense sporoplasm had abundant polyribosomes. The polar tube was almost isofilar - only the last turn (Fig. 35) abruptly tapered to half the diameter of the preceding ones - and was deposited in a single coil of 11 to 23 turns, the mode being 12 and average number 13.6. In aberrant spores, there might be a double row of turns or the turns could be fused together. In cross sections, the tube revealed the usual concentric lucent and dense layers. The exospore was about 13 nm, the endospore about 70 nm thick.

In grown xenomas, mature spores occupied most of their volume. Some of the spores still preserved their separate compartments, the majority, however, were situated in large common vacuoles with distinct limiting membranes, while the granular substance between spore became pale as if dissolving (Fig. 38); the tubules still persisted. There were always some developmental stages left; most of them, however, displayed signs of abortive development.

In mature xenomas, discharged polar tubes were frequently observed. Mostly, however, the discharge did not seem to be complete which perhaps may explain the absence of secondary xenomas in the vicinity of the grown ones. Other evidence of incomplete discharge was seen in the multiple walls of the tubes (Fig. 39). Discharged tubes were observed piercing the xenoma wall and nuclei of the apposed fibroblasts (Fig. 40).

DISCUSSION

Identity with *Glugea acerinae*

The parasite described above is identical with *Glugea acerinae*. We did not observe the large lobose plasmodial cells ("schizonts") with more than one hundred peripheral nuclei reported by Jírovec (1930). He was limited by light microscopy which may perhaps explain the difference; however, in all other respects, including spore size, the identity could be confirmed.

Ultrastructural comparison with species of the genus *Loma*

Xenoma structure

The structural features of the xenoma reveal features typical of the genus *Loma*. All *Loma* species - unlike *Glugea* - produce rather small xenomas, with eventually centrally located hypertrophic host cell nucleus, with developmental stages intermingled with spores throughout the xenoma. The xenoma wall consists invariably of an amorphous, finely granular substance. There is some variation in the thickness of the granular wall in *Loma* spp. and in the presence or absence of deep invaginations of the xenoma plasmalemma, present in *L. branchialis* (= *L. morhua*) (Morrison and Sprague 1981c) in *L. diplodae* (Bekhti and Bouix 1985a); and in *L. acerinae* (this paper). On the other hand, the *Glugea* species examined thus far ultrastructurally all reveal laminar structure of the xenoma wall.

Although the significance of xenoma structure for the generic assignment used to be put constantly in doubt, referring to the possibility that it may reflect character of the host cell rather than that of the parasite. It has not been disproved, however, that the type of xenoma can really depend on the nature of the parasite. First, it is quite possible, that in genera like *Glugea* and *Loma*, the cell turned into xenoma is supposedly the same cell of the fish host, neutrophile granulocyte (Bekhti and Bouix 1985b); the granulocyte may be the same while the parasite differs. In an other group of protists, in the genus *Sarcocystis*, the structure of the cyst, in fact a special type of xenoma, does serve for a definite determination of the parasite, although not at the generic but at the species level. In the same skeletal muscle cells, different *Sarcocystis* species produce cysts of different structure: e.g., in pigs *S. miescheriana vs. S. suihominis*; in sheep, *S. arieticanis vs. S. tenella* and in cattle, *S. cruzi vs. S. hirsuta vs. S. hominis* - see Eckert *et al.* (1992). Thus the importance of xenoma structure should not be underestimated.

Merogony

Morrison and Sprague (1981a, c; 1983) failed to see plasmodial meronts in the type species *L. branchialis* (= *L. morhua*), as well as in *L. fontinalis* and *L. salmonae*.

However, Bekhti (1984) and Bekhti and Bouix (1985a) did observe in *L. salmonae* multinucleate merogonial plasmodia. In *L. dimorpha*, meronts were not sufficiently studied and only uninucleate ones were recorded with certainty (Loubès *et al.* 1984). In all other species, plasmodial meronts occur. Meronts of *L. diplodae* (see Bekhti and Bouix 1985a) and of *L. acerinae* are covered by flat ER cisternae. In *L. boopsi*, the cisternae seem sometimes to be present (see Faye *et al.* 1995), while they are absent in *L. fontinalis* (see Morrison and Sprague 1983). In other species they have not been studied enough. Flat cisternae of ER typically envelop meronts in the genus *Glugea* (e.g., Berrebi 1979; Morrison *et al.* 1985).

Sporogony

Plasmodial sporonts were not observed in *L. branchialis* (=*L. morhua*), *L. fontinalis*, *L. salmonae* by Morrison and Sprague (1981a, b, c; 1983). Disporoblasty claimed by these authors for *L. salmonae* was disproved by Bekhti (1984) who found four spores in the sporogony vacuole. In *L. diplodae* the sporont only has been observed to have two nuclei (Bekhti and Bouix 1985a) while in other *Loma* species sporogonic plasmodia occur. Paramural bodies have only been observed in *L. boopsi* and *L. acerinae*. Sporoblast mother cells which were easily identified with those defined in *Glugea anomala* or *G. pimephales* (Canning *et al.* 1982 and Morrison *et al.* 1985, respectively) have thus far only been recorded in *L. acerinae*. However, one cannot escape the feeling that in most papers sporogony stages have not been examined thoroughly enough.

This certainly applies to the way in which the membrane delimiting the sporogony vacuole - PV or SPV - in *Loma* species arises; the origin of this membrane is not unambiguously known in several species. In one- or two-spore containing PVs, however, like the species *L. branchialis*, it is supposedly of host origin. In *L. fontinalis* the PV membrane was said to originate around sporoblasts by coalescence of host cell vesicles, also forming the tubules in the PV space (Morrison and Sprague 1981c). In other species, a SPV wall was said to be formed from blisters at the surface of the parasite cell origin, in *L. diplodae* (Bekhti and Bouix 1985a), *L. camerounensis* (Fomena *et al.* 1992), which in *L. boopsi* (Faye *et al.* 1995) proceeds in a way typical of *Glugea* (Canning *et al.* 1982). In *L. dimorpha*, the boundary of the sporogony vacuole (PV? SPV?) is perhaps formed by delamination of the sporont wall (Loubès *et al.* 1984). In *L. acerinae*, no participation of the sporont wall in formation of the sporogony vacuole membrane could be detected.

Thus within the genus *Loma* we face the problem of not well defined origin of the sporogony vacuole wall, which also exists in many other genera, e.g., *Cystosporogenes* - see Cali and El Garhy (1991) and their discussion on the sources from which the sporogony vacuole wall may be derived.

Tubular appendages associated in the episporontal space with the surface of sporogonic stages are common in many microsporidian genera (Larsson 1986), having sometimes a quite complicated structure (Larsson 1995). They have also been reported in *Glugea atherinae* (Berrebi 1979), *G. anomala* (Bekhti 1984) and *G. stephani* (Takvorian and Cali, 1983) - in all three species they originate,however, as true sporoblast appendages. In *G. stephani*, there are even three types of tubular appendages, all being produced by the sporoblast plasmalemma. However, this is at variance with the tubules of *Loma*, which do not arise as appendages of sporonts or sporoblasts. In *L. fontinalis* they originate allegedly from the host cell cytoplasmic vesicles (Morrison and Sprague 1981c) and connect spores with the host cell membrane of the PV. Similarly, in *L. dimorpha* the tubules also are attached to spore surface (Loubes *et al.*, 1984). In other species the tubules evidently arise within the granular substance in the SPV, including *L. embiotocia* (see Shaw *et al.* 1997). In view of the obvious origin of this substance from the host cell (see below), they are also not of parasite origin. Their structure has been shown thus far in more detail only in *L. camerounensis* (Fomena *et al.* 1992); according to published micrographs they have a diameter of about 50 nm and show a structure similar to that in *L. acerinae*.

It is interesting to remember that tubular appendages also occur in meronts of *G. atherinae* (Berrebi 1979).

The granular matrix which accumulates between the sporogony stages in *L. acerinae* divides eventually the space of the sporogony vacuole, which we have to term PV, into separate compartments for individual spores (see also Awakura *et al.* 1982). Transport of this material across the PV membrane indicates that it is not an inert product of the sporont surface. Fusing of the membrane with the vesicles coming from host cytoplasm to discharge dense substance into the PV space suggests a kind of active metabolic role. The space within the SPV of *Glugea* species examined ultrastructurally thus far (*G. anomala*,

G. atherinae, G. pimephales, G. stephani, G. weissenbergi) has also been found to contain a granular matrix, in which the sporoblasts and spores are embedded. It is, however, invariably much more thinner than in *Loma*. It is most pronounced in *G. anomala*; even in this species, however, its density is incomparably lighter than e.g., in *L. acerinae* or *L. branchialis*.

Glugea acerinae cannot be preserved within the genus *Glugea*. This is contradicted by the absence of a true SPV membrane produced by the sporont surface in a way typical of e.g., *G. anomala* (Canning *et al.* 1982); the parasite sporogony proceeds in a PV much alike the type species of *Loma, L. branchialis*. Further, the nature of the xenoma is quite different, complying again with what is known in *L. branchialis*. Finally, the dense granular matrix separating the sporoblasts and spores within the PV is much denser than in any species of the genus *Glugea*. Therefore we propose to transfer this species into the genus *Loma* as *Loma acerinae* (Jírovec, 1930) comb. nov..

The above ultrastructural comparisons show that - if all species described are included - the genus *Loma* is a rather heterogenous assembly. Although such a situation is not rare among microsporidian genera - it is true, that *Glugea* comprises species in which developmental stages also reveal considerable ultrastructural differences (e.g., *G. anomala* vs. *G. truttae* as mentioned by Loubès *et al.* 1984) - it should be avoided. Therefore we propose to include into the genus *Loma* Morrison and Sprague, 1981 the species *L. branchialis* (Nemeczek, 1911), *L. acerinae* (Jírovec, 1930), *L. fontinalis* Morrison and Sprague, 1983, *L. salmonae* (Putz, Hoffman and Dunbar, 1965) and possibly, *L. embiotocia* Shaw, Kent, Docker, Brown, Devlin and Adamson, 1997 which do comply with the definition of the type species, *L. branchialis*. The assignment of other *Loma* species, *L. boopsi* Faye, Toguebaye and Bouix, 1995, *L. camerounensis* Fomena, Coste and Bouix, 1992, *L. dimorpha* Loubes, Maurand, Gasc, de Buron and Barral, 1984, and *L. diplodae* Bekhti and Bouix, 1985 in view of the alledged presence of a true SPV warrants further study.

Quite recently, Nilsen *et al.* (1998) have constructed cladograms based on their analysis of the SSU rRNA genes of a number of fish-infecting microsporidia including several species of *Glugea* and *Loma salmonae*. They have clearly shown a great distance between *Loma* (which curiously groups with *Ichthyosporidium*) and *Glugea*, which indicates that they should even be placed in two different families.

These results seem to support our tranfer of *G. acerinae* - different from typical glugeas - to the genus *Loma*.

Comments on ultrastructural relations of *Loma acerinae* to other microsporidia

Spindle ("centriolar") plaques (SP)

There is a wide variety among various microsporidia in SP structure. The depression in nuclear envelope may contain one up to five dense layers (Larsson 1986), e.g. two curved layers in *Trichoctosporea pygopellita* (Larsson 1994) or two discs of twin membranes in *Stempellia* (Desportes 1976). In *L. acerinae* there are no polar vesicles, unlike *Glugea anomala* (Canning *et al.* 1982) or *L. fontinalis* (Morrison and Sprague 1981c). The SP may support the ideas on the kinship of microsporidia with fungi, as the SPs resemble the spindle pole bodies of *Saccharomyces* (Sacchi *et al.* 1997).

Cytoplasmic microtubules have been rather infrequently recorded in various microsporidia, including *G. anomala*. They are most probably residues of the microtubules radiating at mitosis from the cytoplasmic face of the SP, described by e.g., Desportes (1976) and more recently by Sacchi *et al.* (1997). It is difficult to ascribe these detached elements any particular role.

Membrane bound, homogenous dense globules like those of *L. acerinae* can be found in posterior parts of spores or sporoblasts of various microsporidia under varied designations - e.g., "enclave proteique" in *Stempellia mutabilis* (Desportes 1976), "secretion granules" in *L. salmonae* (Morrison and Sprague 1983), in *L. camerounensis* (Fomena *et al.* 1992), in *Trichoctosporea pygopellita* (Larsson 1994). They are most probably proteinaceous bodies originating in Golgi vesicles.

'Paramural bodies can be found in various sporogony stages, from segmenting sporogonial plasmodia up to maturing spores. One of the possible functions of these bodies might be to supply membrane material during sporogony (Larsson 1986). Occurence of similar structures in yeasts might again seem to support the assumptions of phylogenetic kinship between microsporidia and fungi (see Müller 1997 for a recent summary) were it not for occurrence of similar structures in other organisms, higher plants and algae. Curiously enough, residual membranaceous material in spores from which the polar tube has extruded may assume appearance very similar to paramural bodies. Also malformed spores of various microsporidia e.g., *Nosema ceratomyxae* (Diamant and Paperna 1985) reveal contorted tubular formations, pos-

sibly a transformation of membrane material destined for the polaroplast.

Virus-like particles (VLP), common in protozoa (Miles 1988), have been so far encountered in microsporidia only once in the cytoplasm and twice intranuclearly (Vávra *et al.* 1996/97). Ours is the fourth finding in microsporidia. The particles in *L. acerinae* are twice as big as those described by Vávra *et al.* (1986/97) which reached only 25 nm. In the numbers observed, these VLP can hardly inhibit the parasite's development. This is at variance with some massively occurring VLP's like those in *Ichthyophthirius* (Lobo-da-Cunha and Azevedo 1992) in which an adverse action on the host cell can be presumed (Miles 1988).

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Mantoscyphidia fanthami sp. n., an Ectosymbiont (Ciliophora: Peritrichia) from the Gills of the Marine Gastropod *Oxystele* Philippi, 1847 in South Africa

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Summary. Parasitological surveys of the marine gastropod *Oxystele* Philippi, 1847 (Trochidae) revealed a ciliophoran on the gill filaments. This topshell genus comprises 5 species endemic to and distributed along the South African coast. The ciliophorans were found associated with all 5 species and occurred on the southern, western and eastern coastline. This ciliophoran is described as a new species, *Mantoscyphidia fanthami*, based on light and scanning electron microscopy.

Key words: ectosymbiont, *Mantoscyphidia*, marine mollusc, new species, scyphidiid peritrich, sessiline ciliophoran.

INTRODUCTION

Fantham (1930) recorded an unidentified trichodinid from the mantle cavity and foot of the topshell *Oxystele variegata* (Anton, 1838) near Cape Town, South Africa. Later, Sandon (1965) collected specimens of *O. variegata* in the same area and again found this mobiline ciliophoran, which he described as *Trichodina oxystelis* Sandon, 1965. He also recorded the presence of a sessiline ciliophoran co-existing with the trichodinid on the same host, but did not provide a taxonomical description. Basson and Van As (1992) presented a redescription of *T. oxystelis* based on silver impregnated specimens. Along with the material used for this description we also noted the presence of a sessiline ciliophoran, but did not describe it in the 1992 paper.

The genus *Oxystele* Philippi, 1847 is endemic to the southern African marine zoogeographical province and comprises 5 species. According to Wye (1991) this marine province is defined from Walvis Bay on the west coast of Namibia southwards, including the whole coast of South Africa as well as the east coast just north of Maputo (Mozambique). On the south coast region 4 *Oxystele* species co-exist, i.e. *O. variegata*, *O. tigrina* (Anton, 1838), *O. impervia* (Menke, 1843) and *O. sinensis* (Gmelin, 1791). On the west coast one of the four, *O. sinensis*, is absent, whilst on the eastern subtropical coastline only a single species, *O. tabularis* (Krauss, 1848) occurs. This paper is based on collections in all three coastal regions of South Africa.

Until the mid 1980's all cylindrical sessiline ciliophorans that adhered to their substrate by means of a broad scopula

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were placed in the family Scyphidiidae Kahl, 1935. This family comprised only 2 genera, *Ambiphrya* Raabe, 1952 and *Scyphidia* Dujardin, 1841. The former was distinguished by a ribbon-like macronucleus and a permanently ciliated telotroch band, whilst the latter was characterised by a compact macronucleus. Jankowski (1980, 1985) proposed a new system of classification for members of the family Scyphidiidae, dividing the family into 5 genera, based on differences in host or substrate. The genus *Scyphidiella* Guhl, 1979 includes sessiline ciliophorans associated with waterborne Coleoptera; the genus *Myoscyphidia* Jankowski, 1985 is found associated with water plants in pools; while the free-living scyphidiid peritrichs were placed in the genus *Speleoscyphidia* Jankowski, 1980. *Riboscyphidia* Jankowski, 1980 was erected for those scyphidiid peritrichs associated with freshwater and marine fishes, whilst the genus *Mantoscyphidia* Jankowski, 1980 was proposed to accommodate all those scyphidiid peritrichs associated with freshwater and marine gastropods. The sessiline ciliophoran found associated with *Oxystele* species in the present study therefore belongs to the genus *Mantoscyphidia* according to Jankowski 's classification.

The scyphidiid peritrich collected from the endemic *Oxystele* species along the South African coast differs from all the known species based on general body morphology and nuclear apparatus, as well as host and habitat, and is described as a new species below.

MATERIALS AND METHODS

A total of 373 specimens of *Oxystele* were collected at low tide on rocky shores from Margate and Ballito on the east coast; Nature's Valley, Buffel's Bay and Jeffrey's Bay on the south coast as well as Mc Dougall's Bay and the Olifants River mouth on the west coast (see Table 1). Molluscs were taken live to a field laboratory where they were dissected, their gills removed and preparations made for microscopic study. Wet smears with live ciliophorans were examined and photomicrographs taken of fully extended specimens. Wet smears were then fixed in Bouin's and kept in 70% ethanol for later preparation in the laboratory. Smears were stained with Harris hematoxylin for studying the nuclear apparatus and for obtaining body measurements. An adapted method of Wilbert (1975) for Protargol impregnation was followed to reveal details of the infundibulum.

For scanning electron microscopy, fresh gills were fixed in 10 % buffered neutral formalin made up in filtered sea water. In the laboratory the material was washed in tap water, dehydrated in a series of ethanols and critical-point dried. Sputter coating was done with gold, and specimens were examined in a JEOL WINSEM JSM 6400 at 5 kV.

Minimum and maximum values of all body measurements are given, followed in parentheses by the arithmetic mean, standard deviation and number of specimens measured. Accuracy of measurements is to the nearest micrometer. The body length was measured from the scopula to the epistomial disc, and the body diameter at the widest part of the body. Both the macro-and micronucleus lengths were measured from adoral to aboral. Type material is deposited in the collection of the National Museum, Bloemfontein (South Africa).

RESULTS

Mantoscyphidia fanthami sp. n. (Figs. 1-9)

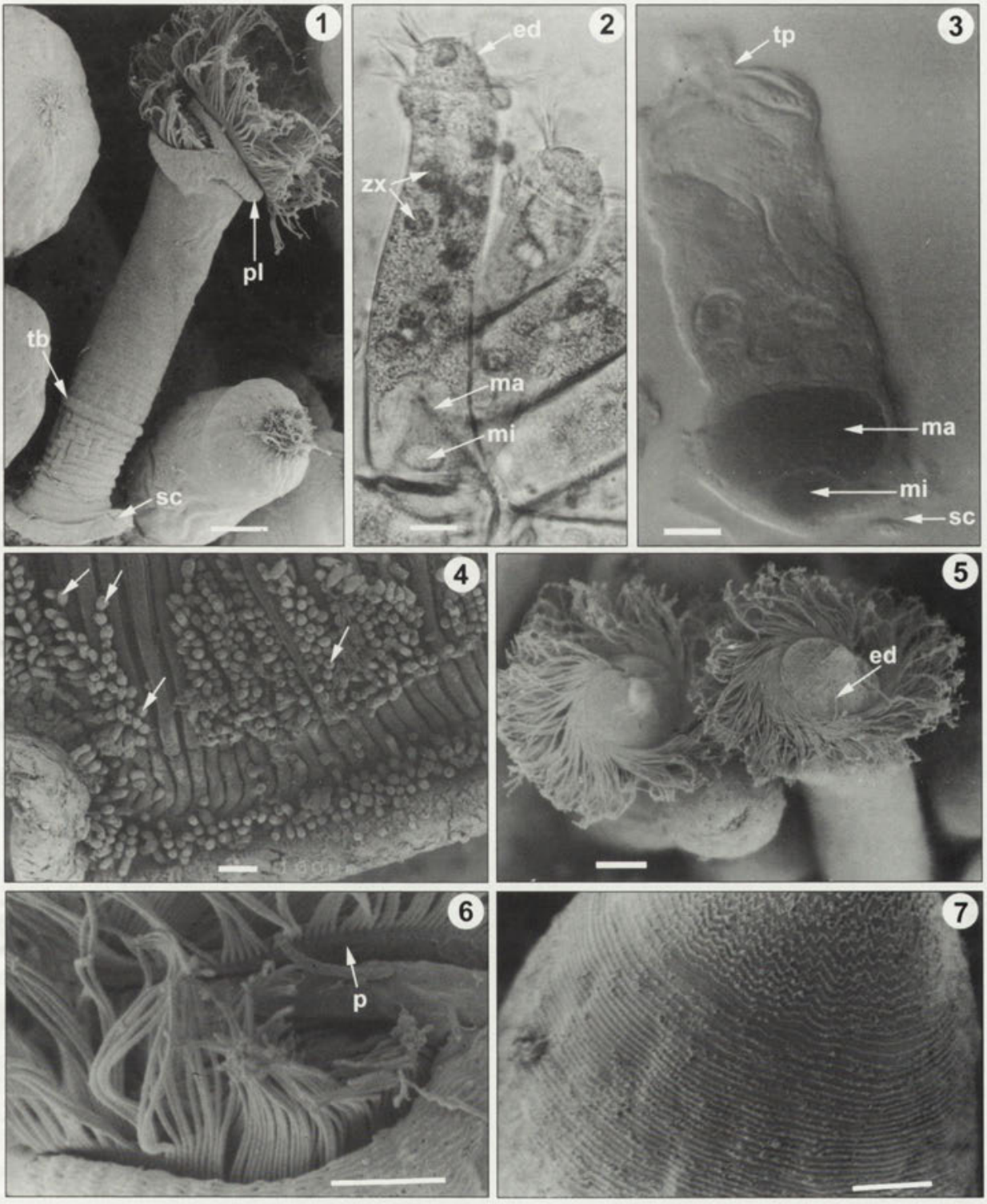
Hosts: *Oxystele sinensis* (Gmelin, 1791), *O. tabularis* (Krauss, 1848), *O. tigrina* (Anton, 1838), *O. variegata* (Anton, 1838) and *O. impervia* (Menke, 1843).

Localities: Margate and Ballito on the east coast; Nature's Valley, Buffel's Bay and Jeffrey's Bay on the south coast and Mc Dougall's Bay and the Olifants River Mouth on the west coast (South Africa) (see Table 1).

Table 1. Prevalence of *Oxystele* species infested with *Mantoscyphidia fanthami* sp.n. from various collection localities in South Africa

	N	Prevalence (%)
Oxystele tigrina		
McDougall's Bay	20	40
Olifants River Mouth	20	15
Nature's Valley	19	95
Buffel's Bay	20	100
Jeffrey's Bay	20	100
Oxystele sinensis		
Nature's Valley	18	94
Jeffrey's Bay	20	60
Oxystele variegata		
McDougall's Bay	20	100
Olifants River Mouth	20	100
Nature's Valley	20	95
Buffel's Bay	20	100
Jeffrey's Bay	18	100
Oxystele impervia		
McDougall's Bay	20	95
Olifants River Mouth	20	100
Buffel's Bay	29	100
Jeffrey's Bay	19	93
Oxystele tabularis		
Margate	20	100
Ballito	20	100

N- number of specimens examined



Figs. 1-7. Scanning electron micrographs (1, 4-7) and photomicrographs of live (2) and hematoxylin stained (3) specimens of *Mantoscyphidia fanthami* sp. n. 1 - fully expanded body with telotroch band located in lower third of body; 2 - expanded body with zooxanthellae; 3 - hematoxylin-stained specimen with partially contracted body, macro- and micronuclei adoral to scopula; 4 - gill filaments of mollusc with numerous attached specimens of *M. fanthami* (arrows); 5 - epistomial discs with prominently pointed apexes and long adoral cilia; 6 - adoral cilia prior to plunging into infundibulum, with row of prominent pores associated with haplokinety; 7 - undulating striations on peristome lip and concentric striations on body. ed-epistomial disc; ma-macronucleus; mi-micronucleus; p-pores; pl-peristomial lip; sc-scopula; tb-position of telotroch band; tp-tapering point; zx-zooxanthellae; Scale bars - 20 µm (1), 10 µm (2, 3, 5), 100 µm (4), 5 µm (6, 7). 1,4-7 - *M. fanthami* from *Oxystele tigrina* (Anton, 1838) collected at Nature's Valley on the south coast. 2, 3 - *M. fanthami* from *O. variegata* (Anton, 1838) collected from Jeffrey's Bay on the south coast

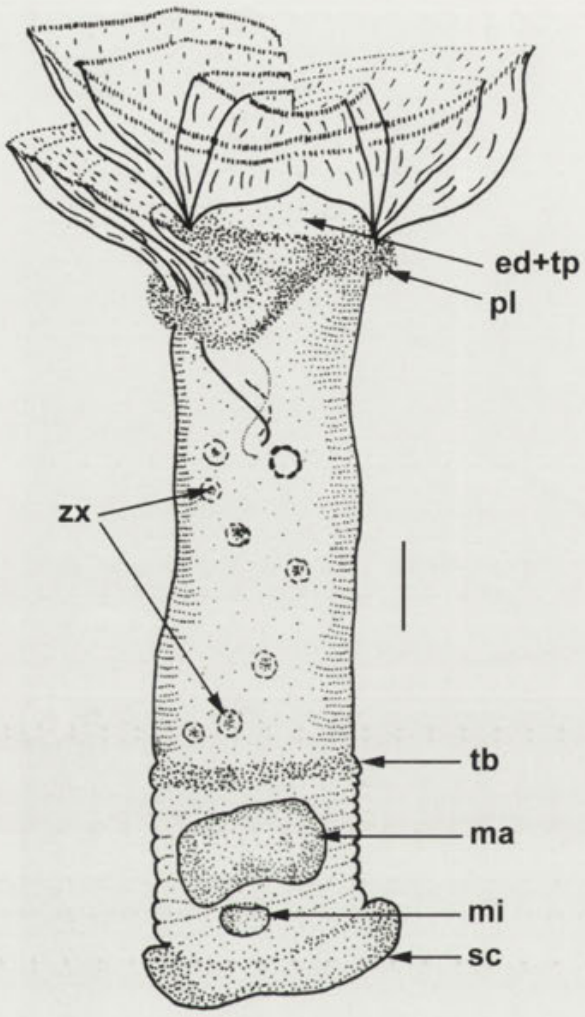


Fig. 8. Microscope projection drawing of *Mantoscyphidia fanthami* sp. n. from the gills of *Oxystele variegata* (Anton, 1838) collected at Jeffrey's Bay. ed+tp-epistomial disc with tapering point; ma-macronucleus; mi-micronucleus; pl-peristomial lip; sc-scopula; tb-position of telotroch band; zx-zooxantellae; Scale bar - 10 μm

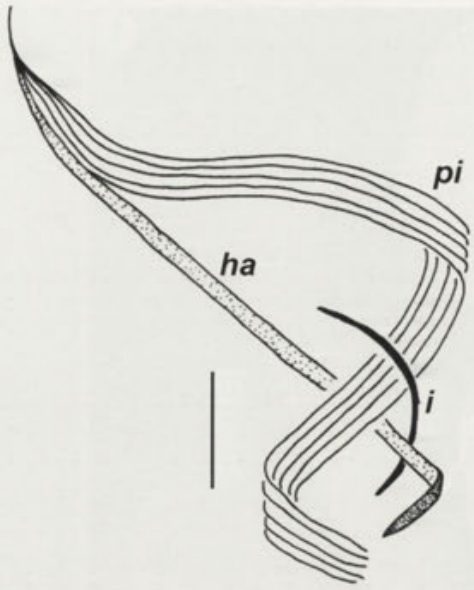


Fig. 9. Buccal infraciliature of *Mantoscyphidia fanthami* sp. n. from the gills of *Oxystele variegata* (Anton, 1838) collected at Jeffreys's Bay, ha - haplokinety; i - impregnable structure; pi - polykinety. Scale bar - 5 μm

Location on host: gills.

Type-specimens: holotype, slide 98/01/10-09 (NMBP 200); paratypes, slides 98/01/10-10 (NMBP 201), 98/01/10-11 (NMBP 202), 98/01/10-12 (NMBP 203), in the collection of the National Museum, Bloemfontein.

Type host and locality: *O. variegata*, Jeffrey's Bay (34° 2.2'S, 24° 56.5'E).

Etymology: the species is named after H.B. Fantham, a pioneering protozoologist from South Africa.

Description

Body slender, elongated when expanded, with peristome broader than rest of body (Figs. 1, 2, 8). Body length 80-130 μm (101.3±12.5, 23), body diameter 25-35 μm (28.9±3.7, 23). In contracted specimens, epistomial disc and adoral cilia completely enclosed in body. In Fig. 1 a specimen is shown with cilia almost completely enclosed, but with tips still clearly visible. Contracted adoral area with prominent elevated tapering point (Fig. 3). Telotroch band (Figs. 1, 8) broad, elevated, situated about one third of body length from scopula. Prominent pellicular folds in area aboral to telotroch band (Figs. 1, 8). Scopula broader than body, diameter 35-50 μm (41.1±4.5, 23).

Peristome, body and scopula with encircling pellicular striations, sometimes bifurcated. Telotroch band with three closely spaced striations. Number of striations adoral to telotroch band 149-179 μm (165±3.3, 10), number of striations aboral to telotroch band 83-107 μm (97±2.5, 10) Pellicular pores located randomly between striations. In contracted specimens peristomial striations undulating, situated slightly closer to one another (Fig. 7). Epistomial disc smooth, convex with prominently pointed apex (Figs. 2, 5). Peristomial lip broad when expanded, oral opening large, leading into infundibulum (Figs. 1, 5).

Adoral zone completes spiral of 540° counterclockwise around epistomial disc before plunging into infundibulum (Fig. 6). Cilia rows consist of single outer row (haplokinety) and triple inner row (polykinety). Pores associated with both haplokinety and polykinety (Fig. 6). Haplokinety and polykinety enter infundibulum separately, with haplokinety plunging directly into infundibulum, completing almost one full turn (Fig. 9). After parting, polikinety completes

another half turn on rim of oral opening before entering infundibulum as peniculus that in turn completes another 360°. Impregnable structure always visible, associated with haplokinety. Both rows complete spiral at level of cytostome with peniculus and haplokinety facing one another.

Single contractile vacuole situated in adoral third of body, associated with lower infundibulum (Fig. 8). Some specimens with large symbiotic zooxanthellae occurring throughout body (Fig. 2).

Macronucleus compact, somewhat rectangular, situated in base of body, length 7-16 µm (11.4±2.5, 18), diameter 13-28 µm (17.1±3.6, 18). Micronucleus oval-shaped, situated on aboral side of macronucleus, sometimes in indentation of macronucleus, diameter 5-10 µm (6.9±1.4, 17), length 3-6 µm (4.6±0.9, 17) (Figs. 3, 8).

DISCUSSION

So far 14 species of the genus *Mantoscyphidia* have been recorded from gastropods. Of these, only 4 have been found associated with freshwater gastropods, i.e. *M. physarum* (Lachmann, 1856), *M. limacina* (Lachmann, 1856), *M. inclinata* (Lom and Corliss, 1968) and *M. capitis* (Boitsova, 1976). Both *M. physarum* and *M. limacina* have elongated macronuclei, whilst *M. inclinata* has an oval macronucleus located in the adoral half of the body. No information is available regarding the nuclear material of *M. capitis*. It is, however, unlikely that any of these scyphidiid peritrichs from freshwater molluscs can be the same as *M. fanthami* collected from endemic marine topshells from the southern African zoogeographical province.

Ten species belonging to the genus *Mantoscyphidia* have been described from marine molluscs. A brief summary of these species was provided by Van As *et al.* (1998). Several of the marine scyphidiid species are smaller than *M. fanthami*. Only *M. acanthophora* (Fish and Goodwin, 1976) is larger, while *M. ubiquita* (Hirshfield, 1949) and *M. fanthami* fall within the same size range. Most of the species of *Mantoscyphidia* have cylindrical body shapes with prominent narrowing just adoral to the scopula, except *M. littorinae* (Issel, 1918) where the cylindrical body shows no constriction towards the scopula. In the case of *M. fanthami*, *M. branchi* Van As, *et al.*, 1998 and *M. marioni* Van As, *et al.*, 1998 slight constrictions are visible adoral to the scopula. In the case of *M. littorinae*, the scopula is a very prominent, plastic feature, whilst in *M. fanthami* the scopula is only slightly wider than the widest part of the body.

The most constant feature of molluscan scyphidiid peritrichs is the position and shape of the nuclear apparatus. The macronucleus in *M. lusitana* Jankowski, 1985 varies from beaded (Hirshfield 1949) to forming a band (Cuénot 1891). *M. hydrobiae* (Kahl, 1933), *M. ubiquita*

Table 2. Biometrical data (in µm) of *Mantoscyphidia fanthami* sp. n. from four *Oxystele* species collected from Jeffrey's Bay, South Africa

Host Species	*Oxystele variegata*	*Oxystele sinensis*	*Oxystele impervia*	*Oxystele tigrina*
Body length	80 - 130 (101.3±12.5, 23)	70 - 140 (96.5±17.4, 17)	90 - 120 (101.5±9.6, 17)	50 - 70 (57.7±5.7, 23)
Body diameter	25 - 35 (28.9±3.7, 23)	20 - 30 (26.2±3.3, 17)	25 - 40 (30.6±4.3, 17)	20 - 25 (20.9±1.5, 23)
Scopula diameter	35 - 50 (41.1±4.5, 23)	25 - 40 (35.3±5.1, 17)	35 - 50 (42.4±5.3, 17)	25 - 30 (27.6±2.4, 23)
Macronucleus length	7 - 16 (11.4±2.5, 18)	7 - 14 (9.5±1.8, 21)	8 - 17 (12.1±2.7, 18)	4 - 11 (7.3±1.8, 25)
Macronucleus diameter	13 - 28 (17.1±3.6, 18)	11 - 16 (13.1±1.8, 21)	11 - 23 (16.6±3.3, 18)	8 - 13 (10.3±1.3, 25)
Micronucleus length	3 - 6 (4.6±0.9, 17)	3 - 6 (4.4±0.8, 21)	4 - 7 (5.1±0.9, 18)	2 - 5 (2.8±0.7, 22)
Micronucleus diameter	5 - 10 (6.9±1.4, 17)	4 - 9 (6.8±1.3, 21)	6 - 11 (8.4±1.5, 18)	2 - 10 (4.5±1.6, 22)

Minimum and maximum values are given, followed in parentheses by the arithmetic mean, standard deviation and number of specimens measured

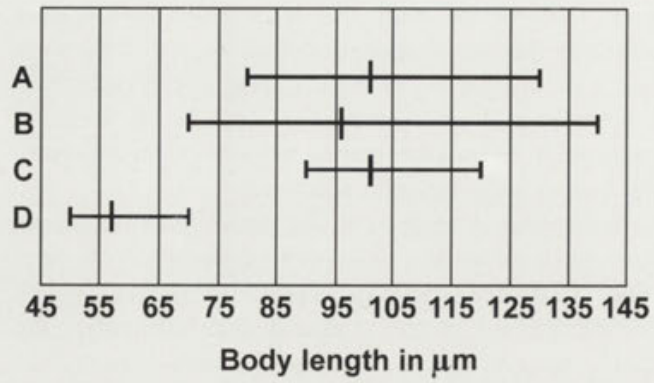


Fig. 10. Graphic representation of the body length from different populations of *Mantoscyphidia fanthami* sp. n. from four *Oxystele* species collected at Jeffrey's Bay.
A - *O. variegata* (Anton, 1838), **B** - *O. sinensis* (Gmelin,1845), **C** - *O. impervia* (Menke, 1843), **D** - *O. tigrina* (Anton, 1838)

and as *M. marioni* have elongated sausage-shaped macronuclei (Kahl 1933, Hirshfield 1949, Van As *et al.* 1998). In the case of *M. fischeri* (Vayssiére, 1885) and *M. bengalensis* (Jamadar and Choudhury, 1988), the macronuclei appear ribbon-like as described by Hirshfield (1949) and Jamadar and Choudhury (1988) respectively. *Mantoscyphidia acanthophora* has a C-shaped macronucleus (Fish and Goodwin 1976). Both *M. littorinae* and *M. branchi* show some resemblance in the shape of the macronucleus to *M. fanthami*, all three species having compact macronuclei. In the smaller body of *M. littorinae*, however, the macronucleus is found higher, sometimes even located in the middle of the body (Raabe 1952). In the case of both *M. branchi* and *M. fanthami* the position of the nuclear apparatus is constant, being immediately adoral to the scopula (Van As *et al.* 1998). In most species the micronucleus is small and round and located close to the macronucleus. The only exception seems to be *M. hydrobiae* where no micronucleus was indicated by Kahl (1933). According to Hirshfield (1949), *M. ubiquita* has two micronuclei, one situated near the peristome and the other near the macronucleus. In the case of *M. fanthami* and *M. branchi*, the micronuclei are oval, relatively large and always found close to and sometimes in prominent indentations of the macronuclei.

Of all the scyphidiids from marine molluscs, *M. branchi* and *M. fanthami* most closely resemble one another morphologically. The main differences between these two species are the following: *M. fanthami* has a longer more slender body, with the telotroch band situated in the lower third of the body, whilst in the case of *M. branchi*, the body is far shorter, with the telotroch band situated almost in the middle. The infundibulum of these two species also differs; in the case of *M. branchi* the polykinety plunges into the infundibulum, followed by the haplokinety 180° later. This is exactly the opposite in *M. fanthami*, in which the haplokinety enters first, followed 180° later by the polykinety. Furthermore, the impregnable structure in *M. branchi* is closely associated with the haplokinety and far longer than that found in *M. fanthami*. In the latter species, the impregnable structure is much shorter and found on a different level than both the haplo- and polykineties.

Mantoscyphidia fanthami shows a number of distinguishing characteristics, such as a very large infundibulum opening as illustrated in Figs. 1 and 6. This species also has a very prominently pointed epistomial area (Fig. 5), exceptionally long adoral cilia (about 25 µm) as well as a number of zooxanthellae distributed in the cytoplasm of some individuals.

Mantoscyphidia fanthami was found associated with all five species of the genus *Oxystele* from the west coast, south coast and east coast of South Africa. This is most likely the same species noted by Sandon (1965). In most cases the prevalence was very high, up to 100%, with only two cases where the prevalence was below 50% (Table 1). Populations from *O. variegata*, *O. impervia* and *O. sinensis* are very similar in size, whilst those from *O. tigrina* are considerably smaller in most body measurements (Fig. 10, Table 2). A possible reason for these size differences could be the influence of the different host species. This, however, needs to be investigated further.

Mantoscyphidia fanthami can, thus, be distinguished from other species of this genus from marine molluscs based on body form of extended specimens, macronucleus and shape of the scopula.

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Short Communication

A New Myxozoan, *Chloromyxum careni* sp. n. (Myxosporea: Chloromyxidae) from the Kidney of *Megophrys nasuta* Schlegel, 1858 (Anura: Pelobatidae) from Indonesia

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Summary. A new species of Chloromyxum from a pelobatid anuran (*Megophrys nasuta*) is described. Spores and plasmodias were found in the kidneys of adult frogs. Fresh spores and sporogenic stages from tissue imprints as well as histological sections are documented and illustrated. Pathological changes in the renal system are discussed. This is the first record of a *Chloromyxum* in anurans.

Key words: Anura, Chloromyxidae, *Chloromyxum careni* sp. n., *Megophrys nasuta*, Myxozoa.

INTRODUCTION

In contrast to the real distribution in amphibians the knowledge about myxozoans in anurans and urodeles is still underdeveloped (Mutschmann 1998). Chloromyxids, very common in fish, were reported from amphibians only three times. At first, Thélohan (1894) described *Chloromyxum caudatum* from the gall bladder of crested newts (*Triturus cristatus*) in France. Due to the caudal appendages of the spores, this species should be placed actually in the genus *Caudomyxum* Bauer, 1948 (Upton *et al.* 1995). A second species, *Chloromyxum protei*, was found and reported by Joseph (1905) in the renal tubes of Proteus *anguineus* near Vienna. In the latest mentioning, Upton *et al.* (1995) described *Chloromyxum salamandrae* from the gall bladder of salamanders (*Eurycea* spp.) from North America. No chloromyxid myxozoan have been reported in anurans.

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MATERIALS AND METHODS

Three freshly imported adult male frogs of the species *Megophrys nasuta* (Asian horned toad) that had been caught in the wild were dissected and examined. Two frogs died during transport to a pet shop and were preserved on ice; the third one was moribund and died after a few hours at my animal practice office. Samples of fresh material from inner organs as well as from heart blood, skin and brain were taken, all organs were placed in 5% buffered formalin. Fresh samples were directly examined under a research microscope. Material was also fixed with absolute methanol and Giemsa stained. Slides were examined as wet mount preparations under the oil immersion lens of the microscopes (Reichert „Zetopan", „Jenamed" Zeiss Jena) using light field, phase contrast and dark field methods. Giemsa stained slides were observed

only in light field. Histological preparations were made from all inner organs and were stained (Unna and Heidenheim, Hematoxilin-Eosin, Ziehl-Neelson). Measurements and photographs were taken using an ocular micrometer (Zeiss) and a photomicroscope (mf-AKS Zeiss, Jena). All measurements are reported in micrometers (µm) as means, followed by the standard deviations and ranges in parentheses.

RESULTS

All frogs were found highly parasited with different kinds of helminthes (Digenea, Nematoda) especially in the gut and lungs. Cysts harboring trematode larva were recognized in skin and skeleton muscles. The gut also contains also a high number of opalinids resembling *Opalina ranarum* Purkinje and Valentin, 1835 for having numerous nuclei. The kidneys (renal tubes) of each frog were infected with chloromyxid myxozoans. The spores were noted in freshly impressed material as well as in Giemsa stained slides. Plasmodias (trophic forms) and other developmental stages were also found in fresh and stained samples. No spores or other forms could be found in organs like liver, gall bladder, uric bladder, gonads, spleen, heart, gut and blood. The spores were small, ovoid or ellipsoid and each spore contains 4 polar capsules located at the apical end. Plasmodias harbor submature and mature spores (from two up to more than 30). No gross pathology was found associated with infected kidneys except for a slightly swollen appearance. Histological examination showed only few pathological changes. No inflammatory reactions were found. The nuclei of epithelia cells of the renal tubules connected with parasitic plasmodia appeared pycnotic. Sometimes the wall of epithelic cells was inapparent, or the cells were degenerated or appeared necrotic. Only few hyaline or fibrinogen-like droplets were found in infected areas.

The chloromyxid found in frog kidneys is described in the following as a new species.

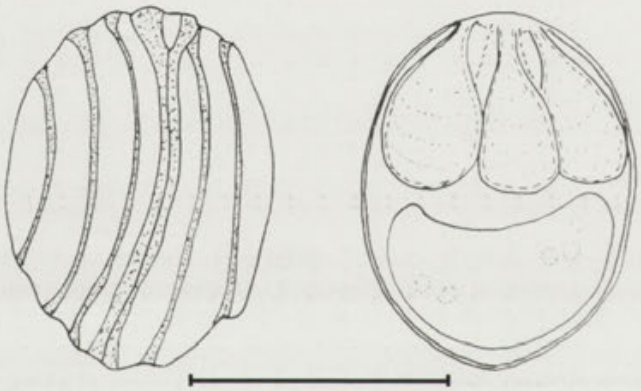
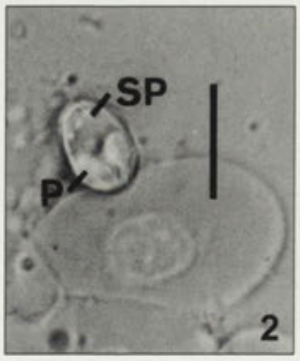
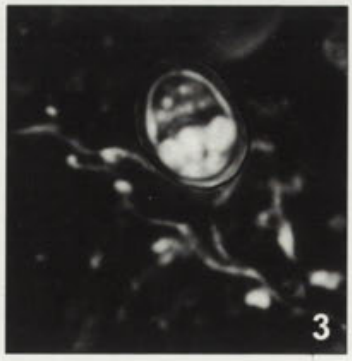


Fig.1. Line drawings of mature spores of *Chloromyxum careni* sp. n. Left figure shows external striations, right - internal details. Scale bar - 5 µm

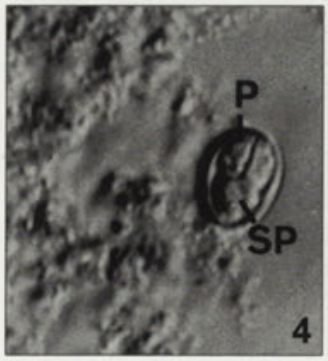
Description

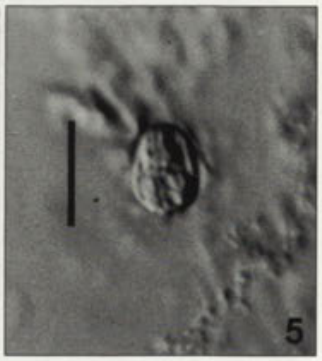
Myxosporea Bütschli, 1881
Bivalvulida Schulman, 1959
Variisporina Lom, 1984
Chloromyxidae Thélohan, 1892



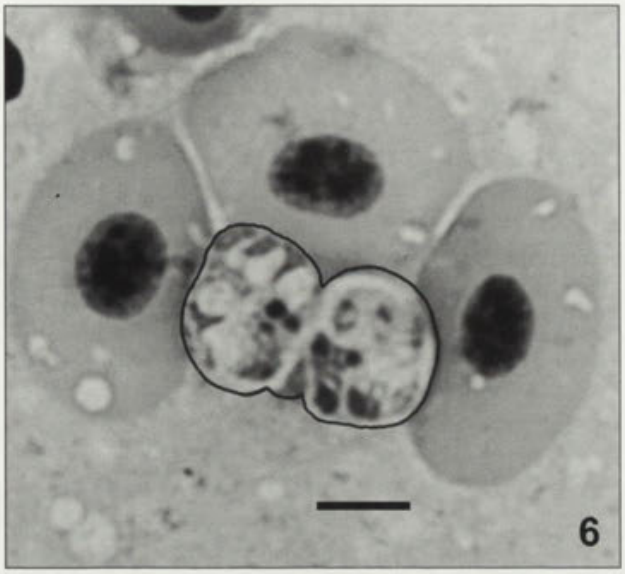


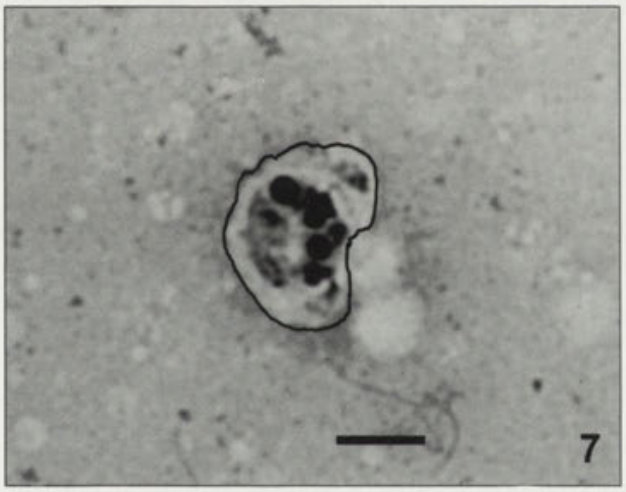
Figs. 2,3. Fresh mature spore of *Chloromyxum careni* in renal imprints of *Megophrys nasutus*. 2 - light field illumination, 3 - dark field illumination. Scale bar - 10 µm. P - polar capsule, SP - sporoplasm



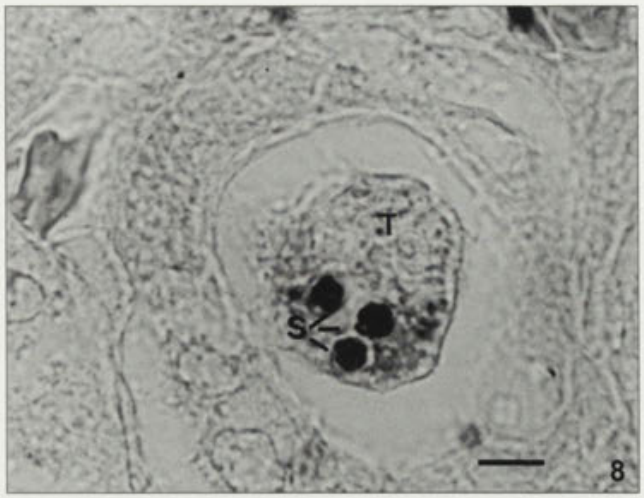


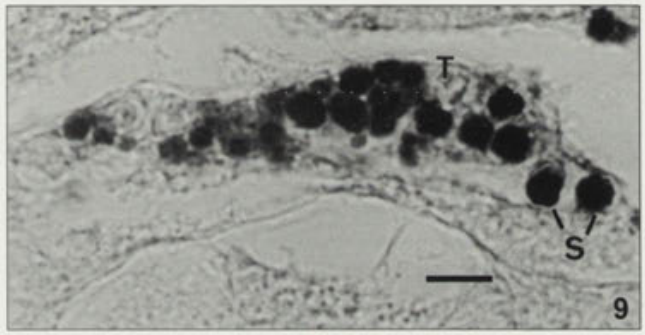
Figs. 4,5. Submature spore of *Chloromyxum careni*. 3 - demonstration of the internal structure, 4 - phase contrast micrograph shows the external striations. Scale bar - 10 µm. P - polar capsule, SP - sporoplasm





Figs. 6,7. Giemsa-stained kidney impression with of *Chloromyxum careni*. 6 - disporoblastic developmental stage, 7 - sporoblast. Scale bar - 10 µm





Figs. 8, 9. Ziehl-Neelson stained sections of kidneys of *Megophrys nasutus* with plasmodia (Trophozoits) of *Chloromyxum careni* in the renal tubules. Scale bar - 10 µm. S - spore, T - trophozoit

Chloromyxum careni Mutschmann sp. n.

Host: *Megophrys nasuta* Schlegel, 1858 (Anura: Pelobatidae)

Locality: Indonesia (no exact information about the origin of the frogs could been obtained).

Site of infection: renal tubuli.

Incidence: 3/3 (100%).

Spore: ovoid to ellipsoid, length: 8 µm ± 0.7 (7.2 - 9.0) (n= 30); width: 5 µm ± 0.4 (4.8 – 5.9) (n= 30); valves: 0.8 ± 0.2 (0.7 - 1.0) (n = 20) with 6 to 8 external striations.

Polar capsules: 4 pyriform capsules in each spore located at the anterior, thicker end; length: 2.8 µm ± 0.5

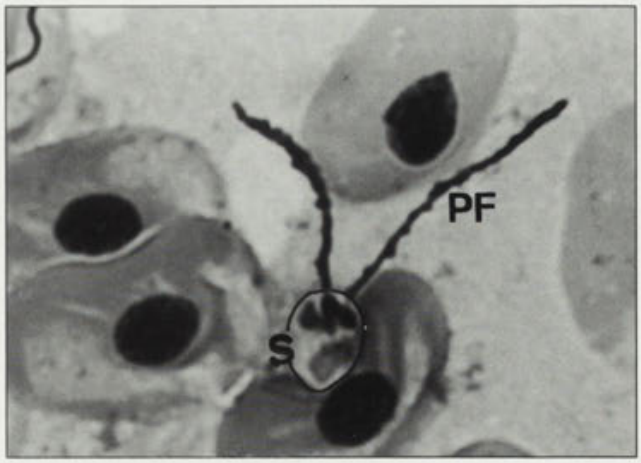


Fig. 10. Giemsa-stained kidney impression, mature spore of *Chloromyxum careni* with discharged polar filaments surrounded by erythrocytes. Scale bar - 10 µm. PF - polar filament, S - spore

(2.0-3.1); width: 1.8 µm ± 0.6 (1.1 - 2.3); number of polar filament turns not to be seen; length of extruded polar filaments 20 µm ± 0.8 (16 - 44) (n = 50).

Sporoplasma (amoeboid germ): irregular in shape containing a granulated mass or nuclei (3 - 5 nuclei in most cases).

Plasmodium: adhering to the epithelium of renal tubules, irregular in shape and size with pointed pseudopodia (Acanthamoeba-like). Largest size seen in histological preparations 128 x 98 µm (end to end). Containing mature and submature spores (2-more than 40), spore development seems to be disporoblastic.

Etymology: „careni" refers to my wife Caren for her cooperation in studies of diseases of amphibians and reptiles.

DISCUSSION

No previous report on chlormyxid myxozoa in anurans could been found in literature. The parasites found in different urodeles species differ from the species described herein. Spores of *Chloromyxum protei* (Joseph, 1905; 1907) are larger (10-13 µm in diameter) and had a spherical shape, as well as *Chloromyxum salamandrae* (Upton, McAllister, Trauth, 1995). The later species is not reported from other organs as the gall bladder of salamanders, had more external striations and larger polar capsules. *Chloromyxum caudatum* (Thélohan, 1894) should be treated as a *Caudomyxum* and had caudal appendages, never seen in spores of the species obtained from *Megophrys nasuta*. The pathological changes in infected kidneys are slight, often restricted to the epithelial surface of renal tubes or inapparent. Sometimes picnotic and necrotic epithelia cells were seen nearby adhered parasitic plasmodia. No large numbers of fibrinogenous or hyaline material inclusions were found in host tissue and renal tubes, this fact is different to other findings related to myxozoan infections of frog kidneys e. g. *Sphaerospora ohlmacheri*-infections in Bullfrog tadpoles reported by Dresser and Lom (1986). It is possible, that pathological findings will increase with the age of infection and the rate of parasitic reproduction. On the other hand, the failure of inflammatory reactions implicates a long lasting phylogenetic coevolution of host and parasite. Further investigation should be done to study the biology of the parasite and its role as pathogen.

Holotype is deposited in my private collection as Coll. No. 410 as well as diapositives and histological sections (Coll. No. 2608).

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