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Didier Chardez
(1924 - 2000)

In the morning of 9 September 2000 Didier Chardez died from a cardiac stroke. He was widely known as a protozoologist working on testate amoebae, and to a lesser degree on ciliates. He was a striking example of what non-professional scientists can perform in a field where basic skills as microscopic observation and identification are still important. Didier was born in Paris on 2 October 1924 from Belgian elders. He came to Belgium at the age of six, and obtained a degree as technical designer at the Ecole Technique Provinciale in Verviers. This was in 1943. At that time, Belgium was occupied by the German troops, and Didier experienced this bitter reality when he was a Nazi's prisoner. But he survived and took up an employment as technical designer, first in an electricity company, later in the Belgian army. After the office hours, he immersed himself in the microscopic world in the laboratory he had installed at his home at Omal.

He was an autodidact, and his interest for protists cumulated in a general note in a regional journal on natural history, the *Revue Verviétoise d'Histoire Naturelle*. This was the first of many to appear in this journal. Maybe one would wish he had chosen a more international orientated journal, which is more readily available in libraries. But on the other hand, here he could submit all kinds of observations, even very short ones, which otherwise would not have been communicated. Luckily he had the habit of sending a copy of his works to colleague protozoologist. In 1959 he became a scientific collaborator in the "Faculté Universitaire des Sciences Agronomiques de Gembloux". He was much solicited by the agronomy researchers for analyzing soil samples coming from all over the world. This led to a real proliferation of his articles, which also began to appear in the *Bulletin de Recherches Agronomiques de Gembloux*. His preference for the soil habitat was intensified that way, and expressed in two survey papers on the testate amoebae from soils in Belgium (1959 and 1960). It is no surprise that his devotion to testate amoebae drew the attention of another well known Belgian rhizopod specialist, Dr. Paul van Oye, who included Didier Chardez in his monograph on the history and development of hydrobiology in Belgium (1967). Didier was always keen to try some techniques, and so he rigged up a micro-separation device to concentrate and isolate

shells of testate amoebae from the soil substratum. It must be said that he knew the tricks, since some other researchers, including myself, did not readily get away with it. From 1977 on he became involved in forensic science, which led to a paper in *Forensic Science International* (1985), describing different ciliate protozoa observed in immersed dead human bodies. In another note (*Thecamoebologie et expertises juridiques*, 1990) he explained the use of testate amoebae in forensic expertise. A good knowledge of the habitat preferences of testate amoebae is in such studies of more than academic interest. In this article he demonstrated his habitat approach to the ecology of testate amoebae. Looking at so many samples from different habitats had provided him a good view on the relation between taxa and the environment. This knowledge is reflected in a paper (1981) on the use of testate amoebae as bio-indicators.

As a result of acquiring samples from all over the world, he compiled many protistological lists. In most cases these lists include many “first records” from that region. Another consequence is that he described a lot of new taxa. Of course we are heading now in muddy waters, since the species concept in testate amoebae is at the moment almost solely based on the pseudopodia shape and on morphological considerations of the shell. It could be expected that Didier with his detailed knowledge made a lot of lower rank taxa. Later in discussions he questioned himself the validity of some of these. From his experiments he knew that some morphological traits could change when the protist is kept in a culture, e.g. he thus reported a reduced shell size and number of spines in *Centropyxis discoides*. But maybe it is better to split up than ignoring characteristic traits. Among his major works I would reckon “*Histoire naturelle des Protozoaires Thecamoebiens*”, a general introduction to the systematic of the testate amoebae with a list of taxa known in 1967, and most taxa illustrated with a drawing. For such a work it is a pity that most of these drawings were strongly reduced in size. In recording his observations, he indeed made generously use of his drawing skills. Another important list was published twenty years later: “*Catalogue des thecamoebiens de Belgique*”. Since biodiversity became a hot topic, such lists are now urgently needed, and his latest paper, which appeared after his death, is related to protist diversity in the Polar Regions. His final list of publications goes well above 150 titles, mostly on testate amoebae, and a lot of them in a range of international journals.

To those who knew him personally, Didier was a warm and humoristic individual. On a note he recently send me, he had written: “I expect to see you with some testate amoebae”. This characterizes his never-ending enthusiasm, albeit a bit tempered last years by his illness. He is survived by the companion of his life Maggy Heuschen. He will be remembered.

Louis Beyens

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Taxonomy and Phylogeny of Heliozoa. III. Actinophryids

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Summary. The diversity, relationships and classification of the actinophryid heliozoa (protists) are reviewed. Descriptions of two new species (*Actinophrys salsuginosa* and *Ciliophrys azurina*) are presented. The actinophryid heliozoa are revised to include six species: *Actinophrys sol* (Müller, 1773) Ehrenberg, 1830, *A. pontica* Valkanov, 1940, *A. tauryanini* Mikrjukov et Patterson, 2000, *A. salsuginosa* sp. n., *Actinosphaerium eichhornii* (Ehrenberg, 1840) Stein, 1857, and *A. nucleofilum* Barrett, 1958. *Echinosphaerium* /*Echinosphaerium* Hovasse, 1965 and *Camptonema* Schaudinn, 1894 are regarded as junior subjective synonyms of *Actinosphaerium* Stein, 1857. The relatedness between actinophryid heliozoa and pedinellid helioflagellates is discussed. The new species, *Ciliophrys azurina*, exhibiting characters (tapering axonemes and peripheral location of heterochromatin) previously only reported in the actinophryids. This allows a proposition for the sequence of character acquisition and a new group of stramenopiles - the actinodines - uniting pedinellids, ciliophryids and actinophryids.

Key words: actinodines, Actinophryida, *Actinophrys salsuginosa* sp. n., *Actinophrys tauryanini* nom. nov., *Actinosphaerium*, *Camptonema*, *Ciliophrys azurina* sp. n., *Echinosphaerium*, *Echinosphaerium*, heliozoa, Pedinellales, protista, protozoa, stramenopiles.

INTRODUCTION

The heliozoa are a polyphyletic assemblage of protists having arisen from different evolutionary origins but have developed a similar life style and body form (Smith and Patterson 1986; Patterson 1988, 1994; Mikrjukov 1998, 2000a; Mikrjukov *et al.* 2000). Despite its historical use as a taxon, the term "heliozoa" is now used only colloquially to describe organisms with a round body, no internal skeleton but with radiating stiff pseudopodia. Most organisms previously classified as

heliozoa are now assigned to the centroheliozoa (Centrohelida Kühn 1926), desmothoracids (Desmothoracida Hertwig and Lesser 1874), gymnosphaerids (Gymnosphaerida Poche 1913) or to the actinophryids (Actinophryida Hartmann 1913). We believe these groups to be monophyletic (Smith and Patterson 1986, Patterson 1999). Other genera of heliozoon-like protists have been placed within the nucleariid filose amoebae (Mikrjukov 1999a). Various helioflagellates are to be found in the dimorphids (*Dimorpha* and *Tetradimorpha*) and pedinellids (*Ciliophrys*, *Parapedinella*, *Actinomonas*, *Pteridomonas*, *Pedinella* and *Pseudopedinella*). A few genera (*Wagnerella*, *Actinolphus*, *Servetia*, *Sticholonche*, and *Pseudodimorpha*) remain of uncertain affinities (Mikrjukov 2000d, Mikrjukov *et al.* 2000).

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Actinophryid heliozoa are unflagellated organisms with tubular mitochondrial cristae. They can be distinguished by having axopodial axonemes formed of double polygonal spirals of microtubules, two types of extrusomes, with cysts having a layer of siliceous scales and within which autogamy occurs. The nuclei divide as a semi-open orthomitosis (Mignot 1979, 1980a, b, 1984; Patterson 1979, 1986). The most characteristic species are *Actinophrys sol* and *Actinosphaerium eichhornii* (Fig. 1).

There are only a few species of actinophryids, but they are the most frequently occurring heliozoa in freshwater habitats (see: Rainer 1968, Siemensma 1991). Actinophryids are recorded occasionally in soils and mosses (Sandon 1927, Geltzer 1993), or in marine and brackish environments (Jones 1974, Golemanski 1976, Bovee and Sawyer 1979, Mikrjukov 1996a, Mikrjukov and Patterson 2000). Like other heliozoa, they are passive predators, consuming motile prey which adhere to the axopodia (Patterson and Hausmann 1981, Grębecki and Hausmann 1993).

Major lineages of protozoa are identified by electron microscopy (Krylov *et al.* 1980; Corliss 1994; Patterson 1994, 1999; Mikrjukov 1999c). The actinophryids are one of the most intensively studied groups of free-living protists. Classical light microscopical observations (Hertwig 1899; Penard 1904; Bělař 1923, 1924) have been extended with ultrastructural studies on general morphology (Andersen and Beams 1960; Hovasse 1965; Tilney and Porter 1965; Patterson 1979, 1986; Shigenaka *et al.* 1980; Jones and Tucker 1981; Mikrjukov 1996a), growth and feeding (Ockleford and Tucker 1973, Suzaki *et al.* 1980a, Patterson and Hausmann 1981, Hausmann and Patterson 1982, Linnenbach *et al.* 1983, Pierce and Coats 1999, etc.), locomotive mechanisms (Ockleford 1974, Grębecki and Hausmann 1992, 1993), cyst formation (Patterson 1979, Patterson and Thompson 1981, Shigenaka and Iwate 1984, Shigenaka *et al.* 1985, Newman and Patterson 1993, etc.), asexual cell fusion (Toyohara *et al.* 1977, Shigenaka and Kaneda 1979, etc.), mitosis (Suzaki *et al.* 1978, Mignot 1984), and of autogamy in the cyst (Mignot 1979, 1980a, b). As freshwater actinophryids may often be maintained in culture with relative ease (Sakaguchi and Suzaki 1999), they have been exploited for a number of studies on microtubules (e.g. Tilney and Byers 1969; Roth *et al.* 1970, 1975; Roth and Pihlaja 1977; Suzaki *et al.* 1980b; Patterson and Hausmann 1982; Shigenaka *et al.* 1982;

Matsuoka and Shigenaka 1985; Suzaki *et al.* 1992, etc.). The actinophryid heliozoa can be regarded as a well studied group.

We here extend the revision of the taxonomy of heliozoa (Roijackers and Siemensma 1988; Siemensma and Roijackers 1988a, b; Siemensma 1991; Mikrjukov 1996b, c, 1997, 1999a, 2000c, d) to the actinophryids. Despite the extent of investigations of actinophryid biology and ultrastructure, species identities are very unclear. Individuals exhibit considerable variation in form- especially as a result of recent feeding history. In the absence of type material for any of the species, many of the identities remain ambiguous. We believe that it is now appropriate to review the composition of this family, removing those species which cannot be unambiguously identified. We use this opportunity also to comment on two ideas about the origins of the actinophryids - that they are related either to filose amoebae or to the pedinellid flagellates (Patterson 1986, 1989).

MATERIALS AND METHODS

Actinophrys salsuginosa was isolated from the brackish-water pond Swanpool (Falmouth, England) with salinity varying from 1 to 19‰. The heliozoon was isolated from an organically enriched sample from the shore line, and was maintained in 20% sea-water in Evian water and fed twice weekly with washed *Tetrahymena* or *Colpidium*. Light-microscopy and electron-microscopy was carried out as described elsewhere (Patterson 1979, 1980), except that fixatives etc. for electron-microscopical investigations were made up in distilled water or in 20% calcium-free sea water.

The growth of the new species and of *A. sol* was investigated in a range of salinities corresponding to 0-40% sea water. Nine replicate cultures, each initiated with five heliozoa, were made up with 0.5 ml medium of each salinity, to which were added equal numbers of washed *Tetrahymena vorax* cells. The food organisms did not survive at salinities greater than 40% sea water. The cultures were kept in humid chambers at 16°C, and the number of heliozoa counted daily. The size of uncompressed cells was measured microscopically at the end of study. A fixed sample of the cultures from which the type-series of *A. salsuginosa* was taken has been deposited as type material at the Natural History Museum, London (Department of Palaeontology) as a resin-embedded block of material (N PR 138). Living material was lodged at the Culture Collection for Algae and Protozoa, England.

Samples containing the *Ciliophrys azurina* were collected from East Point and Lee Point, Darwin (Northern Territory, Australia) in September 1994 using the procedures outlined by Larsen and Patterson (1990) and Patterson and Simpson (1996).

We refer to photographs as reference type material for new species. This practice is accepted under the guidelines of the International Code for Botanical Nomenclature, but not under the guidelines of the International Code for Zoological Nomenclature. We have

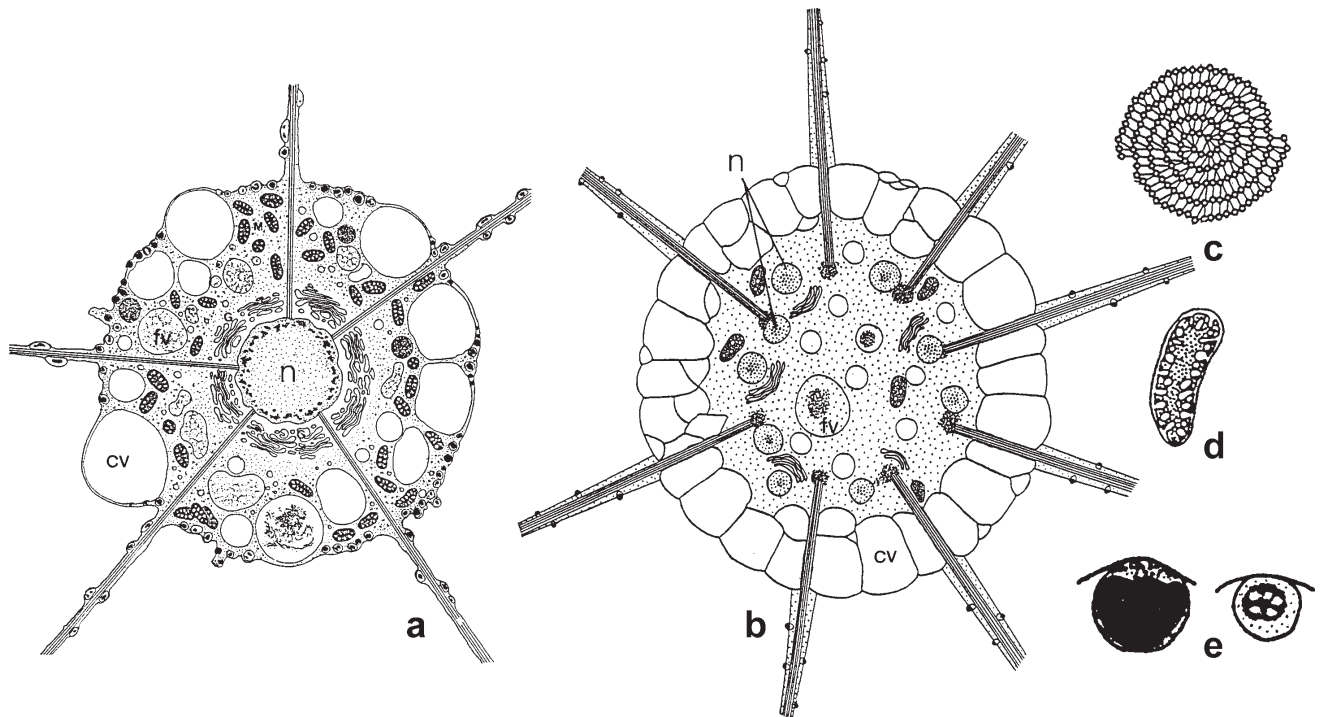


Fig. 1. General structure of actinophryid heliozoa: **a** - *Actinophrys sol* (after Siemensma 1991); **b** - *Actinosphaerium eichhornii*; **c** - axonemal lattice; **d** - mitochondrion; **e** - two types of extrusomes. cv - contractile vacuole, fv - food vacuole, n - nucleus

adopted the practise of using uninterpreted illustrations as type material as we know of no effective alternative of providing unambiguous identities for these small protists, and because we believe that the use of uninterpreted records is compliant with the spirit of the ICZN.

TAXONOMY

Diagnostic criteria

As noted above, species identities among actinophryid heliozoa are not well established, and are mostly based on variations in size and vacuolation of the cell. Different isolates of actinophryids exhibit subtle yet persistent differences in appearance (Sondheim 1916, Shigenaka *et al.* 1980, this study). There is considerable intraspecific variation for this group, especially associated with feeding and excystment (e.g. Patterson and Hausmann 1981).

Identities of taxa cannot be corroborated by reference to the biological species concept. The only form of sexual activity recorded for actinophryid heliozoa is a process of the autogamy involving a fusion of gametes

within the cyst (Bělař 1923, 1924; Peters 1964, 1966; Mignot 1979, 1980a, b). Each cell represents an independent genetic lineage, comparable to asexual organisms. Biological species concepts are not applicable. We therefore apply the concept that species are groups of more than one individual which can be distinguished unambiguously and consistently from other groups of individuals by discontinuities in one or more intrinsic attributes, but which contain no groups which satisfy the same definition. In this case the discontinuities are established by the microscopical appearance of the trophic and encysted organisms.

We attach importance to the word “unambiguously” in the definition above, and place in the same entity, taxa which cannot be easily distinguished. Entities with an appearance which falls within known intraspecific variation do not meet the condition of allowing those nominal taxa to be readily identified. Previous authors have also come to the same conclusion and the taxonomic history of this taxon is characterized by extensive synonymies (e.g. Leidy 1879, Rainer 1968). We recognize only four previously described species which can be distinguished by one or more characters that are exclusive to them.

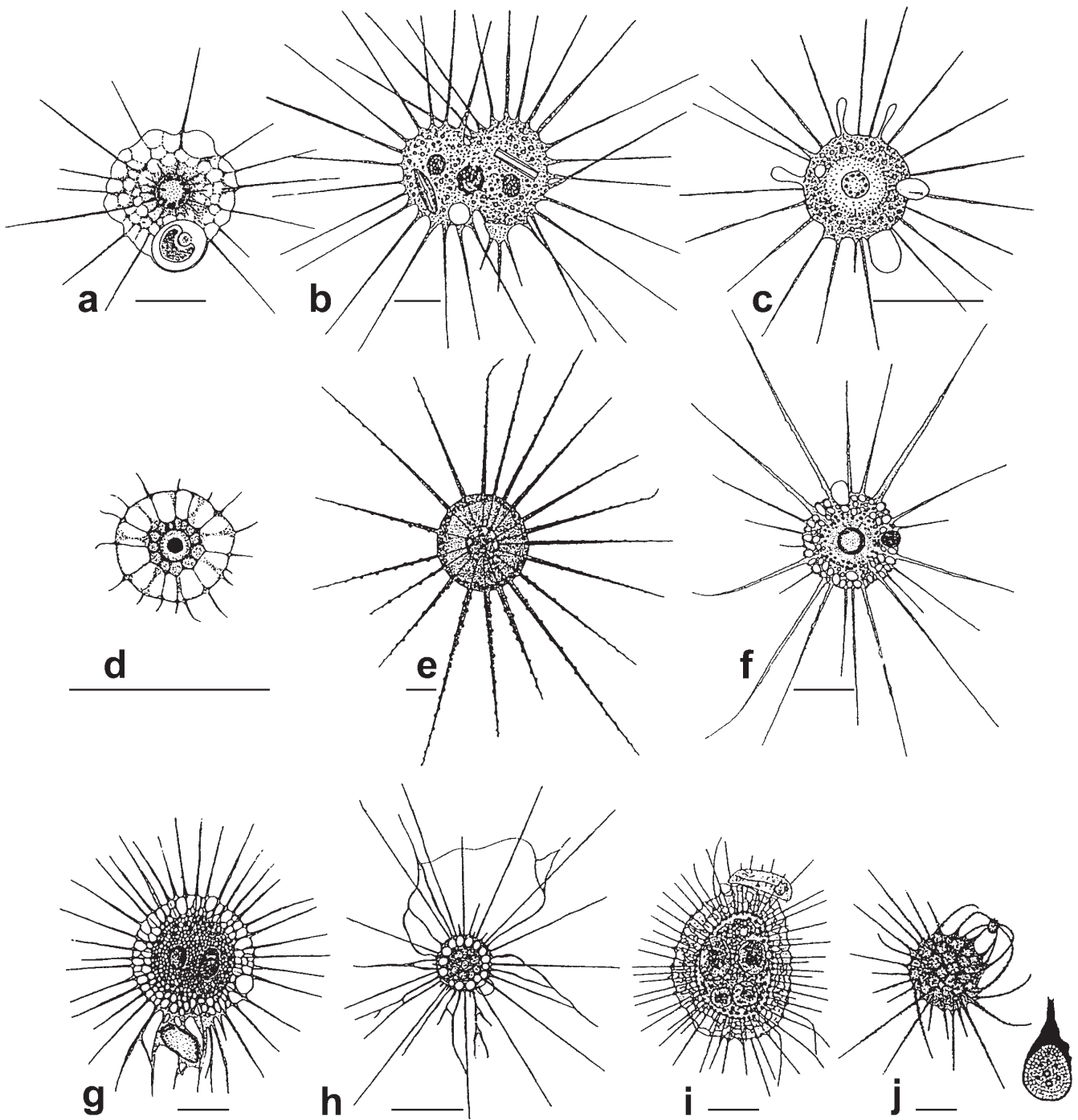
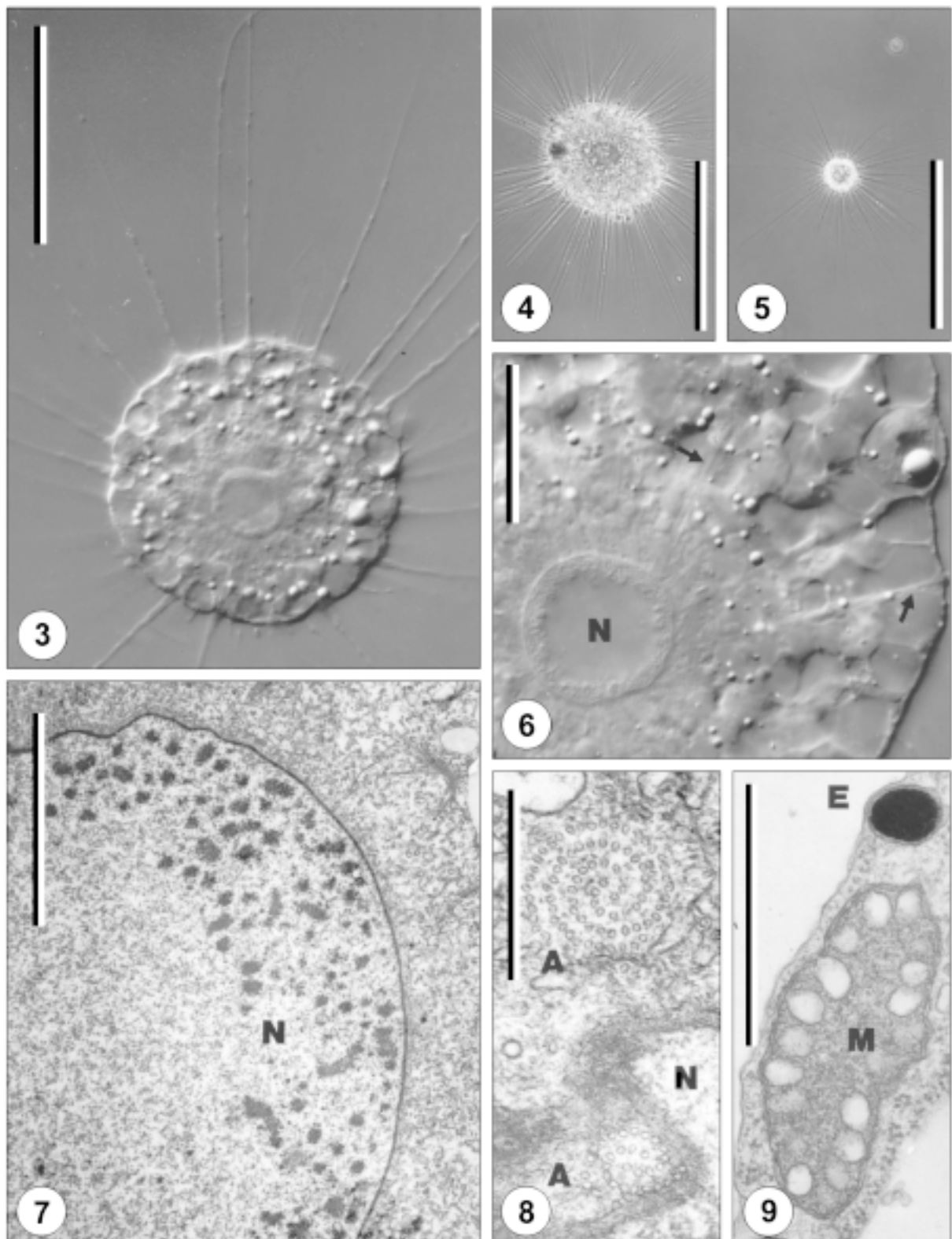


Fig. 2. Actinophryid heliozoa: **a** - *Actinophrys sol* (after Grenacher 1869); **b** - *A. subalpina* (after West 1901); **c** - *A. vesiculata* (after Penard 1901); **d** - *A. pontica* (after Valkanov 1940); **e** - *A. tauryanini* (after Mikrjukov 1996 a); **f** - *A. salsuginosa* sp. n.; **g** - *Actinosphaerium eichhornii* (after Rainer 1968); **h** - *A. arachnoideum* (after Penard 1904); **i** - *A. nucleofilum* (after Barrett 1958); **j** - *Camptonema nutans* and base of axoneme (after Schaudinn 1894). Scale bars - **a-f** - 50; **g-j** - 100 µm



Figs. 3-9. *Actinophrys salsuginosa* sp. n.; 3-5 - general views, differential interference microscopy (3) and phase contrast microscopy (4, 5) of living cells; 6 - the nucleus (N), the cortical cytoplasm, and axonemes (arrows) in a living cell, differential interference microscopy; 7 - transmission electron micrograph showing nucleolar material as a layer of small aggregations at the periphery of the nucleus; 8 - axonemes (A); 9 - mitochondrion. E - an extrusome with dark homogeneous contents. Scale bars - 3 - 50; 4 - 100; 5 - 300; 6 - 10; 7 - 35; 8, 9 - 2 μ m

The history of Actinophryidae Dujardin, 1841

The first observations of heliozoa are probably those of Joblot (1718), although the kind of heliozoon he observed is not clear. The earliest unambiguous descriptions of actinophryid heliozoa were made by Ehrenberg (1830) of *Actinophrys sol*. This is the type-genus for the family and type-species for the genus (Rainer, 1968). Ehrenberg used a specific name for organisms of uncertain identity described by Müller (1773, 1786) under the name *Trichoda sol*. The recognition of the actinophryid type of organization as distinctive is ascribable to Dujardin (1841) who was the first to use the root “actinophry-” in the name of a suprageneric taxon. He did not employ a latinised name (referring to the “family Actinophryiens”), but it is to him that we assign nomenclatural authority for (all) the suprafamilial ranks based on this root. The first use of a latinised family name (Family Actinophryina) is that of Claparède and Lachmann (1858). The actinophryids are now typically assigned ordinal rank in traditionalist classification schemes (e.g. Levine *et al.* 1980, Cachon and Cachon 1982, Febvre-Chevalier 1985, Siemensma 1991). The first use of this rank is attributable to Hartmann (1913). The taxon has been placed of class by Krylov *et al.* (1980), Karpov (1990), Corliss (1994), and Kussakin and Drozdov (1998), and has been incorporated in unranked schemes by others (Patterson 1994, 1999).

Generic composition of the actinophryids

The most recent review of heliozoan taxonomy (Siemensma 1991) included two genera in the actinophryids. They are the uninucleate *Actinophrys* Ehrenberg, 1830 and multinucleate *Actinosphaerium* Stein, 1857. We agree with this view. Other reviews refer to four or five actinophryid genera.

Hovasse (1965) divided the genus *Actinosphaerium*, creating *Echinosphaerium* or *Echinosphaerium* (the paper is ambiguous in respect to the preferred spelling) on the basis of whether all axopodia terminate on nuclei (*Echinosphaerium*/*Echinosphaerium*) or not (*Actinosphaerium*). This distinction is maintained by Shigenaka with co-authors (1980).

Trégouboff (1953) recognised four actinophryid genera: *Actinophrys*, *Actinosphaerium*, *Camptonema* Schaudinn, 1894 and *Vampyrellidium* Zopf, 1887. *Vampyrellidium* has been shown to be a nucleariid (Patterson *et al.* 1987). *Camptonema* was recognised

by Rainer (1968), but as the axonemes of this monotypic genus terminate on the nucleus, we regard it as a junior synonym of *Actinosphaerium* Febvre-Chevalier (1985) recognised *Actinophrys*, *Actinosphaerium*, *Echinosphaerium* and *Camptonema*.

Levine *et al.* (1980) and Sleight *et al.* (1984) include the helioflagellate genus *Ciliophrys* Cienkowski, 1876.

What makes actinophryid heliozoa distinctive

Actinophryid heliozoa are round bodied unicellular organisms. There are no cilia or flagella. There is a single central nucleus or many small nuclei located in the central part of the cell (the endoplasm). Numerous stiff arms or axopodia, noticeably tapering from the base to the tip, radiate from the whole body surface. The axopodia are supported internally by microtubules arranged in a double hexagonal spiral and terminate in electron-dense material located on the nuclear envelope or near a nucleus. Mitochondrial cristae are tubular (bleb-like) and have an electron-dense matrix. There are two types of simple extrusomes - a larger osmiophilic type and a smaller granular type. The surface of trophic cells is naked. Actinophryids feed mainly by predation, often accompanied by fusion of several cells. Cysts may form which have multiple walls, one of which is comprised of siliceous elements. Reproduction is mainly by binary fission. Sexuality is limited to autogamy and occurs in the cyst and is accompanied by the formation and subsequent fusion of amoeboid gametes.

Diagnoses and discussion of the genera and species

Actinophrys Ehrenberg, 1830

Diagnosis. Uninucleate actinophryid heliozoa in which the axonemes terminate on a central nucleus.

Remarks. The taxonomic history of the genus and of its type species is confused. Müller’s (1773) original reference to it as *Trichoda sol* reappears in a later work (Müller 1786) but there is no specific indication that the drawings were made from the same material as used for the original description. These drawings might well (but not certainly) relate to a uninucleate actinophryid. The species name was reassigned to the genus *Peritricha* by Bory de St. Vincent (1824), but without any further new observations. Ehrenberg (1830) provided the first unambiguous description of this organism, identifying it with the organism described by Müller. The status of the

genera *Trichoda* and *Peritricha*, and of the numerous species originally included in them, is obscure. Corliss (1979) regards *Trichoda* as a *nomen oblitum*. Neither generic name appears to have been in contemporary use and we are unaware of the designation of any type-species for either genus. Both names are held to be *nomina dubia*, in that the taxa are not well circumscribed and it is no longer clear to what organisms these taxa refer. For this reason, and in order not to introduce nomenclatural confusion to a well circumscribed genus, the generic name *Actinophrys* is retained.

Recent accounts include different number of species in this genus. Rainer (1968) includes four species: *A. sol* (Müller, 1773) Ehrenberg, 1840, *A. subalpina* West, 1901, *A. vesiculata* Penard, 1901, and *A. pontica* Valkanov, 1940. Siemensma (1991) considers only *A. sol*, and regards *A. vesiculata* and *A. subalpina* as synonyms of it. He makes no comments in respect of *A. pontica*. Mikrjukov (1996a) described a new marine species *A. marina* using a species name preoccupied by Dujardin (1841) for species previously synonymized with *A. sol* by Rainer (1968) and for which we introduce *A. tauryanini*.

***Actinophrys sol* (Müller, 1773) Ehrenberg, 1840 (Fig. 2a)**

Diagnosis. *Actinophrys* with a body measuring about 50 (19-90) µm in diameter, with heterochromatin forming a continuous layer under the nuclear envelope; cyst wall with flat siliceous scales.

Remarks. The species to which the name *Actinophrys sol* refers to is ambiguous because of the absence of type material. This problem is compounded by phenotypic variability of actinophryids - there being considerable variation of form as a function of feeding history. Many nominal species assigned to this genus are now regarded as synonyms of this species (for lists see: Rainer 1968). Despite being extensively studied (e.g. Bělař 1923, 1924; Ockleford and Tucker 1973; Mignot 1979, 1980a, b, 1984; Patterson 1979; Patterson and Hausmann 1981; Newman and Patterson 1993), there have to date been no features which allow the unambiguous separation of species in this genus. As indicated below, we now rely on the appearance of plates in the cyst to distinguish *A. sol* from *A. salsuginosa*. In the absence of previously designated type material, we apply *Actinophrys sol* to organisms which satisfy

the description based on culture LB 1502/2 from the Culture Collection for Algae and Protozoa (Patterson 1979).

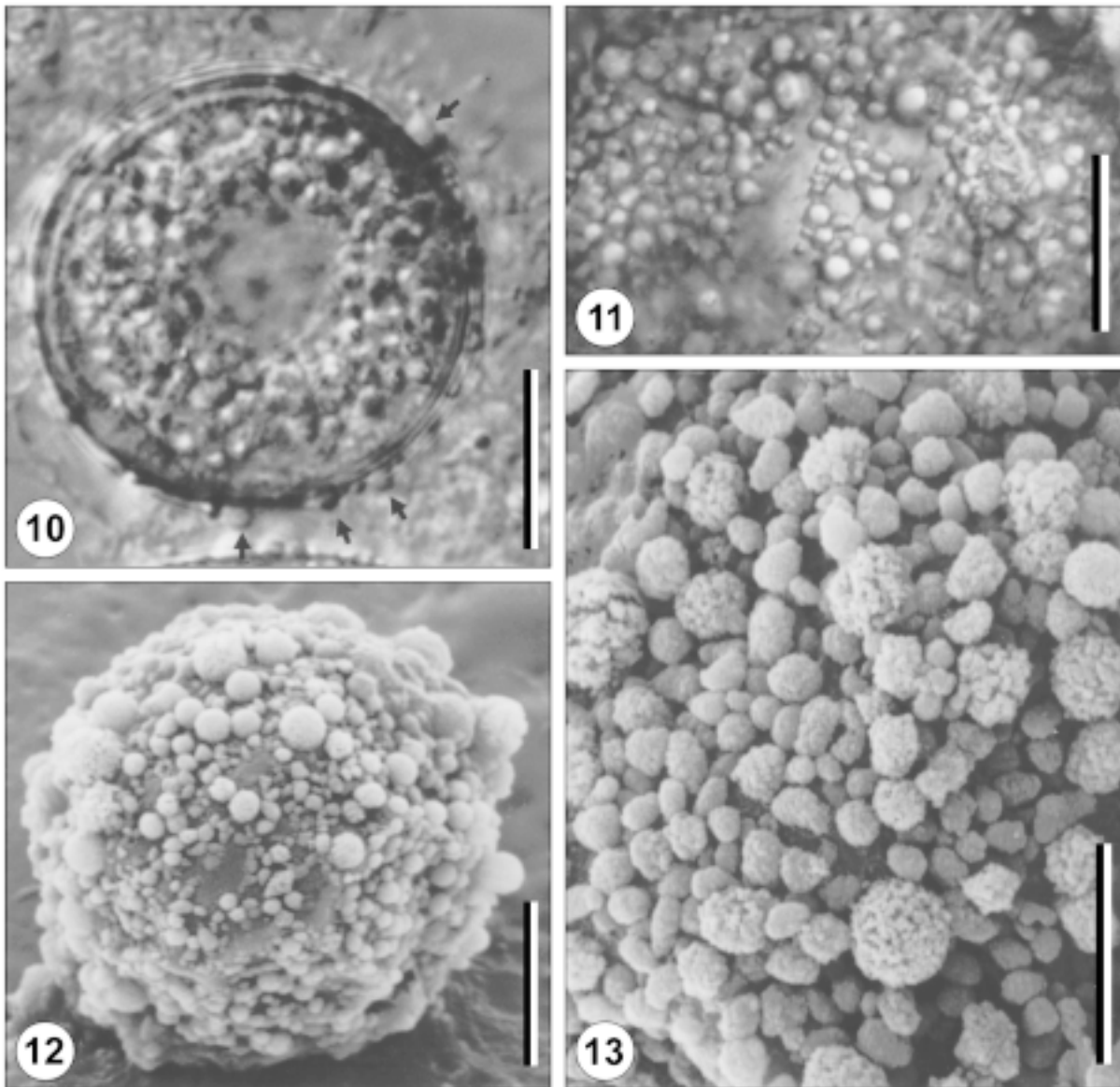
West (1901) described *A. subalpina* (Fig. 2b) as a species of *Actinophrys* having a spherical body 42-61 µm in diameter, and finely granular cytoplasm and no peripheral vacuoles. As peripheral vacuolisation is a function of the recent feeding history of the organism (Patterson and Hausmann 1981), this description could equally well apply to individuals of *A. sol*. No type material designated in the original description. It was published with a single figure (plate 30, Fig. 36) herein designated as lectotype. *A. subalpina* cannot be unambiguously distinguished from *A. sol*, and in agreement with Penard (1904), we regard *A. subalpina* as a subjective junior synonym of *A. sol*.

Penard (1901) described *A. vesiculata* (Fig. 2c) as a species of *Actinophrys*, 25-30 µm in diameter, with pendulous vacuoles and nucleoli in the form of condensed spheres. Penard (1904) subsequently questioned his own observations on the nucleoli. No other original observations have been made on this species. No type material was designated in the original description, which was published with three figures [Figs. 2-4 (by Penard)] of which Fig. 2 (by Penard) is herein designated as lectotype. The «pendulous» vacuoles would be mechanically unstable structures and we concur with Rainer (1968) that they were probably caused by pressure from the cover-slip and that Penard (1901) observed *A. sol*. *A. vesiculata* is held to be a junior subjective synonym of *A. sol*.

***Actinophrys pontica* Valkanov, 1940 (Fig. 2d)**

Diagnosis. *Actinophrys* species measuring about 12 µm, the nucleus with a central spherical nucleolus.

Remarks. Valkanov described this organism from the Black Sea brackish-water habitats, and it was subsequently redescribed by Jones (1974) and recorded by Febvre-Chevalier (1990). Neither account is explicit as to whether living material was observed. There was no designation of type material in the original publication, but there were two figures (1 and 2), of which figure 1 is herein designated lectotype. By virtue of its small size, and distinctive nucleolar location, this species may be distinguished from *A. sol*. This species is said to have very marked peripheral vacuolization, but this may reflect recent feeding history and we do not regard this as



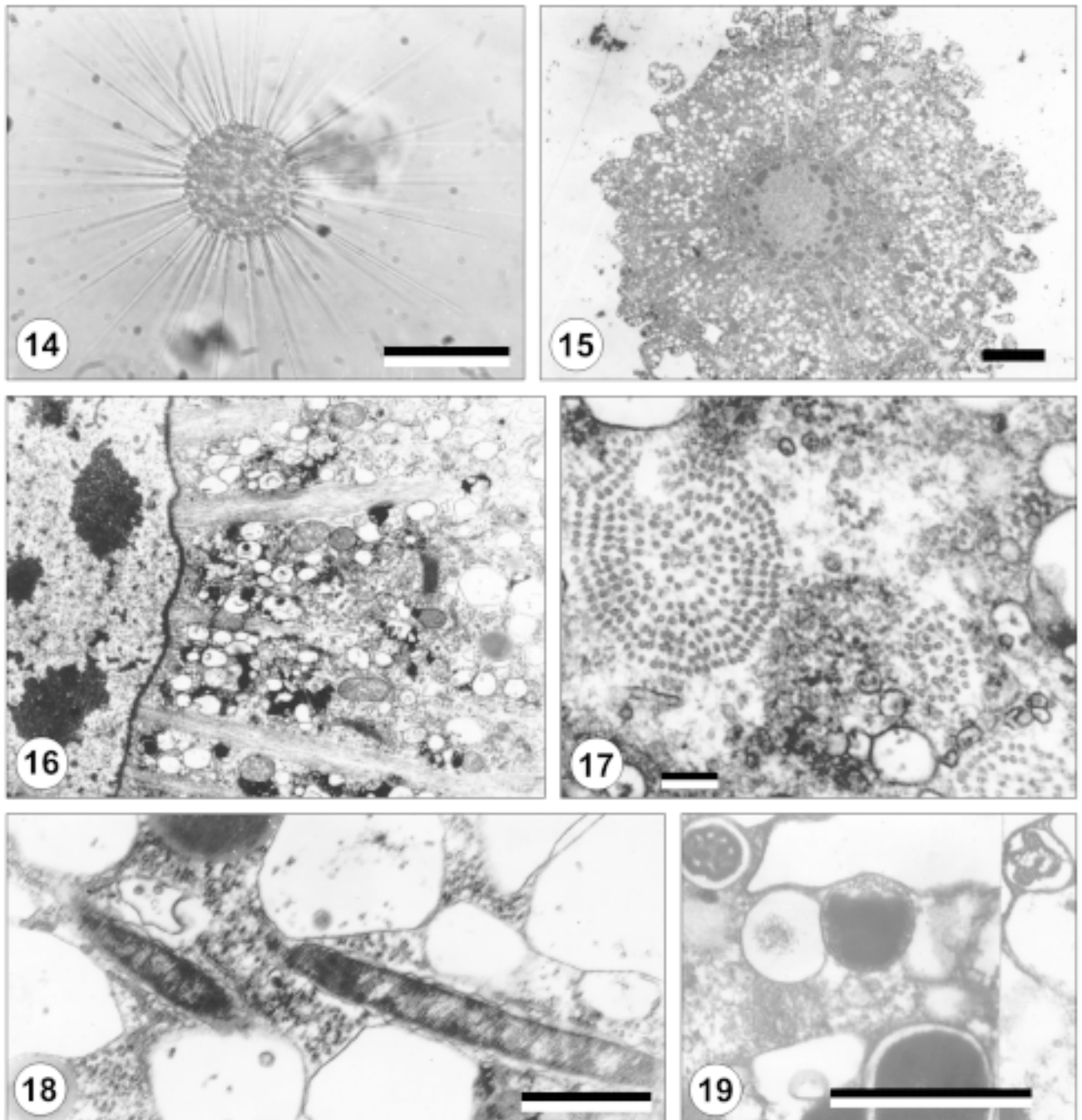
Figs. 10-13. Cysts of *Actinophrys salsuginosa* sp. n.; **10** - general view; **11-13** - spherical nature of siliceous elements of the wall; **10,11** - light microscopy; **12, 13** - scanning electron microscopy. Scale bars - **10, 12** - 10; **11** - 5; **13** - 2.5 μm

a reliable diagnostic feature. Further work is required, specifically to ensure that individuals of *Ciliophrys* were not observed. Mikrjukov (1999b) observed *Actinophrys* sp. in coastal Black Sea water (with a salinity of 1.8%), about 30 μm in diameter, but corresponding more to the characteristics of *A. sol*.

***Actinophrys salsuginosa* Patterson, sp. n. (Fig. 2f).**

Diagnosis. *Actinophrys* species measuring about 29-114 μm , with nucleolar material forming a peripheral layer of small aggregates, and with a cyst incorporating spherical siliceous elements.

Description. The size of the trophic organism is quite variable. The average diameter of non-feeding cells, under the culture regime described above, is 44.2 μm . The arms extend about 150-200 μm from the body. The dimensions of the body vary depending upon the recent feeding history and the salinity of the medium. During feeding, very large masses of cells may form, but they do not adhere strongly to the substrate. After feeding, uninucleate cells separate from the fused masses. Initially these have a diameter of about 95-100 μm , but after a day or so, the majority of the cells have a body diameter about half this value. Consequently, a fre-



Figs. 14-19. *Actinophrys tauryanini*; **14** - general view by light microscopy; **15** - cross-section through the median part of the cell; **16** - central area of the cell showing a peripheral part of the nucleus with nucleoli as large aggregations and the inner parts of the axonemes; **17** - axonemes in cross-section; **18** - rod-like ectoplasmic bacteria; **19** - two types of extrusomes: large ones with a homogeneous content, and smaller ones with a heterogeneous, microgranular content. **15-19** - transmission electron microscopy. Scale bars - **14** - 100, **15-16** - 10; **17-19** - 1 μ m

quency distribution histogram of body diameters in a population tends to be bimodal. The extreme dimensions encountered for the body were 29 μ m and 114 μ m. Cells grown in a medium of zero salinity had an average

diameter of 61 μ m, while the average diameter of cells grown in 40% sea water was 40.5 μ m. Higher concentrations were not investigated because the prey became moribund in these salinities. The nucleolar substance/

heterochromatin forms a layer 3-4 μm thick under the membrane of the centrally located nucleus (Figs. 3, 6, 7). Ultrastructural observations confirm that: the axonemes radiate from the nuclear envelope and are formed of double interlocking spirals of microtubules (Fig. 8), there are electron-dense extrusomes, and mitochondria have bleb-like cristae and a dark matrix (Fig. 9). As with other actinophryids, *A. salsuginosa* is able to form cysts with multiple layers in the wall (Fig. 10). Sintered siliceous beads mostly 0.3-1.0 μm in diameter form a layer of the cyst wall (Figs. 11-13).

Remarks. The organism isolated from Swanpool was identified as a member of the genus *Actinophrys* because of the stiff radiating arms, with axonemes comprised of double interlocking spirals of microtubules and terminate on a large central nucleus, and because of the siliceous material in the cyst (Fig. 6). Calkins gives a figure of a marine *Actinophrys* from Woods Hole which is similar to *A. salsuginosa* (Calkins 1902).

Actinophrys salsuginosa resembles *A. sol* closely in general appearance and size of trophic individuals. It can be distinguished from *A. sol* (*sensu* Patterson 1979) because of the arrangement of the nucleolar material and by the spherical (as opposed to flattened) shape of the siliceous elements of the wall. The body size of *A. salsuginosa* is similar to that of *A. sol*, although the new species has a more vacuolated outer region, and more delicate (longer and thinner) arms. The two species differ in their tolerance of saline conditions. *A. sol* did not survive in salinities greater than 20% sea water. *A. salsuginosa* continued to grow actively in 40% salinity. Under similar salinity conditions, the new species was slightly larger, more vacuolate, with fine arms, formed larger masses during feeding; these masses did not adhere to the substrate to the same extent as those of *A. sol*. *A. salsuginosa* differs also from the marine *A. tauryanini* (see below) because it is usually half the size, has a different nucleolar morphology, is tolerant of low salinity regimes, and may have a layer of large peripheral vacuoles. *A. salsuginosa* can be distinguished from *A. pontica* by the nucleolar configuration.

Two ultrastructural characteristics of *A. salsuginosa* suggest a close relationship to *Actinosphaerium nucleofilum*. They are: location of the nucleolar substance as a peripheral layer of small grains (Anderson and Beams 1960, Shigenaka *et al.* 1980), and siliceous components of the cyst wall of both are sintered spheres (Patterson and Thompson 1981).

***Actinophrys tauryanini* (Mikrjukov, 1996) nom. nov. (Figs. 2e, 14-19)**

Diagnosis. Marine *Actinophrys* species measuring 70-90 μm ; the nucleus with large peripheral clumps of the nucleolar material; without contractile vacuoles.

Remarks. This species was found in the White Sea (18-40 m depth), at salinities of 2.7-2.9‰, and was first reported as *A. marina*. This name was preoccupied by one introduced by Dujardin (1841) and a replacement name was required.

In size and with peripheral vacuoles, it resembles the taxon described as *A. subalpina* (Fig. 2 b). *A. tauryanini* grows well at oceanic salinities (35‰), but dies at salinities below 22-23‰. Organisms were maintained from 1992-1996 in the laboratory using marine diatoms and *Bodo* sp. as food. No peripheral layer of vacuoles was observed during feeding. Cells in culture are always solitary and did not fuse. Attempts to obtain cysts of this species were not successful. *A. sol* was recorded at the same time from in estuarine bays of the White Sea with salinities not exceeding 14 ‰ (Mikrjukov 2000b). Estuarine *A. sol* measured about 30-40 μm in diameter, had a transparent cytoplasm with large peripheral vacuoles, some of which behaved as contractile vacuoles. *A. tauryanini* can be distinguished from *A. sol* not only by being double the size, by having a fine vacuolisation of the cytoplasm, no spongiome nor contractile vacuoles, and by its tolerance of saline conditions. The marine species *A. tauryanini* differs from the brackish-water *A. salsuginosa* and *A. pontica* by the appearance of the nucleolar material. The species has also been recorded in the Tasman Sea (Mikrjukov and Patterson 2000).

The ultrastructure of *A. tauryanini* (Figs. 15-19) is similar to that of *A. sol* but it differs in having large clumps of the peripheral nucleolar material (Fig. 16) and by having rod-like cytoplasmic bacteria (Fig. 18).

***Actinosphaerium* Stein, 1857**

Diagnosis. Multinucleate actinophryid heliozoa in which the axonemes may or may not end on the nuclei.

Remarks. The genus *Actinosphaerium* was erected by Stein (1857) to accommodate *Actinophrys eichhornii* Ehrenberg, 1840 and was distinguished by the presence of large number of nuclei. Of the species which have been assigned to this genus, current reviewers (e.g. Trégouboff 1953; Rainer 1968; Febvre-Cheva-

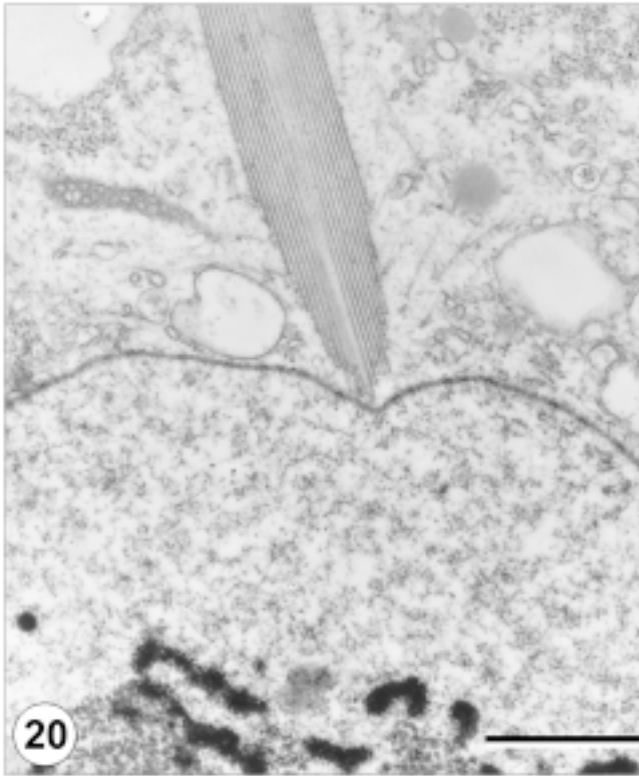


Fig. 20. Termination of an axoneme adjacent to the envelope of a nucleus of *Actinosphaerium eichhornii*. The central position of the nucleolus is clearly seen. Scale bar - 1 μm . Transmission electron micrograph by J. Robertson

lier 1985, 1990; Siemensma 1991) also accept *A. arachnoideum* Penard, 1904. Barrett (1958) added *A. nucleofilum*. Some aspects of the ultrastructure of *A. nucleofilum* were provided by Anderson and Beams (1960). Using this information, Hovasse (1965) erected a new genus for *A. nucleofilum*. The name was spelled *Echinosphaerium* and *Echinosphaerium* in the original paper, and is probably more commonly referred to under the latter spelling (Tilney and Byers 1969, Matsuoka *et al.* 1985). The appropriateness of Hovasse's action is discussed under *A. nucleofilum* below. Subsequently, and incorrectly, Shigenaka *et al.* (1980) assigned all of the above-named species to the genus *Echinosphaerium* and added two new species, *E. akamae* and *E. ikachiensis*, which were synonymized with *A. eichhornii* by Siemensma (1991) without any comments. The status of *A. portuum* Kufferath, 1952 included by Shigenaka *et al.* (1980) has received little attention. Kufferath's description makes no mention or inference of the number of nuclei, and cannot be admitted to a discussion of the genus *Actinosphaerium*, nor indeed, on the basis of the information provided, is there

a good case for regarding the organism as an actinophryid. In the discussion which follows we include the genus *Camptonema* Schaudinn, 1894 as it is a multinucleated heliozoan.

The genus has been subject to extensive ultrastructural study (Anderson and Beams 1960; Hovasse 1965; Kitching and Craggs 1965; Tilney and Porter 1965; Tilney and Byers 1969; Shigenaka *et al.* 1975; Schliwa 1976, 1977; Shigenaka 1976; Shigenaka and Kaneda 1979; Shigenaka *et al.* 1979, 1980; Toyohara *et al.* 1977, 1978, 1979; Suzaki *et al.* 1980a; Patterson and Thompson 1981, *etc.*). Similarities in the packing pattern of microtubules in axopodia, in extrusome morphology, mitochondrial appearance and cyst morphology, confirm that this genus is very closely related to *Actinophrys* and is probably derived from it (Smith and Patterson 1986).

***Actinosphaerium eichhornii* (Ehrenberg, 1840) Stein, 1857 (Fig. 2g)**

Diagnosis. *Actinosphaerium* species measuring typically 200-300 μm , with numerous nuclei, each usually 13-17 μm in diameter, with nucleoli clustered centrally in each nucleus.

Remarks. Originally described as *Actinophrys eichhornii* Ehrenberg, 1840, Borowsky (1910) has shown the number and dimension of nuclei to be sensitive to the recent feeding history, and a range of sizes from 11 to 21 μm has been reported in the literature (Cash and Wailes 1921, Penard 1904). The nucleolar material (heterochromatin) lies as a cluster of granules in the centre of each nucleus. At the moment, this is the only useful diagnostic criterion. Shigenaka and co-workers (1980) proposed several morphometric criteria by which species might be distinguished. They included the ratio of the diameter of endoplasm to the thickness of ectoplasm having to be always more than 2:1. There is no electron microscopical information on the structure of the cyst wall in *A. eichhornii*.

Penard (1904) described *A. arachnoideum* Penard, 1904 (Fig. 2h) based on observations of six cells, and stated that the body measures 70-80 μm , and the cell had 4-12 nuclei measuring 7-8 μm in width. Penard believed that *A. arachnoideum* was distinct from *A. eichhornii* because the cell had some pseudopodia without axonemes. The dimensions of the body fell within the range encountered in *A. eichhornii*. The second type of pseudopodia noted by Penard may be seen in many actinophryids, particularly if compressed by a cover slip. The small size and number of nuclei is the only means of identifying the organism. However, excysting specimens

of *Actinosphaerium* are uninucleate (Hertwig 1899). There is uncertainty as to the dimensions of the nuclei (Cash and Wailes 1921 vs Penard 1904). The number of nuclei varies according to the recent feeding history of the organism (Borowski 1910). In the absence of the other diagnostic features, there is no sound basis for retaining this as a valid species. The name is held to be a junior synonym of *A. eichhornii*.

***Actinosphaerium nucleofilum* Barrett, 1958 (Fig. 2i)**

Diagnosis. *Actinosphaerium* species measuring 230–400 µm, with small and numerous nuclei, 4–8 µm in diameter, with peripherally located nucleolar substance, and with a cyst wall incorporating spherical siliceous elements.

Remarks. Barrett (1958) distinguished this species from *A. eichhornii* because in *A. nucleofilum* some axopodia terminate on the surface of nuclei (see remarks to *Echinosphaerium* below), and the nuclei have the nucleolar material located peripherally. The diameter of the nuclei is very small, and this was confirmed by Shigenaka *et al.* (1980). The cysts have spherical siliceous elements in the wall (Patterson and Thompson 1981). We consider *A. nucleofilum* Barrett, 1858 as the second species of the genus *Actinosphaerium*. Currently, this is the most extensively studied of the multinucleated actinophryids.

Hovasse (1965) introduced the generic name *Echinosphaerium* / *Echinosphaerium* (both spellings were used in his paper) for actinosphaerids with axopodia terminating on the surface of nuclei - on the assumption that in *Actinosphaerium* axopodia do not end on nuclei. This is supported by observations of some authors (Bütschli 1882, Penard 1904, Valkanov 1940). Stein's original description of *Actinosphaerium* included no details on this feature. Hovasse (1965) identified *E. nucleofilum* (Barrett, 1958) Hovasse, 1965 as the type species of a new genus. Tilney and Porter (1965) provided ultrastructural evidence that some axopodia do end on the nuclei, but this is not always the case (Jones and Tucker 1981). The situation in *A. eichhornii* appears to be the same. Several workers have shown that axopodia end close to the nuclei (Roskin 1925, Rumjantzew and Wermel 1925). That some axopodia terminate against nuclei with central heterochromatin (i.e. are micrographs of *A. eichhornii*) has been confirmed ultrastructurally (Fig. 20). Electron micrographs similar to Fig. 20 have been obtained from *Actinophrys*

and *A. nucleofilum* (Allison *et al.* 1970, Shigenaka and Kaneda 1979, Tilney and Porter 1965, Toyohara *et al.* 1977, etc.). As *A. eichhornii* and *A. nucleofilum* may have axopodia terminating against nuclei or ending freely in the cytoplasm, and we do not believe that Hovasse's reasoning for erecting a new genus is justified. The generic names *Echinosphaerium* and *Echinosphaerium* are held to be synonymous with *Actinosphaerium* Stein, 1857.

Shigenaka *et al.* (1980) described *Echinosphaerium ikachiensis* Shigenaka, Watanabe et Suzuki, 1980 as a species of *Echinosphaerium*, with body diameter 186–436 µm, and with nuclei (diameter 9–15 µm) with peripheral clots of the nucleolar material. Some details of the description of this taxon, especially the ratio of endoplasm to ectoplasm (3–16:1) generate the same uncertainties as raised with *A. akamae* (see below). No information was given on axonemal termination nor on the structure of the cysts. Fig. 3c from the work by Shigenaka *et al.* (1980) shows sparse peripheral clots of the nucleolar material in a nucleus, and in this character the taxon is similar to *A. nucleofilum*. Despite consistent differences between isolates observed by Shigenaka *et al.* (1980) we do not believe that this taxon could be unambiguously distinguished from *A. nucleofilum*, and we regard *E. ikachiensis* as a junior synonym of *A. nucleofilum*.

***Actinosphaerium akamae* (Shigenaka, Watanabe et Suzuki, 1980) nov. comb.**

Diagnosis. A species of *Actinosphaerium* measuring 82–244 µm, with nuclei (diameter 8–13 µm) with nucleolar material as a central cluster of granules, and with a cyst wall not incorporating siliceous components.

Remarks. Much of the information provided about this species relates to the dimensions of the cell, and proportions of endoplasm and ectoplasm (morphometric characters). The proportions of endoplasm to ectoplasm (2–14:1) appear to have been calculated (rather than measured) using the minimum of one value against the maximum of the other. If the values are summed, they give sizes for cells outside the range described in the paper. The cyst of *A. akamae* does not incorporate siliceous components, but is composed of several organic (i. e. mucous, granular, fibrillar and electron-dense ones) layers (Shigenaka *et al.* 1985). Originally named *Echinosphaerium akamae* but, for reasons given above, now assigned to *Actinosphaerium*. The appearance of nuclei is similar to that of *A. eichhornii* although they

are reported as slightly smaller. In view of the discrepancies over reports of nuclear size in *A. eichhornii* and in view of the variability of this character (see above), this aspect requires reinvestigation. While there is little doubt that different stocks investigated by the Japanese workers exhibited consistent and identifiable differences from which species, there are no absolute characters by which *A. akamae* can be identified except the absence of siliceous elements from the cyst. Given the overall similarity of this taxon to *A. eichhornii*, the uncertainty over some of the distinguishing characters, we treat the taxon as *nomen dubium* until the unusual nature of the cyst is confirmed and/or other discriminatory characters emerge.

***Camptonema* Schaudinn 1894**

The genus *Camptonema* was created by Schaudinn (Schaudinn 1894) to accommodate a single marine species, *C. nutans* Schaudinn, 1894 (Fig. 2J). This organism has not been recorded since its original description. It has body with a diameter of 120-180 µm, vacuolated ectoplasm, and granular endoplasm. At the periphery of the endoplasm there are about 10 oval nuclei, about 15 µm long. This species is normally not admitted to the genus *Actinosphaerium* because a cone of dense material surrounds the axoneme where it terminates from the nucleus. However, cone-like aggregations of material around *Actinosphaerium*, and the species name *C. nutans* a junior subjective synonym of *A. eichhornii*. *A. eichhornii* has been recorded in estuarine bays of the White sea with a salinity not exceeding 1.0‰ (Mikrjukov 2000b).

The genus *Ciliophrys* Cienkowski, 1876

Several authors (Levine *et al.* 1980, Cachon and Cachon 1982) include the flagellated genus *Ciliophrys* Cienkowski, 1876 among the actinophryids. This follows arguments of the close affinities of these two groups (Davidson 1972, 1982). Although the case for such affinity is attractive, it is quite clear that *Ciliophrys* has more characters in common with the pedinellids - sharing with them flagellar and cytoskeletal organization (Zimmermann *et al.* 1984, Patterson and Fenchel 1985, Preisig *et al.* 1991 - see below). As discussed below we do not believe that the ciliophryids are a subset of the actinophryids, rather the converse. Our discussion and diagnosis of actinophryids (above) does not include this

genus. We describe here a new species which contributes to our understanding of relationships between these groups.

Ciliophrys is a naked and heterotrophic pedinellid with either no stalk or a short stubby stalk (unpublished ultrastructural information). It is distinguished from other pedinellid taxa without plastids because the arms radiate from the whole cell surface, and, while in the heliozoan state, has weakly active flagellum held in a figure of 8 configuration. The cell may convert into an arm-less form at which time the flagellum becomes more active and the pseudopodia are withdrawn. These arm-less cells usually swim with the flagellum directed to the front. The fine, non-tapering axopods are supported by single triads of microtubules. As with actinophryids and other pedinellids, the interior ends of these axonemes are associated with nuclei. The composition of the genus was discussed by Larsen and Patterson (1990). We currently admit two species, and here add a third.

***Ciliophrys infusionum* Cienkowski, 1876 (Fig. 21, a) (Syns: *C. marina* Caullery, 1909; *Dimorpha monomastix* Penard, 1921)**

This species is distinguished because it has non-tapering arms; the nucleus has a large central nucleolus, and because the arm-less form swims actively. *Ciliophrys infusionum* has been found in marine sites in SE North America, subtropical and tropical Australia, Denmark, England, English Channel, Fiji, Gulf of Finland, Hawaii, Mediterranean, Norway and equatorial Pacific (Lee and Patterson 2000).

***Ciliophrys australis* Schewiakoff, 1893**

This species is distinguished because it is not motile in the arm-less state. This species has not been observed since its original description. We suspect that this may prove to be the same as *C. infusionum*. The spelling *C. australiensis* by Larsen and Patterson (1990) is incorrect.

***Ciliophrys azurina* Patterson, sp. n. (Figs. 21b; 22, 23)**

Diagnosis. *Ciliophrys* with tapering arms; nucleus with a central nucleolus and additional peripheral heterochromatin.

Description. Cell 15 µm in diameter, with radiating arms with extrusomes. The single flagellum is held in

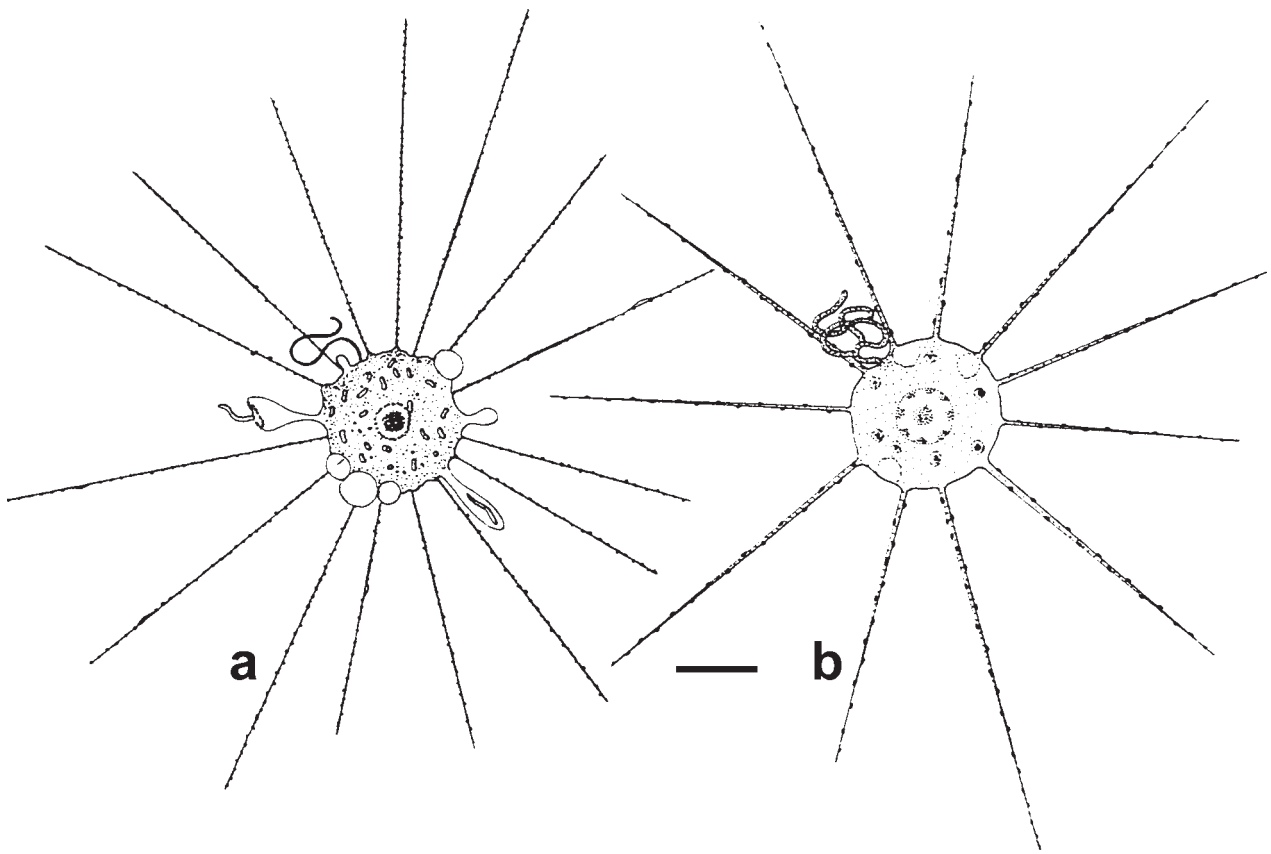
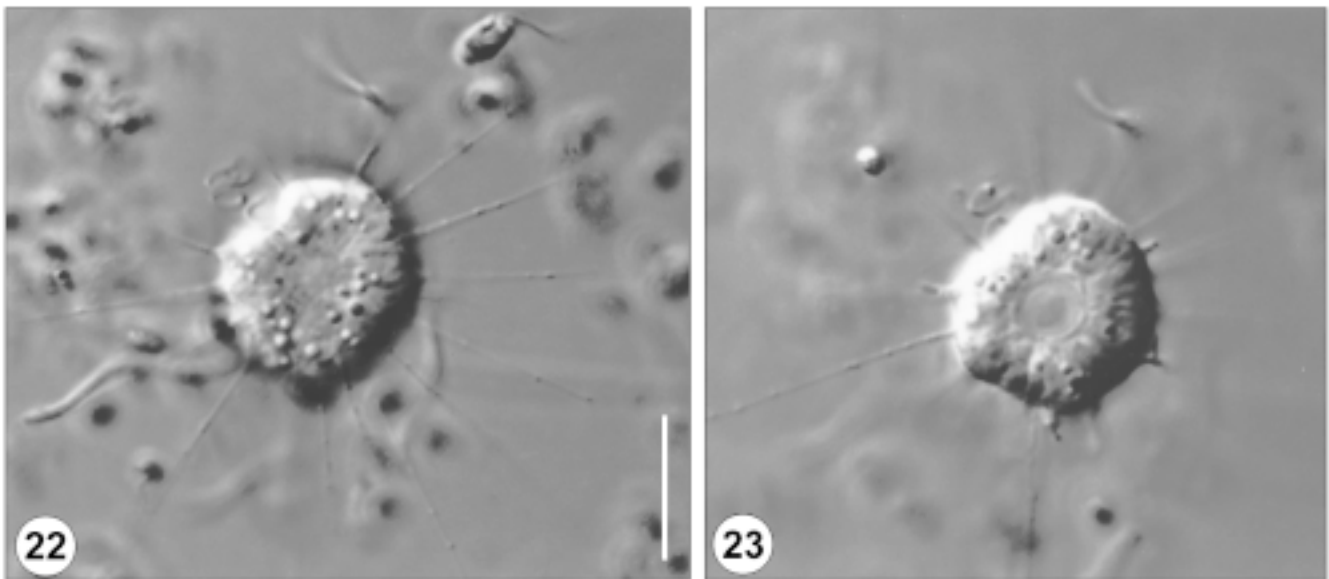


Fig. 21. Line drawings of (a) *Ciliophrys infusionum* Cienkowski, 1876 (after Siemensma 1991) and (b) *C. azurina* Patterson, sp. n.). Scale bar - 10 μ m



Figs. 22,23. *Ciliophrys azurina* sp. n., live cells viewed with differential interference microscopy; showing the double "figure of 8" flagellum, nucleus with a central nucleolus and additional peripheral aggregates of heterochromatin. Scale bar - 10 μ m

front of swimming cells, and in non-swimming (feeding) cells the flagellum is held tightly curled, typically in a double “figure of 8”. The nucleus is large, prominent and has a nucleolus and clumps of material located around the inner face of the nuclear envelope. Observed consuming diatoms.

Remarks. *Ciliophrys azurina* can be distinguished from the other well described species in the genus, *C. infusionum*, by being considerably larger (15 µm vs 5 µm, although we note that *C. infusionum* has been reported as up to 20 µm long). More importantly, *C. azurina* can also be distinguished because the flagellum is longer and held in a double “figure of 8”, because the arms taper from base to tip, and because of the existence of peripheral clumps of heterochromatin in the nuclei. These two characters are held in common with *Actinophrys* - and there is especial similarity with *Actinophrys pontica*. We interpret the tapering arms and peripheral heterochromatin as being apomorphic characters for a previously unrecognised clade which includes *C. azurina* and the two genera of actinophryids and which we here refer to as the heliomonads

Summary of the composition of actinophryids

Actinophrys Ehrenberg, 1830

A. sol (Müller, 1773) Ehrenberg, 1830

Synonyms: *A. difformis* Ehrenberg, 1830; *A. marina* Dujardin, 1841; *A. stella* Perty, 1852; *A. oculata* Stein, 1854; *A. tenuipes* Claparède and Lachmann, 1858; *A. fissipes* Lachmann, 1859; *A. longipes* Lachmann, 1859; *A. tunicata* Lachmann, 1859; *A. limbata* Lachmann, 1859; *A. paradoxa* Carter, 1864; *A. picta* Leidy, 1879; *A. alveolata* Schewiakoff, 1893; *A. subalpina* West, 1901; *A. vesiculata* Penard, 1901.

A. pontica Valkanov, 1940

A. salsuginosa Patterson, n. sp.

A. tauryanini Mikrjukov et Patterson, 2000

Actinosphaerium Stein, 1857

Synonyms: *Camptonema* Schaudinn, 1894, *Echinospaerium* Hovasse, 1965

A. eichhornii (Ehrenberg, 1840) Stein, 1857

Synonyms: *A. arachnoideum* Penard, 1904; *C. nutans* Schaudinn, 1894;

A. nucleofilum Barrett, 1958

Synonym: *E. ikachiensis* Shigenaka, Watanabe et Suzuki, 1980.

The evolution of the actinophryids

The evolutionary relationships of the actinophryid heliozoa among the protists has not previously been

resolved (Patterson 1994, 1999). Polyphyly of the taxon Heliozoa has been clearly established (Febvre-Chevalier 1982; Smith and Patterson 1986; Patterson 1988; Mikrjukov 1998, 2000a *inter alia*). There have been some arguments that heliozoa with axonemes terminating on the nucleus (i.e. actinophryids, desmothoracids, taxopodids) should be grouped together and separated from those (centrohelids, gymnosphaerids and dimorphid helioflagellates) with an axoplast or centroplast as a microtubule organizing centre. On the basis of this argument, the former have been grouped (sometimes with the ciliophryids) as the Cryptaxohelida (Febvre-Chevalier and Febvre 1984), or as the Actinophryidea (Karpov 1990) or the Nucleohelea (Cavalier-Smith 1993). As other characters, such as cell topology, organization of mitochondria, extrusomes, microtubule packing pattern, cyst morphology, life cycle, do not suggest that these taxa are closely related (Smith and Patterson 1986), we are of the view that the nuclear termination of axonemes is a homoplasious character (convergence) (Patterson 1999, Mikrjukov 2000a). We do not support an explicit or implicit argument that the affinities of the actinophryids lie with other heliozoa.

Two other proposals as to the affinities of the actinophryids have been discussed: (1) with filose amoebae; or (2) with the helioflagellate *Ciliophrys* and the other pedinellid flagellates (Patterson 1986, 1988).

The evolutionary affinity with filose amoebae was suggested by Trégouboff (1953). The amoebae generally and rhizopods are polyphyletic and are being replaced by a larger number of more restrictively circumscribed groups (Patterson 1999). Two types of amoebae have a gross similarity to the heliozoa - the vampyrellid and nucleariid filose amoebae.

The vampyrellid filose amoebae include *Vampyrella* (Hausmann 1977, Hülsmann 1982) and *Lateromyxa* (Hülsmann 1993, Röpstorff *et al.* 1993). Like actinophryids, they have mitochondria with tubular cristae. They have a number of additional features not found in actinophryids. They contain large electron-dense bodies which probably account for their orange colour. They have elaborate ribosomal arrays, often associated with digestion vacuoles. Vampyrellids have a peculiar mode of feeding which involves perforating the walls of algae and fungi, they produce digestion cysts which lack the actinophryid wall structure, and they do not undergo autogamy. These lack any clear affinity with the heliozoa.

The nucleariids include *Nuclearia*, *Vampyrellidium* and some taxa previously linked to heliozoa such as *Pompholyxophrys* and *Pinaciophora*. Nucleariids have

discoidal cristae in the mitochondria, no extrusive organelles, no siliceous elements in the cyst, no axonemes, and nuclear division profiles unlike actinophryids (Mignot and Savoie 1979; Patterson 1983, 1985; Cann 1986; Mikrjukov 1999a, c; Mikrjukov and Mylnikov 2000). Recent molecular data suggests affinities of nucleariids with other lamellicristate taxa (Mikrjukov and Mylnikov 2000, Amaral pers. comm.). No particular character or characters support a relatedness between nucleariids and actinophryids (Patterson 1986).

The suggestion of a phylogenetic link between actinophryids and *Ciliophrys* and other heterotrophic pedinellid helioflagellates has been discussed on several occasions (Davidson 1972, 1982; Patterson and Fenchel 1985; Patterson 1986, 1989). *Ciliophrys* has been regarded by some as a distinct type of heliozoon (Febvre-Chevalier 1985, Siemensma 1991) or as a type of actinophryid (Levine *et al.* 1980, Sleight *et al.* 1984). *Ciliophrys* is undoubtedly related to the pedinellid helioflagellates. Shared characters include: (1) the common presence of microtubular triad axonemes, (2) the axonemes ending on the nucleus, (3) the axonemes being linked by strands of fibrous material, (4) mitochondria with tubular cristae, (5) homogeneous extrusomes, (6) a single apical flagellum with adjacent barren basal body, (7) basal bodies attaching almost directly to the nucleus; (8) paraxonemal inclusions; (9) tripartite flagellar hairs; and (10) transitional helix or rings below the transitional plate (data on heterotrophic genera of pedinellids from Larsen 1985, Patterson and Fenchel 1985, Pedersen *et al.* 1986, Mylnikov 1989; data on plastidic genera: Swale 1969a, Throndsen 1971, Ostroff and van Valkenburg 1978, Zimmermann *et al.* 1984, Koutoulis *et al.* 1988, Thomsen 1988, Daugbjerg 1996a). Based on these characters, *Ciliophrys*, the other genera of heterotrophic pedinellid helioflagellates (e.g. *Pteridomonas* and *Actinomonas*), and those taxa with plastids (e.g. *Pedinella*, *Pseudopedinella*, *Apedinella*, and *Mesopedinella*) form a well circumscribed group of stramenopiles called the pedinellids. We now regard the pedinellids as a paraphyletic group and in line with arguments presented elsewhere (Patterson 1994, 1999) prefer to use this name only in its colloquial sense.

Pedinellids and actinophryids resemble each other in the nuclear termination of the axonemes, in having mitochondria with tubular cristae, and having extrusomes with electron-dense unstructured contents. The pedinellids and actinophryids differ in several major respects. Firstly, the actinophryids have no flagellum nor any flagellated stage in the life cycle. Secondly, the axopodial axonemes

in pedinellids and *Ciliophrys* are supported by triads of microtubules and not by a double polygonal spiral as is observed in all actinophryids. Thirdly, the cristae in the mitochondria of *Ciliophrys* and other pedinellids are tubular and not-bleb like; there are fine wisps of material within the cristae and crystalline deposits may be seen in the matrix of the mitochondria. Neither feature has been observed in the actinophryids. Indeed, the mitochondria of actinophryids more closely resemble those of many chrysophytes (*sensu* Hibberd 1976, 1986) than those of *Ciliophrys*. Fourthly, the extrusomes of *Ciliophrys* are smaller than the larger extrusomes of *Actinophrys* (Davidson, 1980; Patterson, unpubl.) but are similar to the smaller ones of *Actinophrys* (Linnenbach *et al.* 1983, Patterson 1986, Mikrjukov 1996a, Fig. 19). This type of extrusomes does not appear to have been recorded in other taxa except for those under consideration here, but large dark homogeneous structures have been observed in *Pseudospora* (Swale 1969b), xanthophytes (Hibberd 1981), *Olisthodiscus* (Leadbeater 1969), gymnosphaerid heliozoa (Mikrjukov unpubl.), etc. The value as phylogenetic markers is therefore debatable. Fifthly, both groups form cysts, but those of actinophryids have a complex envelope including a layer of siliceous artefacts, whilst the cyst envelope of pedinellids is purely organic (Hibberd 1986, Thomsen 1988). No sexuality has been reported in the cysts of pedinellids.

Despite the differences between actinophryids and ciliophryids, the new data on *C. azurina* gives credibility to the argument that ciliophryids and actinophryids are related. *C. azurina* combines features previously thought to be exclusive to the pedinellids (one hairy flagellum held in a "figure of 8", radiating actinopods which may be withdrawn in swimming cells) and features previously thought to be exclusive of the actinophryids (tapering arms with substantial axonemes, and clumps of condensed material around the inner periphery of the nucleus). We presume that the tapering axopodia have more than 3 microtubules. We conclude that there is a clade that includes some pedinellids and which has tapering axopodia and peripheral heterochromatin as apomorphic. This clade includes *Ciliophrys azurina* and the actinophryid heliozoa (Fig. 24).

Classification of the actinophryids and pedinellids

The only authors to previously use a taxon to house the actinophryids and all pedinellids are Karpov (1990) and Kussakin and Drozdov (1998). Both exploit a 'heterokont phylum' the Pedinellomorpha. However,

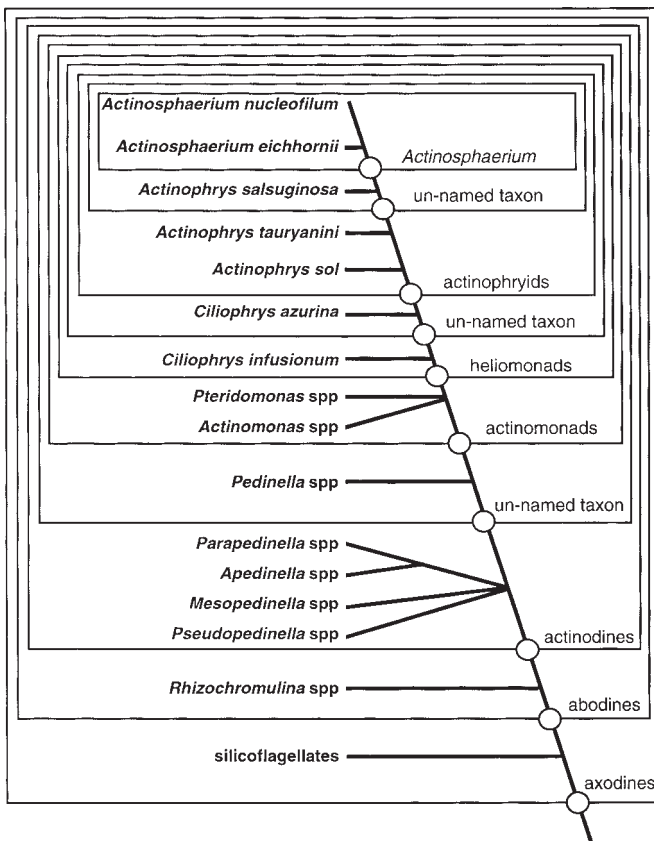


Fig. 24. Suggested relationships among the axodines with names for monophyletic and holophyletic groups, the taxa contain all of the descendants from the circle adjacent to the clade name. For details see the text

they include other heliozoa (desmothoracids and taxopodids) with the group. We do not regard these types of heliozoa as being related (Smith and Patterson 1986) and so regard this taxon as mis-conceived, and not identical to the clade which includes pedinellids and actinophryids.

We hold the view that nomenclature will be made less ambiguous if we introduce a new clade name as a new clade is identified. We propose the new term "actinodines" to refer to the pedinellids (including the ciliophryids) and actinophryids. Within this is a further clade with the synapomorphy of microtubule supported axopodia arranged with radial symmetry and which we refer to as the heliomonads. Our current hypothesis as to the inter-relationships among the actinodines is shown in Figure 24.

Affinities of the actinodines

The phototrophic pedinellid genera have traditionally considered as a taxon of various rank within the (strict)

chrysophytes (Pascher 1910, Christensen 1980, Lee 1980, Zimmermann *et al.* 1984, Cavalier-Smith 1986), or as a group with a more distant affinity to the strict chrysophytes (Hibberd 1976, 1986; Cavalier-Smith *et al.* 1995; Moestrup 1995). This latter view is based on a more sophisticated and defensible argument that the pedinellids differ from ochromonadine chrysophytes by virtue of the number of flagella, absence of rootlet structures such as the rhizoplast or the quadripartite microtubular root system, non-flagellar axonemes, number of plastids, absence of photosensory apparatus (stigma), absence of stomatocysts, etc.

On the basis of molecular and structural comparisons, the pedinellids have been linked with the silicoflagellates and the Rhizochromulinales (Cavalier-Smith *et al.* 1995, Moestrup 1995, O'Kelly and Wujek 1995, Cavalier-Smith and Chao 1996, Medlin *et al.* 1997). All have microtubular axonemes ending on the nucleus. These groups have been united as the axodines by Patterson (1994, 1999), the class Dictyochophyceae (Moestrup 1995, Preisig 1999), and as the class Actinochrysophyceae (Cavalier-Smith *et al.* 1995). Of these, the axodines are conceived as defined by synapomorphy and this concept is unaffected by the inclusion of the actinophryids.

Of the two other axodine groups, *Rhizochromulina marina* is amoeboid and has plastids (Hibberd and Chretiennot-Dinet 1979, O'Kelly and Wujek 1995) and seems to be most closely related to actinodines. Zoospores of *Rhizochromulina* resemble pedinellid cells in (1) the presence of a non-flagellated kinetosome, (2) lacking microtubular kinetosomal rootlets, (3) the position of the helix (or of two rings) underneath the transitional plate of the kinetosomes, (4) the posterior position of the Golgi apparatus. The close relatedness of pedinellids with *Rhizochromulina* is supported by molecular data (Cavalier-Smith *et al.* 1995, Cavalier-Smith and Chao 1996). The microtubules in pseudopods of *Rhizochromulina* are not fixed in number and not gathered in axonemes, and hence we do not consider rhizochromulinids inside the actinodines. We refer to the group (*Rhizochromulina* + actinodines) as the 'abodines'. The synapomorphy of abodines is the posterior location of the dictyosomes.

Silicoflagellates differ from the abodines because their dictyosomes are located to the sides of the nucleus and not posterior to it; and they have a well developed intracellular siliceous skeleton (Deflandre 1953, van Valkenburg 1971, Moestrup and Thomsen 1990). Like the abodines, they have microtubule-supported pseudopods with axonemes terminating on the nuclear envelope; a double ring below the transverse septum (van

Valkenburg 1980; Moestrup and Thomsen 1990; Moestrup 1992, 1995; O'Kelly 1993). Some pedinellids and silicoflagellates have a flagellar wing supported by a dense paraxial rod. Silicoflagellates have an unusual ring-like structure (perhaps a ring of opaque bodies) outside the axoneme at the level of the distal end of kinetosome.

The Pelagophyceae is the most probable sister taxon to the axodines (Andersen *et al.* 1993, Honda *et al.* 1995, Cavalier-Smith and Chao 1996, Potter *et al.* 1997). The pelagophytes include unflagellated and coccoid algae. The axodines and the pelagophytes are unflagellated stramenopiles with two rings inside the basal body, but there is no evidence of microtubular axonemes in the body of the pelagophytes. They probably form the next most proximate group of stramenopiles. Saunders *et al.* (1997) argue that the diatoms are the sister group to this cluster. Honda *et al.* (1995) include *Sulcochrysis* within this territory. Medlin *et al.* (1997) consider diatoms, pelagophytes, silicoflagellates and pedinellids as a separate group of stramenopiles which they call as "reduced flagellar apparatus group"; this group is characterised by (1) a flagellar transitional region with two transitional plates and a small transitional helix (or two rings ?) below the major plate, (2) a flagellar apparatus that lacks microtubular roots, (3) basal bodies positioned on or very near the nucleus, (4) a paraxial rod which is common in some members.

Cavalier-Smith *et al.* (1995) are of the view that the plastidic stramenopiles gave rise to the aplastidic taxa. The most primitive stramenopiles identified in molecular studies are the bicosoecids and oomycetes both of which are heterotrophic (Leipe *et al.* 1994, 1996). The bicosoecids appear to be related to the heterotrophic *Caecitellus* and pseudodendromonads, but whether the two latter taxa form a sister structure to the stramenopiles or fall within the stramenopiles is unclear. Overall, this suggests that the first stramenopiles were heterotrophic. The molecular data do indicate that the pedinellids were derived early in stramenopile evolution. Given that the most likely sister groups to the actinodines, and the sister groups to the axodines contain plastids, it seems probable that actinodines were ancestrally with plastids and subsequently lost them. This point of view is supported by cladistic analysis on ultrastructural data of pedinellids (Daugbjerg 1996b) which suggests that the most primitive pedinellid is a species of *Pseudopedinella*.

There are two recent schemes of conventional classification considering the position of pedinellids and related taxa. That of Moestrup (1995) is:

Class Dictyochophyceae Silva, 1982

Order Pedinellales Zimmermann *et al.*, 1984

Order Rhizochromulinales O'Kelly et Wujek, 1995

Order Dictyochales Haeckel, 1894.

The classification of Cavalier-Smith and co-workers (Cavalier-Smith *et al.* 1995, Cavalier-Smith and Chao 1996) infers that the rhizochromulinids should be regarded as a pedinellid, creates a paraphyletic taxon by segregating the Ciliophryida from the other pedinellids, and intrudes a monotypic taxon:

Superclass Dictyochia Haeckel, 1894, Cavalier-Smith, 1993

Class Pelagophyceae Andersen et Saunders, 1993

Class Actinochrysophyceae Cavalier-Smith, 1995

Subclass Pedinellidae Cavalier-Smith, 1986

Order Pedinellales

Order Ciliophryida

Order Rhizochromulinales

Subclass Silicophycidae Rothmaler, 1951

We have been unable to emerge with a single scheme of classification which protects familiar groupings and rank for convenience, as well as reflects our understanding of relationships. We present two schemes. The first supported by one of us (KM) and reflects traditional conventions. The second is supported by the other author (DP) and reflects a desire to create a phylogenetic classification using conventions discussed elsewhere (Patterson 1994, 1999). The defining attributes of new taxonomic concepts are:

Abodines: Axodines with posterior dictyosomes

Actinodines: Abodines with non-flagellar axonemes terminating on the nucleus and arranged with radial symmetry

Actinomonads: Actinodines without plastids

Heliomonads: Actinomonads with radial axopodia

Classification 1

Superclass Dictyochia Haeckel, 1894

Class Pelagophyceae Andersen and Sanders, 1993

Class Actinochrysophyceae Cavalier-Smith, 1995

Subclass Silicophycidae Rothmaler, 1951

Subclass Abaxodinae subcl. n.

Superorder Rhizochromulina O'Kelly and Wujek, 1995

Superorder Actinodinea superord. n.

Order Pedinellales Zimmermann *et al.*, 1984

Order Ciliophryida Febvre-Chevalier, 1985

Order Actinophryida Hartmann, 1913

Classification 2

Axodines

Silicoflagellates

Abodines

Rhizochromulinids

Actinodines

Pseudopedinella sm*Mesopedinella* sm*Apedinella* sm*Parapedinella* sm

Un-named taxon

Pedinella sm

Actinomnads

*Pteridomonas**Actinomonas*

Heliomonads

Ciliophrys

Actinophryids

Actinophrys

*Actinophrys sol**Actinophrys tauryanini**Actinophrys salsuginosa**Actinosphaerium**Actinosphaerium eichhornii**Actinosphaerium nucleofilum*

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A Novel ORF-containing Group I Intron with His-Cys Box in the LSU rDNA of *Naegleria*

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Summary. While group I introns are prevalent in the nuclear small subunit ribosomal DNA (SSU rDNA) in the amoebflagellate genus *Naegleria* they have been detected in the nuclear large subunit ribosomal DNA (LSU rDNA) in only two lineages of this genus, *N. morganensis* and an as yet unnamed *Naegleria* sp. In different strains of the unnamed *Naegleria* sp. we have found different combinations of the presence and absence of introns in the SSU rDNA and LSU rDNA. Furthermore, group I introns present in the LSU rDNA of these strains are of two different sizes. The shorter intron is 474 nt in length and has no open reading frame (ORF). The longer (868 nt) intron contains an ORF encoding 143 amino acids with a His-Cys box. This ORF is different from the ORF (which encodes 175 amino acids) with His-Cys box in the second (919 nt) LSU rDNA group I intron of *N. morganensis*. The results of the phylogenetic analyses of the His-Cys box in the ORFs support the hypothesis that the group I introns in the LSU rDNA of both *N. morganensis* and the unnamed *Naegleria* sp. were acquired by horizontal transfer. The different E26' locations of the short and the long group I intron in the unnamed *Naegleria* sp. supports the hypothesis that they were also acquired separately by horizontal transfer.

Key words: endonuclease, group I intron, His-Cys box, LSU rDNA, *Naegleria*.

INTRODUCTION

Naegleria spp. are free-living amoebae that can transform into flagellates. The genus is important to medical science because one species, *N. fowleri*, causes meningoencephalitis, almost invariably leading to death (De Jonckheere 1998). In recent years the presence of group I introns in the nuclear ribosomal DNA of many *Naegleria* spp. has attracted the interest of molecular biologists. Many *Naegleria* species contain a group I

intron at location 516 of helix 21 of the secondary structure within the small subunit ribosomal DNA (SSU rDNA) (De Jonckheere 1994). These introns have attracted special attention because they constitute a novel class of introns (Jabri *et al.* 1997, Einvik *et al.* 1998a), known as twintrons as they contain two distinct group I ribozymes (Einvik *et al.* 1998b). Apart from *Naegleria*, similar twintrons have been found only in the SSU rDNA of the myxomycete *Didymium* (Einvik *et al.* 1998b). Group I introns are also found in the large subunit ribosomal DNA (LSU rDNA) in *Didymium*, and in the LSU rDNA of *Naegleria*. However, group I introns appear to be much more rare in the LSU rDNA of *Naegleria* than they are in the SSU rDNA. During rDNA sequence analyses of 46 *Naegleria* lineages, we

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detected group I introns in the LSU rDNA of only two strains representing two different lineages (De Jonckheere and Brown 1998). Another major difference between the group I introns in the SSU and LSU rDNA of *Naegleria* spp. is that those in the SSU rDNA seem to have been passed vertically (De Jonckheere 1994) while the ones in the LSU rDNA seem to have been acquired horizontally (De Jonckheere and Brown 1998). Because a group I intron in the LSU rDNA of a *Naegleria* strain appears to be a rather unusual finding, we investigated more isolates that have isoenzyme patterns similar to those of the two strains in which LSU rDNA introns were detected. Of the lineage described as *N. morganensis* (Dobson *et al.* 1997), represented by strain NG236, only one more strain has been isolated, and from the same location in South Australia as NG236. The other lineage is an unnamed *Naegleria* sp., represented by strain NG872, which was found to be very closely related to *N. lovaniensis* (De Jonckheere and Brown 1997). Eight more strains belonging to this unnamed *Naegleria* sp. have been isolated from different places in Australia. We present here the results of polymerase chain reaction (PCR) amplifications of SSU rDNA and LSU rDNA, and the sequencing results of the LSU rDNA group I introns found in this unnamed *Naegleria* sp.

MATERIALS AND METHODS

Eight strains that belong to the same unnamed *Naegleria* sp. (based on allozyme comparisons) as NG872 were obtained from B. S. Robinson (Australian Centre for Water Quality Research, Salisbury, 5108 South Australia). The strains originate from different parts of Australia (Table 1).

Strains were cultured on agar plates streaked with *Escherichia coli*. When the agar plates were totally covered with amoebae the cells were harvested and concentrated by centrifugation. The DNA was extracted using a guanidinium thiocyanate-Sarkosyl method (De Jonckheere and Brown 1998). The SSU rDNA and LSU rDNA was amplified using conserved primers at the 5' end and the 3' end of the SSU rDNA (De Jonckheere and Brown 1997) and LSU rDNA (De Jonckheere and Brown 1998) respectively. Amplification conditions were 1 min. at 94°C, 1 min. at 50°C and 2 min. at 72°C for 30 cycles, with 10 min. at 72°C at the end. Products were visualised on 0.7% agarose gels. According to the strain the approximate length of the PCR product was either 2.0 kb or 3.3 kb for the SSU rDNA and either 3.0 kb, 3.5 kb or 3.9 kb for the LSU rDNA. Before sequencing the PCR products were purified using the enzymes supplied with the Sequenase PCR product sequencing kit (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England). After purification the LSU rDNA PCR products were sequenced using primers corresponding to sequences that were surrounding the introns and internal primers

(De Jonckheere and Brown 1998). Sequencing reaction products were separated on 6% acrylamide-urea sequencing gels and autoradiographed overnight at room temperature. The amino acid sequences of the His-Cys boxes were aligned by eye using the Eyeball Sequence Editor (ESEE) (Cabot and Beckenbach 1989). Phylogenetic trees were constructed from these aligned sequences using the PROTPARS (parsimony) and PROTDIST (distance matrix) of the PHYLIP (version 3.572c) package (Felsenstein 1989). The distance matrix was calculated using the categories model designed by Felsenstein. The distance matrix was ran in the NEIGHBOR (Neighbor joining and UPGMA), FITCH (Fitch-Margoliash), KITSCH (Fitch-Margoliash with evolutionary clock) and SEQBOOT (bootstrapping) programs.

The nucleotide sequence of the NG874 LSU rDNA intron reported in this paper is in the EMBL, GenBank and DDBJ nucleotide sequence databases under the accession number AJ271406.

RESULTS

Six of the eight strains of the unnamed *Naegleria* sp. contain a group I intron in the SSU rDNA (Table 1). One of the strains without a group I intron in the SSU rDNA also lacks an intron in the LSU rDNA. Of the seven others, three have an intron in the LSU rDNA with identical length to that reported in NG872 (De Jonckheere and Brown 1998) and four have introns that are longer than the one in NG872. The long and short group I introns are in slightly different positions in the E26' region of the LSU rDNA (shown for strain NG 872 and NG874, respectively, in Table 2). Neither the long nor short introns are confined to strains from one geographical location of this unnamed *Naegleria* sp.; either can be found in strains from the same location in West Australia, or in South Australia (Table 1). The longer introns from three (NG874, NG877 and NG927) out of four strains were sequenced and aligned. These sequences were identical to each other, except that strain NG927 differs from the two others by an indel of 6 nt (TAATAA) in loop P6 (Table 1). Therefore, in loop P6 TAA is repeated only twice in strain NG927, while it is repeated four times in the two other strains. In contrast to the shorter intron, the longer intron has an ORF encoding 143 amino acids with a His-Cys box (shown for strain NG874 in Table 3). Alignments of the His-Cys box reveal that the NG874 amino acid sequence is not closely related to the His-Cys box sequence in the long LSU rDNA intron of *N. morganensis* although they are at the same location, the P1 helix of the secondary structure (De Jonckheere and Brown 1998). In all phylogenetic trees generated from the His-Cys box alignments, NG874 always clusters with *Physarum polycephalum* whichever treebuilding method is used

Table 1. Presence of group I introns in the SSU and LSU rDNA of *Naegleria* strains related to NG872

Strain	Origin	SSU intron	LSU intron
NG872	Kununurra (WA)	+	+ (s) ^a
NG874	Derby (WA)	+	+ (l) ^b
NG876	Kununurra (WA)	-	-
NG877	Derby (WA)	+	+ (l)
NG878	Coolgardie (WA)	+	+ (s)
NG881	Derby (WA)	-	+ (s)
NG887	Hope Valley (SA)	+	+ (l)
NG889	Hope Valley (SA)	+	+ (s)
NG927	Howard Springs (NT)	+	+ (l)

WA - Western Australia; SA - South Australia; NT - Northern Territory; ^a s - short intron 474 nt; ^b l - long intron 868 nt (874 nt in NG927 due to indel in loop P6)

Table 2. Location of the short and long group I intron in the E26' of the secondary structure of the LSU rDNA in *Naegleria*

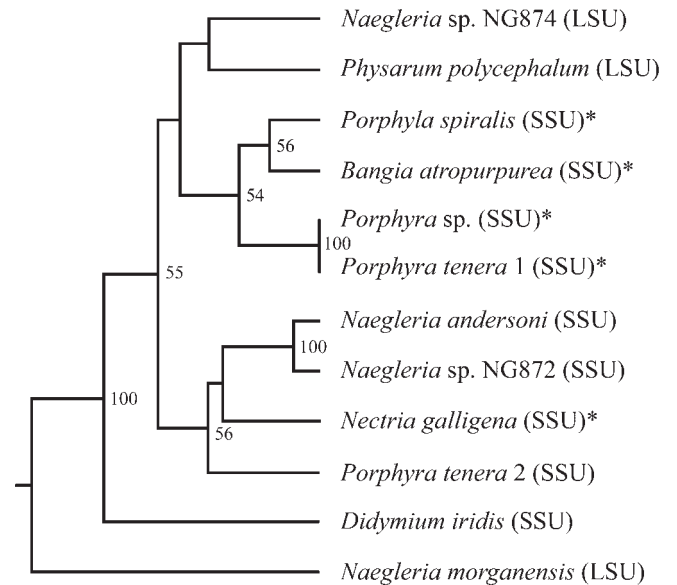
Strain	Length	Location in E26'
NG872	474 nt	GACT↓CTCTTAAGG
NG874	868 nt	GACTCTCTT↓AAGG

Table 3. Amino acid sequence alignment of the His-Cys box in the ORF in group I introns

<i>Naegleria andersoni</i>	SSU	T I S H L C - G N G G C A R P G H - L R I E K K T V N D E R T H C H
<i>Naegleria</i> NG872	SSU	. . . H . C - C . C . . H . - S . K H C .
<i>Naegleria</i> NG874	LSU	. V . H R C - H . E H C L N . D H - . V V . P L Q . . Q S . N T C T
<i>Naegleria morganensis</i>	LSU	V . R H T C - . C K D C C N . E H - . K L G T . S D . E Y D K G I .
<i>Physarum polycephalum</i>	LSU	. A . H . C - H . T R C H N . L H - . C W . S L D D . K G . N W C P
<i>Didymium iridis</i>	SSU	H S . H . C K . D . S C M E L K H T . . V P A Q . N L A . H E L C P
<i>Porphyra spiralis</i>	SSU	E A . H T C - H . A K C V N K A H - . T L . S G D L . K S . I Y C R
<i>Nectria galligena</i>	SSU	?? . H . C - . . P L C L E . G H - I H V . P . . A . E A . K G C ?
<i>Porphyra</i> sp.	SSU	E A . H . C - . D K R C V . . S H - M T L . S G A L . K T . S Y C A
<i>Porphyra tenera</i> 1	SSU	?? . H . C - . D K R C V . . S H - M T L . S G A L . K T . S Y C A
<i>Bangia atropurpurea</i>	SSU	E A . H R C - H . A K C V N . L H - M A F . S G D . . K S . L Y C A
<i>Porphyra tenera</i> 2	SSU	? . . H . C - . T H H C L A A H H - M M L . P . H D . V . C .

. identical amino acid; - gap; ? unknown

(shown in Fig. 1 for Fitch-Margoliash and least-squares distance method with evolutionary clock) although bootstrap values are low, while *N. morganensis* appears to have the most distantly related His-Cys box sequence. The His-Cys boxes of these three organisms are the only ones to have been detected in the ORFs of LSU rDNA introns. All other His-Cys boxes are in ORFs present in SSU rDNA introns. The His-Cys box in the strain

**Fig.1.** Phylogenetic tree inferred from the amino acid alignments of the His-Cys box using the Fitch-Margoliash and least-squares distance method with evolutionary clock (KITSCHE). * - indicates that the group I introns have the potential homing-endonuclease-like protein with His-Cys box on the complementary strand. Numbers at the nodes indicate the frequency at which a cluster appeared in a bootstrap test of 100 runs. Only bootstraps values higher than 50 are shown

NG874 ORF is as different from the one in strain NG236 of *N. morganensis* as the His-Cys box of group I intron ORFs in SSU rDNA of myxomycetes, red algae (Haugen *et al.* 1999) and an ascomycete (Johansen and Haugen 1999).

Also, the sequences of the group I introns of strains NG874 and NG236 outside the ORF are very different. Because of the difficulty in aligning the sequences of the

group I introns accurately no attempt was made to analyse the sequences phylogenetically. Instead, the decision was made to analyse the His-Cys region, accepting that this might have its limitations. It has been shown that intron ORFs in mitochondria also behave as autonomously mobile entities (Sellem and Belcour 1997, Saguez *et al.* 2000). If this is also true for ORFs in nuclear group I introns with the His-Cys motif, then the tree (Fig. 1) might represent the phylogeny of the ORFs rather than of the group I introns. However, the group I intron sequences of strains NG874 and NG236 seem to be as unrelated as their His-Cys boxes, which is consistent with horizontal transfer.

DISCUSSION

Different combinations of presence and absence of group I introns in the SSU rDNA and LSU rDNA have been found in eight strains of the unnamed *Naegleria* sp. In addition, introns of two different sizes are found in the LSU rDNA of different strains belonging to this lineage. The shorter intron has no ORF and is identical to the one previously reported in strain NG872 (De Jonckheere and Brown 1998). The longer intron contains an ORF encoding 143 amino acids. This is different from the ORF encoding 175 amino acids in the second intron in the LSU rDNA of *N. morganensis* (represented by the type strain NG236), which is the only other *Naegleria* lineage known to have a LSU rDNA group I intron. Both ORFs in the *Naegleria* LSU rDNA group I introns are in the P1 helix of the secondary structure. In the SSU rDNA twintrons of *Naegleria* spp. the ORF is inserted between the two ribozymes and encodes 245 amino acids. Notwithstanding the different lengths of these ORFs they all contain a His-Cys box. Many *Naegleria* lineages have a group I intron in the SSU rDNA so it is remarkable that group I introns are found in the LSU rDNA in only two lineages of this genus.

In the NG872 lineage we find two different group I introns, one with and one without an ORF, depending on the strain investigated. The two introns, for example in NG872 and NG874, differ considerably in sequence and in length and are located in slightly different places in the E26' region of the secondary structure (Table 2). This is consistent with the horizontal transfer hypothesis. This horizontal acquisition might be the reason why there are very few group I introns in the LSU rDNA of *Naegleria* spp. In contrast, the SSU rDNA intron has been demon-

strated to be present as a consequence of vertical transmission after ancestral acquisition (De Jonckheere 1994). Consequently, many *Naegleria* spp. have this SSU rDNA group I intron, only some of the species having lost the intron since its ancestral acquisition. Loss of the SSU rDNA group I intron appears to have been rare since speciation within the genus *Naegleria*. To date, no *Naegleria* sp. has been described in which some strains have the group I intron within their SSU rDNA and some do not. The *Naegleria* lineage under investigation here might be the only exception. Therefore, the SSUrDNA and ITS sequences of all the strains presumed to belong to this lineage need to be analysed in order to confirm that they all belong to the same species.

Because the group I intron of the LSU rDNA of strain NG874 contains an ORF encoding a His-Cys endonuclease, it is possible that it was inserted by homing. The intron of strain NG872 does not encode this endonuclease. Either it might have lost the ORF or the intron might have been introduced by reversed splicing (Roman and Woodson 1998). The introns of NG872 and NG874 are not in exactly the same place, which might favour the hypothesis that they were introduced by reversed splicing and that the endonuclease in NG874 has nothing to do with the horizontal transfer. Indeed, it has been demonstrated experimentally that horizontal transfer through reverse splice reaction has a lower specificity than intron homing (Roman and Woodson 1998). This experiment was performed with the *Tetrahymena* group I intron which is located in precisely this same area (E26') in the secondary structure of the LSU rDNA as the intron in the unnamed *Naegleria* sp. The lower specificity of reverse splicing would also explain why the intron-containing sites at E26' are diverse amongst the organisms (De Jonckheere and Brown 1998). This E26' area of the LSU rDNA seems to be special as it is also invaded by group I introns in slime molds (De Jonckheere and Brown 1998), fungi (De Jonckheere and Brown 1998, Johansen and Haugen 1999) and red algae (Haugen *et al.* 1999), while this E26' area has been invaded at least twice by horizontal transfer in this unnamed *Naegleria* sp., as found in strain NG872 and NG874, respectively.

In strain NG236 of *N. morganensis* the LSU rDNA has been invaded by two different group I introns, also one with and one without an ORF, but neither of them is in the E26' area. However, both locations, E28 and G19' in the LSU rDNA of strain NG236, have also been

invaded in other organisms, in different slime molds at location E28 and in different fungi at location G19' (De Jonckheere 1998).

Surprisingly, group I introns have not been reported yet in any other member of the family Vahlkampfiidae (De Jonckheere and Brown 1998), to which the amoebflagellate genus *Naegleria* belongs. There are far more group I introns with the motif LAGLI-DADG than the His-Cys motif in the homing endonucleases (Johansen *et al.* 1997). In *Naegleria*, all the different homing endonucleases detected to date have the His-Cys motif; one in the SSU rDNA of different species and two different ones in the LSU rDNA of two different species.

Homing endonucleases in nuclear rDNA introns with His-Cys boxes are rare. Therefore, they are now still considered special, but it is predicted that there are more in protists and fungi awaiting detection (Einviik *et al.* 1998b). Indeed, more have been detected recently in red algae *Porphyra* and *Bangia* (Haugen *et al.* 1999) and in the ascomycete *Nectria galligena* (Johansen and Haugen 1999). However, in the majority of the latter organisms the group I introns have the potential homing-endonuclease-like protein with His-Cys box on the complementary strand.

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Fine Structure of the Cyrtophorid Ciliate *Chlamydodon mnemosyne* Ehrenberg, 1837

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Summary. *Chlamydodon mnemosyne* is a bottom-dwelling cyrtophorid ciliate found in brackish water ponds. The morphology of non-dividers is described based upon light microscopy of living and silver stained cells. In addition we provide a detailed description of the ultrastructure of the cells. *C. mnemosyne* is dorsoventrally flattened and ciliated only on the ventral surface which is separated from the dorsal surface by a peculiar transversally striped band at the margin of the cells. This band consists of regularly arranged C-shaped clasps with a complex multilayered fine structure. The somatic ciliature is subdivided in right and left fields of kineties flanking a medial field of 4 postoral kineties. The somatic monokinetids are associated with tubular and fibrillar cytoskeletal elements according to the phyllopharyngid pattern, the most pronounced feature being a pack of subkinetal microtubules. Similar to other cyrtophorid ciliates the oral ciliature consists of an inner and an outer circumoral kinety as well as a preoral kinety all of which are built up from dikinetids with their anterior kinetosome bearing the cilium. The oral apparatus consists of an inner ring of cytopharyngeal lamellae and an outer ring of 9 large and 2 small anteriorly positioned nematodesmal rods resulting in the bilateral appearance of the cytopharyngeal basket. The rods are capped with so called dentes which harbour a barren kinetosome each. The detailed ultrastructural description provided here serves as a basis for studies on the morphogenesis of *C. mnemosyne*, part of which are presented in an accompanying paper (Bardele and Kurth 2001).

Key words: *Chlamydodon mnemosyne*, Ciliophora, Cyrtophorida, cytopharyngeal basket, fine structure, Phyllopharyngea, somatic cortex.

INTRODUCTION

Chlamydodon mnemosyne Ehrenberg, 1838 is a bottom-dwelling marine ciliate which feeds on filamentous cyanobacteria. The Cyrtophorida Fauré-Fremiet, 1956 belong to the class Phyllopharyngea de Puytorac

et al., 1974 which are characterised by a cytopharyngeal basket made of prominent microtubular rods. Inside the basket there are leafs (in Greek = phyllon) of cytopharyngeal microtubular lamellae which led to the name of the group.

The class Phyllopharyngea comprises four subclasses, the Cyrtophoria, Chonotrichia, Rhynchodia and Suctoria (Deroux 1994). While the majority of the members of the latter three subclasses are sessile or parasitic organisms, the Cyrtophorida are free-swimming ciliates assigned to three orders (with representative genera in parentheses), the Chilodonellida (*Chilodonella*,

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Trithigmostoma), the Chlamyodontida (*Chlamyodon*) and the Dysteriida (*Brooklynella*).

The characteristic feature of the genus *Chlamyodon* is the cross-stripped band, which is located at the periphery of the flattened cell separating the dorsal from the ventral side of the cell. MacDougall who gave the first detailed description of this enigmatic structure called it "railroad track" since its C-shaped clasps look like cross-ties in flattened specimens (MacDougall 1925). In the early sixties Kaneda performed several light and electron microscopical studies on *Chlamyodon pedarius* found in Japanese coastal waters (Kaneda 1953; 1960a, b, c; 1961a, b; 1962). After more than 30 years *Chlamyodon* has gained new interest, positive phototactic behaviour was discovered in *C. mnemosyne* (Kuhlmann and Hemmersbach-Krause 1993, Selbach and Kuhlmann 1999, Selbach *et al.* 1999).

Our interest in *Chlamyodon* came from morphogenetic studies on the closely related genera *Chilodonella* and *Trithigmostoma* (Hofmann 1987, Hofmann and Bardele 1987). These studies have led to important insights on the merotelokinetal stomatogenesis in phyllopharyngid ciliates in particular as far as the events in the opisthe are concerned. In addition to the studies on *Trithigmostoma* and *Chilodonella* a very careful study on the morphogenesis and fine structure of *Brooklynella hostilis* (Dysteriida), a gill parasite of marine fishes, was carried out by Lom and Corliss (1971).

However, a detailed analysis of the morphogenesis and ultrastructure of a member of the Chlamyodontida is so far not available. Preliminary work done in our laboratory on *C. mnemosyne* has shown that this ciliate is particularly suited to investigate the oral reorganisation which occurs in the proter during divisional morphogenesis. The current study on the fine structure of the somatic and oral cortex of *C. mnemosyne* is a prerequisite for the morphogenetic studies part of which have recently been finished (Bardele and Kurth 2001).

MATERIALS AND METHODS

The euryhaline ciliate *Chlamyodon mnemosyne* was kindly supplied by Dr. H.-W. Kuhlmann, Münster, who isolated it from a sample taken from a brackish water pond near Carolinensiel, Germany. Since 1993 we grew *C. mnemosyne* in a mixture of 50 % natural sea-water and 50 % "Volvic" (French mineral water from the Auvergne with low carbonic acid) and feed them twice a week with the filamentous cyanobacterium *Phormidium inundatum*. Observations of living cells were done with phase contrast and Nomarski differential interference contrast optics. Pyridinated silver carbonate

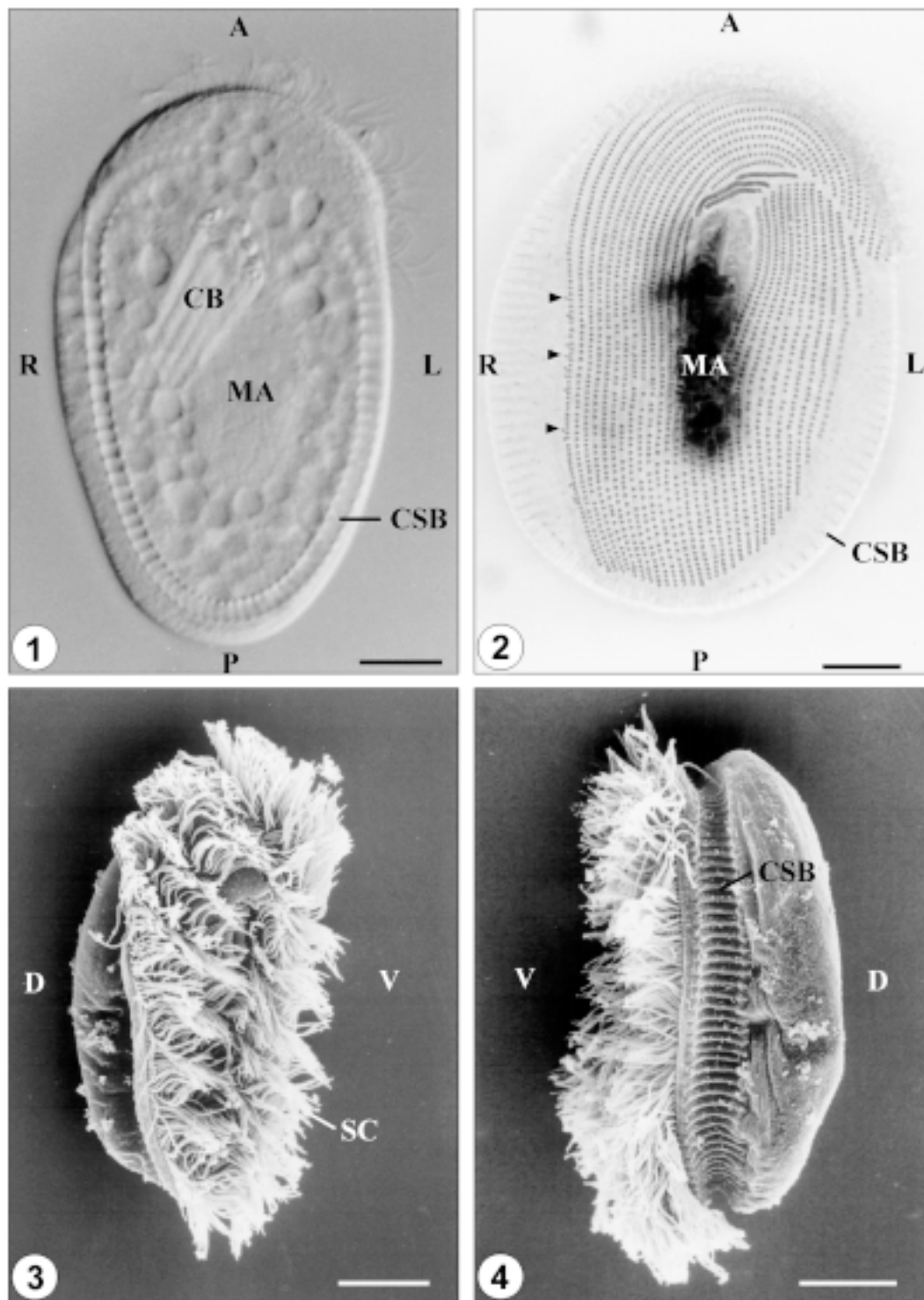
technique was successful only after slight modification of the original recipe (Fernández-Galiano 1976). Cells were prefixed in 1 % formaldehyde for 10 min and then transferred to 0.6 % formaldehyde. One aliquot of this material was mixed with 3 aliquots of the Fernández-Galiano solution on a microscopical slide, mixing was by gentle stirring with a fine glass rod; specimens were put for 3 min on a 50 °C warming plate after the mixing step. Well-stained preparations were sealed with paraffin and stored in moist chambers in the refrigerator for several days. For scanning electron microscopy cells were fixed in the Párducz fixative (Párducz 1967), dehydrated in a graded series of ethanol, mounted on polylysine-coated coverslips, critical point dried in a Polaron CP-dryer, sputter-coated with gold-palladium in Balzers sputter coater and viewed in a Cambridge Stereoscan Mk 2. For transmission electron microscopy cells were fixed in the Shigenaka fixative (Shigenaka *et al.* 1973) on ice for 20 min, washed twice in the recommended phosphate buffer, bloc-stained with saturated uranyl acetate in 70 % ethanol, further dehydrated, embedded in Epon 812, sectioned and stained with lead according to Reynolds (1963). Thin sections were viewed in a Siemens Elmiskope 102. All micrographs (except Figs. 10, 11) are printed as seen from outside the cell. Anterior (A), posterior (P), dorsal (D), and ventral (V) side of the cell are labelled.

RESULTS

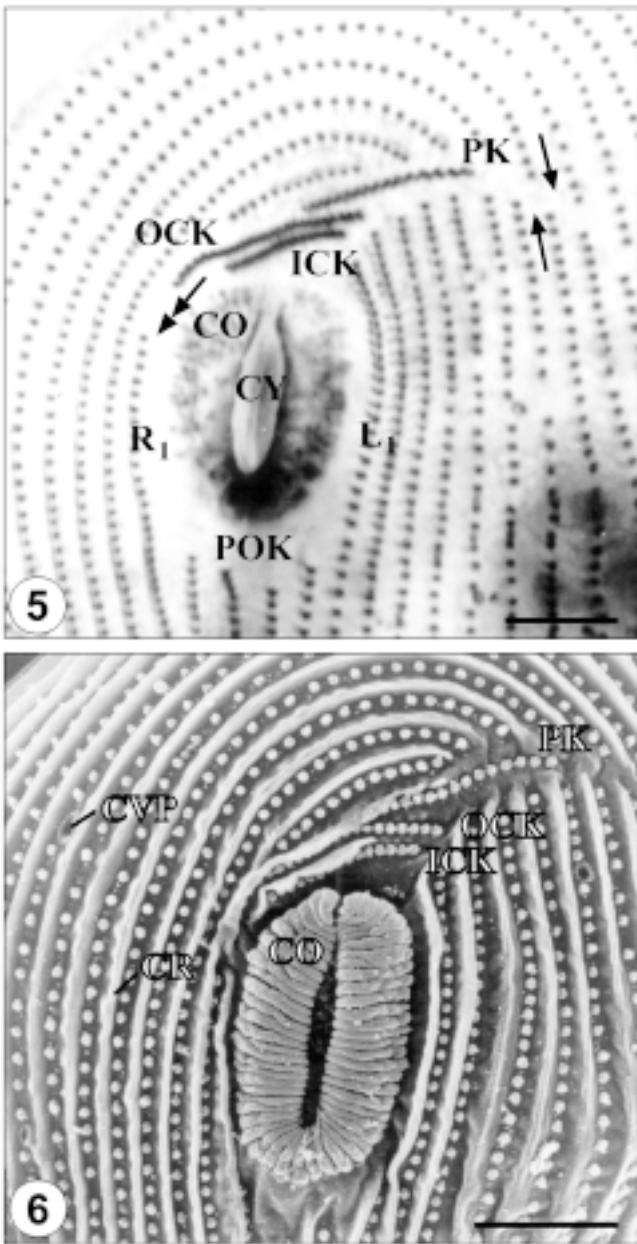
General morphology

Medium-sized cells of *Chlamyodon mnemosyne* measure 70 x 45 µm and are dorsoventrally flattened. Depending on the feeding conditions the cell size may vary considerably (40-100 by 23-65 µm). The ventral side of the cell is uniformly ciliated (Fig. 3). There is neither a non-ciliated area posterior to the cytostome as in several chilodonellid ciliates (e.g. *Chilodonella cyprini*), nor a special field of thigmotactic cilia or an attachment organelle or podite in the posterior part of the cell as in dysteriid ciliates. At the anterior end of the cell the ciliated zone passes a little bit over to the dorsal side so that the anterior part of the right somatic kineties can not be seen if the cell is viewed from the ventral side (Fig. 2). A cross-stripped band lies at the borderline between the dorsal and ventral face of the cell (Fig. 1). This band forms a tube-like girdle around the periphery of the living cell. Some 90 C-shaped clasps resembling tracheal cartilages, probably stiffen the tube which appears to be closed in living cells. The opened tube seen in Fig. 4 is most probably a fixation artefact.

Like other cyrtophorid ciliates *Chlamyodon* possesses a prominent cytopharyngeal basket or "cyrtos", which is very large in proportion to the size of the cell and made of 9-11 nematodesmal rods (Fig 1). These rods are not of equal length and the proximal end of the



Figs. 1-4. 1 - Nomarski interference contrast micrograph of *Chlamydonon mnemosyne* focused to the middle of the cell. The cross-striated band (CSB), macronucleus (MA), and cytopharyngeal basket (CB) are labelled. The cell contains numerous unlabelled food vacuoles. 2 - slightly squashed cell stained with Fernández-Galiano technique to show the kinetome on the ventral surface of the cell. The three more densely stained kineties in the upper half of the cell represent the perioral kineties more clearly seen in (5). The small arrowheads mark several irregularly spaced barren basal bodies of the outer right somatic kinety. 3, 4 - scanning micrographs of *Chlamydonon*. The somatic ciliature (SC) is restricted to the ventral surface (V) of the cell whereas the dorsal side (D) is nonciliated. The oral ciliature is difficult to see. 4 - shows the cross-striated band (CSB) in a swollen cell. Under these circumstances the tube-like channel which surrounds the entire cell is opened artificially; compare with (7). Scale bars - 10 μ m



Figs. 5, 6. Oral area of *Chlamydomonas* in a Fernández-Galiano preparation (5) and in an artificially deciliated cell as seen in the scanning microscope (6). The cytostome (CY) is surrounded by a U-shaped collar (CO) of fine ridges. The perioral ciliature consists of three kineties, the inner circumoral kinety (ICK), the outer circumoral kinety (OCK) and the preoral kinety (PK) are labelled in both micrographs. The ends of the perioral kineties have an inverted polarity (based on transmission electron microscopy, not shown) with respect to the right somatic kineties as indicated by the double-headed arrow in (5), the anterior ends of the former point towards the left side of the micrograph. There are four postoral somatic kineties (POK), a right and a left field of somatic kineties separated by cortical ridges (CR) most clearly seen after deciliation. The opposing arrows in the upper right corner of (5) indicate the polarity of the right and the left somatic kineties at the anterior suture. One of the several contractile vacuole pores (CVP) is labeled in (6). R₁ and L₁ demarcate the first right and left somatic kineties, respectively. Scale bars - 5 μm

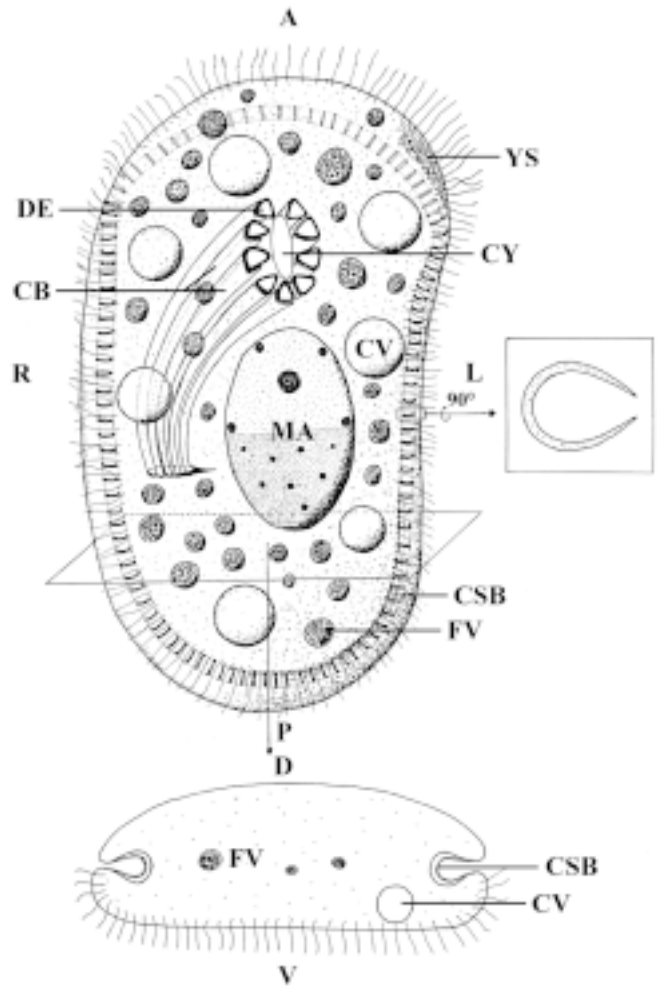
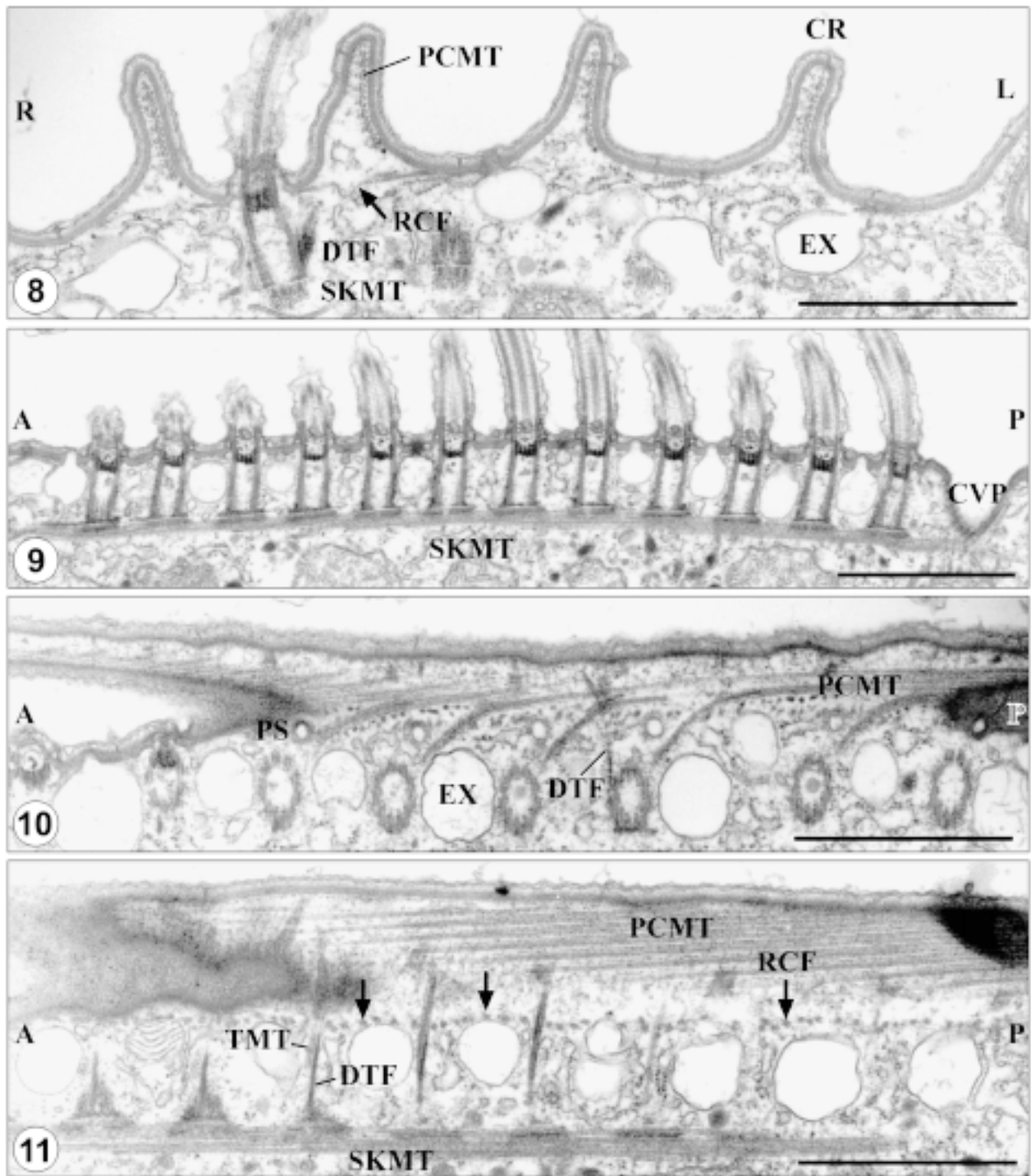


Fig. 7. Schematic drawing of *Chlamydomonas*. The upper drawing shows the ventral side of the cell. The lower drawing represents a cross-section. The dorsal side is non-ciliated. One of the clasps of the cross-striated band (CSB) is shown at higher magnification on the right. In the anterior left of the cell there is a yellow spot (YS). Seven contractile vacuoles (CV), several food vacuoles (FV) and the heteromeric macronucleus (MA) are labelled as well as the cytostome (CY) and the cytopharyngeal basket (CB). In this cell the basket is formed of nine microtubular rods or nematodesmata each of which carries a dens (DE). A -anterior; D-dorsal; L-left; P-posterior; V-ventral

basket has the shape of an obliquely cut straw with three rods being longer than the rest (not shown). The thin inner end of the basket is bowed to the left side of the cell when seen in ventral view. Near their distal end the rods are capped each with a so-called dens. There is no permanent oral opening. A summary of the features seen in living cells is depicted in Fig. 7. While in traditionally prepared SEM specimens the oral area is hidden by the cilia its position is clearly seen in silver-impregnated cells by the course of the somatic kineties (Figs. 2, 5). There



Figs. 8-11. Various aspects of the ventral somatic cortex of *Chlamydomon mnemosyne*. **8** - is from a cross-section through the cell showing several cortical ridges (CR) with postciliary microtubules (PCMT) in the left slope, arranged in a triangular fashion. There is only one cilium seen in this micrograph, its kinetosome is associated with a dense transverse fiber (DTF) and subkinetal microtubules (SKMT). Near the bases of the cortical ridges there are fibrous strands (RCF) perpendicular to the longitudinal axis of the cortical ridges. EX, extrusome. **9** - is a longitudinal section of a somatic kinety with its subkinetal microtubules (SKMT). CVP represents an obliquely cut pore of a contractile vacuole. **10** - shows another somatic kinety. Its kinetosomes seem to be pushed apart slightly by discharged extrusomes (EX). Postciliary microtubules (PCMT), parasomal sacs (PS) and the left dense transverse fiber (DTF) associated with each monokinetid are labelled. **11**- is a slightly oblique section of a somatic kinety to show the transverse microtubule (TMT) and a better view of the dense transverse fibers (DTF) associated with the kinetosomes which are not in the plane of this section. Underneath the postciliary microtubules is the array of dense fibrous strands (RCF, arrows) which run perpendicular to the cortical ridges. Scale bars - 1 μ m

is a right and a left field of somatic kineties. The left field has 9-12 (most often 11) more or less straight kineties. The left-most kinety is the shortest, it hardly reaches down to the middle of the cell which is of some importance during binary division of the cell (Bardele and Kurth 2001). The right field has 12-15 somatic kineties. The anterior part of these kineties bends over to the anterior left quarter of the cell where they abut against the left somatic kineties forming the anterior suture. The leftmost of the right somatic kineties, R₁, is split into two segments by the right end of the outer circumoral kinety as seen in Figs. 2 and 5. Posterior to the oral area (see Fig. 5) there are the four additional somatic kineties, called postoral kineties. These are involved in stomatogenesis. In the anterior part of L₁ through L₅ the kinetosomes are more densely spaced compared to the kinetosomes of the right somatic field, in particular where these kineties take a stretched semicircular course in front of the oral area (Fig. 5). As clearly seen in deciliated cells, and verified by thin sections, all somatic kineties consist of monokinetids. Scanning electron micrographs show prominent cortical ridges between the somatic kineties (Fig. 6). Deciliated specimens also allow to map the position of the excretory pores of the 5-11, most often 6-8, contractile vacuoles which all open to the ventral surface of the cell. Their openings are positioned in two more or less straight fields, one in the area of R₄-R₇, the other in the area of L₃-L₇. Though not shown in our micrographs there is a short outer right kinety (Lom and Corliss 1971), called "cinétique droite externe" in the French literature (Deroux 1994). This kinety may have some relation to the irregularly spaced barren kinetosomes seen near the outer right kinety (arrowheads in Fig. 2).

Light micrographs of silver-stained cells (Figs. 2, 5) give the correct impression that the kinetosomes of the three perioral kineties are spaced more densely than in the somatic kineties. In contrast to the latter they consist of dikinetids. The three perioral kineties are called inner circumoral kinety, outer circumoral kinety, and preoral kinety (Kaczanowska and Kowalska 1969). While the scanning electron micrographs suggest monokinetids for the oral kineties it is known from thin sections that they are formed of dikinetids in which only the anterior kinetosome is ciliated (see below). The inner circumoral kinety is shorter than the outer circumoral kinety. As in *Trithigmostoma* and *Chilodonella* (Hofmann 1987, Hofmann and Bardele 1987) all three are inverted kineties as indicated in Fig. 5 by the double-headed arrow; the anterior ends of all three perioral kineties point toward the right side of the cell (data not shown). The posterior

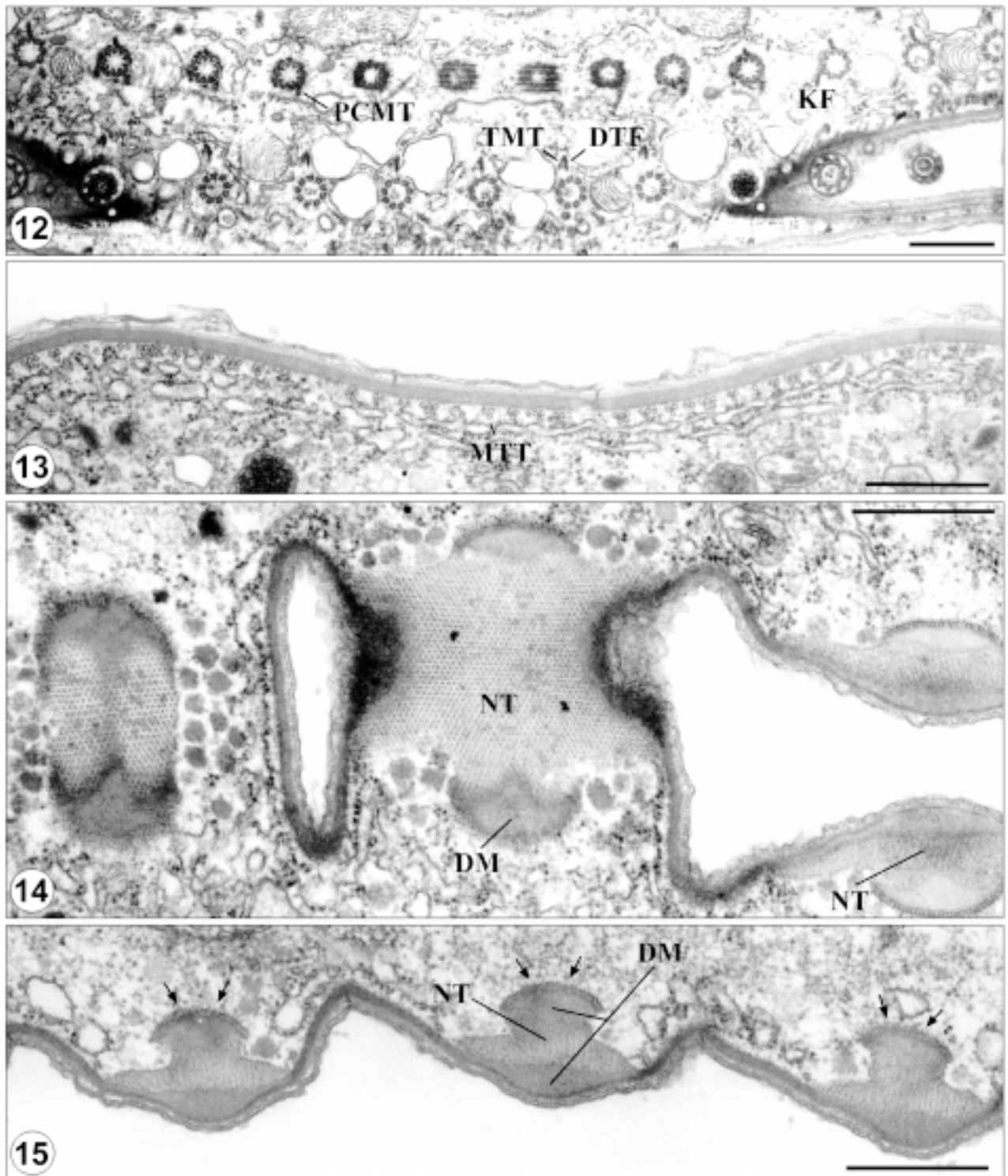
end of the preoral kinety enters the anterior suture, a region where right and left somatic kineties abut each other with their anterior ends (Fig. 5). Surrounding the area where the cytostome forms in feeding cells there is a U-shaped array of up to 60 parallel ridges forming the so-called collar. The collar is particularly apparent in deciliated cells (Fig. 6).

Chlamydodon has an ovoid heteromeric macronucleus 20 µm by 15 µm in size (Figs. 2, 7) which often lies close to the oral area and then hampers a good view of this area in silver-stained specimens. The heteromeric nucleus consists of an anterior protomere and a posterior orthomere, studied so far only by Kaneda (1960c; 1961a, b). Moreover, there is a single micronucleus, 3-4 µm in diameter, close to the macronucleus.

Fine structure of the somatic cortex

Except for the cilia the dominating structures of the ventral surface of *Chlamydodon* cells are the interkinetal crests or cortical ridges, as we will call them (Fig. 8). As might be expected these ridges show the plasma membrane, pellicular alveoli, and a layer of epiplasm. In the left slope of the ridges are numerous postciliary microtubules in triangular array originating from kinetosomes of somatic cilia, which arise from the furrows between the ridges.

From numerous micrographs similar to those in Figs. 8-12 a schematic drawing was prepared to give a three-dimensional view of the major constituents of the ventral somatic cortex (Fig. 16). Details of the kinetid pattern of *Chlamydodon* are summarised in Fig. 17. The kinetosome of every monokinetid is associated with three or four postciliary microtubules accompanied by a faint fibrillar lamina. As can be deduced from Figs. 8, 10, and 11 postciliary microtubules of consecutive kinetosomes overlap eight to ten ciliary territories. One of the four postciliary microtubules which starts from the proximal end of the kinetosome ends at the level where the other three make a sharp bend, rearrange themselves to form a triad and run in a posterior direction. As seen in Fig. 16 the triad at the bottom of the cortical ridge is coming from the nearest kinetosome while triads further up in the ridge come from kinetosomes located more anteriorly with respect to the kinety. A single transverse microtubule orientated perpendicular to the kinetal axis is accompanied by a dense transverse fiber, which enters the next cortical ridge to the left. This dense transverse fiber has a split root, which originates from triplets no. 3 and 4. The dense transverse fiber is longer and more clearly seen than the transverse microtubule itself. The



Figs. 12-15. 12 - this section of two somatic kineties shows in addition to the kinetosome associated fibers mentioned in the forgoing micrographs the rather inconspicuous kinetodesmal fiber (KF). For explanations for labels PCMT, TMT, and DTF see (8-11 legend). 13 - is a cross-section of the dorsal surface of the cell with microtubular triads (MTT) in addition to other arrays with up to seven microtubules. 14 - is an oblique section through the cross-stripped band with three clasps cut at different levels. 15 - is a cross-section through three clasps. Underneath the epiplasma there is a layer of electron dense material (DM) followed by a layer of nanotubules (NT) arranged in a hexagonal array and topped by a bowed sheet of very densely spaced microtubules (arrows). Scale bars - 0.5 μ m

kinetodesmal fiber is very short and inconspicuous as in other phyllopharyngid ciliates (Fig. 12). The most characteristic feature of the somatic infraciliature of the Phyllopharyngea is the pack of subkinetal microtubules. Five of these microtubules start as a flat sheet from underneath every somatic kinetosome and overlap in anterior direction three adjacent kinetid territories. There is an electron dense plate at the proximal end of the kinetosome followed by a small zone of translucent material before, further down, the subkinetal microtubules start from another zone of dense material as seen most clearly in Fig. 9. This micrograph is from the anterior end of one of the left somatic kineties where the kinetosomes are more closely spaced than in other kineties. Judged from the occurrence of an obliquely cut pore of a contractile vacuole in this kinety it ought to be one of the kineties L_3 through L_7 . Between the kinetosomes in Fig. 9 but seen also in Figs. 10 and 11 there are the remainders of extrusomes which regularly explode when the cell is fixed chemically. In unpublished freeze-fracture replicas of the somatic cortex of *C. mnemosyne* numerous attachment rosettes made of 8-11 intramembranous particles were seen in the P-face of the plasma membrane. Such structures are known from many ciliates as e.g. *Tetrahymena* (Satir *et al.* 1973).

As stated above, the dorsal surface of the cell is free of cilia. Underneath the plasma membrane there is a thin layer of flat pellicular alveoli, polygonal in shape, their abutting borderlines form a narrow-meshed silver line system. These observations have been verified by freeze-fracture studies (not shown). Underneath the pellicular alveoli there is a prominent layer of epiplasm followed by longitudinal microtubules which often are arranged in a triangular fashion and one or two layers of rough endoplasmic reticulum (Fig. 13).

The cross-stripped band, actually a hollow "tube" containing the extracellular medium, is the most characteristic feature of the genus *Chlamydomon*. The tube is about 1,5 μm in diameter and as seen in oblique and cross-section the major components of the cross-stripped band are the "C"-shaped clasps, which are about 0.5 μm in width and 1-1,5 μm apart from each other. In a medium-sized cell some 80-90 clasps are counted. These elements are closely associated with the epiplasm and formed of four different zones (Figs. 14, 15). Sandwiched between two layers of electron dense material, a layer of small tubular structures arranged in a hexagonal array and orientated perpendicular to the cell surface can be seen. We call them "nanotubules" because their diameter of 10-12 nm is roughly the half of regular

microtubules. The clasps are topped by a bowed sheet of very densely spaced microtubular structures. We are hesitant to call them true microtubules, since although having the same diameter they are spaced more closely than typical microtubules.

Finally, there are numerous strands of electron dense fibers running criss-cross through the cytoplasm of the cell, encircling the macronucleus, making contact to the somatic and perioral kineties, the cross-stripped band and the nematodesmal rods. So far no obvious pattern of these strands could be detected.

Fine structure of the oral area

For illustration of the fine structure of the oral area a slightly oblique section was chosen, alternatively several cross-sections at different depths would have been necessary to show all aspects (Fig. 18). The cytopharyngeal basket is made of 9-11 massive microtubular rods, also called nematodesmata, very rarely 13 large rods were observed. No matter how many major rods are counted there are always two anterior thin rods which appear to be fused (Fig. 19). Thus the basket has a bilateral organisation. At the proximal end the nematodesmal microtubules, arranged in a hexagonal array, start from a layer of electron dense material. Near the proximal end of the oval array of the nematodesmata there are numerous parallel microtubules seen between the individual rods as well as between the rods and the inner tube of cytostomal microtubular lamellae. Further down these non-patterned microtubules concentrate between the nematodesmal rods and become embedded in some electron dense material (Fig. 19). The larger nematodesmal rods are capped with a dens. The three-dimensional shape of a hollow looking dens is difficult to describe. In cross-section and near to its tip its fibrous wall appears to be closed, but as longitudinal sections show there are two openings in the wall, one in the lateral slope where a single barren kinetosome is found and another hole in the bottom of the dens (Fig. 20). As this figure shows the dens is fixed to the nematodesmal rod by a hinge-like strand of dense fibrous material. The entire area between the upper end of the rods, the dentes and the corrugated ridges of the collar is filled with less dense filaments (FF in Figs. 18, 20). Inside the basket there is an inner tube of cytostomal lamellae made of some 25 microtubules each (Fig. 19). The cytostomal lamellae are also called Z-microtubules. Immediately underneath the cytostome small subcytostomal lamellae made of so-called Y-microtubules are positioned between the cytostomal lamellae. Z- and Y-microtubules

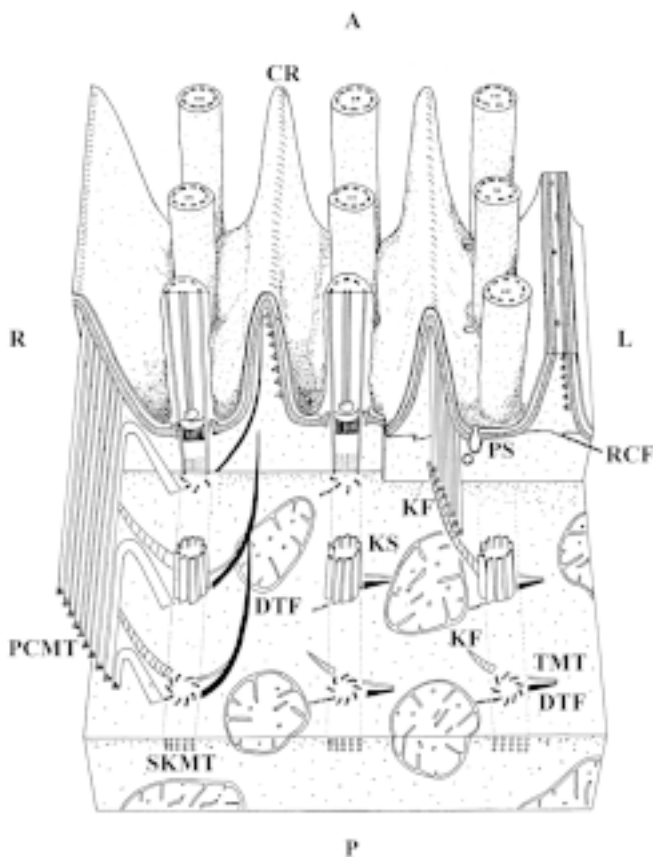


Fig. 16. Schematic drawing of the somatic cortex of *Chlamydomon*. The single transverse microtubule (TMT) together with a dense transverse fiber (DTF) extends in the right slope of the cortical ridge (CR). The triads of the postciliary microtubules (PCMT) and the kinetodesmal fiber (KF) are seen in the left slope of the ridge. A parasomal sac (PS), fibrous strands (RCF) perpendicular to the cortical ridges and the subkinetal microtubules (SKMT) are labelled. The extrusomes have been omitted for sake of clarity. KS- kinetosome

are arranged perpendicular to each other (Fig. 20, better seen in Pyne and Tuffrau (1970); for general discussion of the entire set of oral microtubules in nassulid and phyllopharyngid ciliates see Eisler (1988)). Small ovoid electron dense granules are seen within and around the cytopharyngeal basket. They might represent storage granules of digestive enzymes. The occurrence of mitochondria within the cytostomial tube as seen in Fig. 19 is not a regular phenomenon. In non-feeding cells the site where the cytostome will open when feeding has a slit-like shape. The oral slit is bordered by a prominent collar made of some 60-80 corrugated ridges. At the level of these ridges there are no pellicular alveoli between the plasma membrane and the epiplasm (Fig. 20). The ridges are filled with small granules of unknown nature. Only

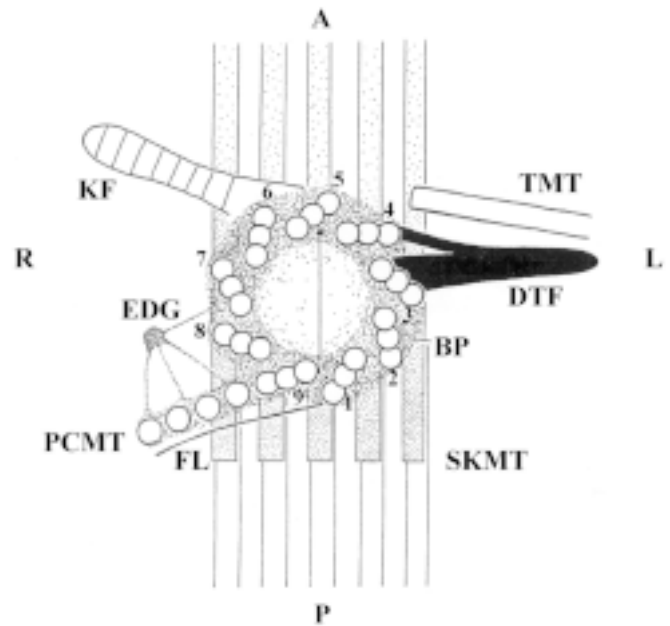


Fig. 17. Kinetid pattern of a somatic monokinetid of *Chlamydomon*. The kinetosome sits on a ring-shaped electron dense basal plate (BP). Four postciliary microtubules (PCMT) are associated with triplet #9. Starting from #1 a fine lamina (FL) lays behind triplet #9 and the PCMT. From the latter fine fibrillar strands run to an electron dense granule (EDG) at the height of triplet #8. There is a short kinetodesmal fiber (KF) associated with triplet #6 and 5. On the left side there is a prominent dense transverse fiber (DTF) with a forked root at triplet #4 and 3, and a single transverse microtubule (TMT) in front of this dense fiber. Finally, there are the subkinetal microtubules (SKMT), a characteristic feature of the phyllopharyngid kinetid pattern, starting from behind and underneath the kinetosome. These microtubules run in anterior direction and are underlain by two or three other sheets of SKMT coming from posterior kinetosomes

small segments of the inner and outer circumoral kinety are seen in Fig. 18. Although not presented here, sections displaying the organisation of the circumoral and preoral kineties reveal that these kineties in every detail look identical to the corresponding kineties in *Trithigmotoma* as described by Hofmann and Bardele (1987).

DISCUSSION

It was Christian Gottfried Ehrenberg who discovered *Chlamydomon mnemosyne* in the Baltic Sea near Wismar/Germany in 1834. This euryhaline species withstands salt concentrations between 5,4-25 ‰ (Fauré-

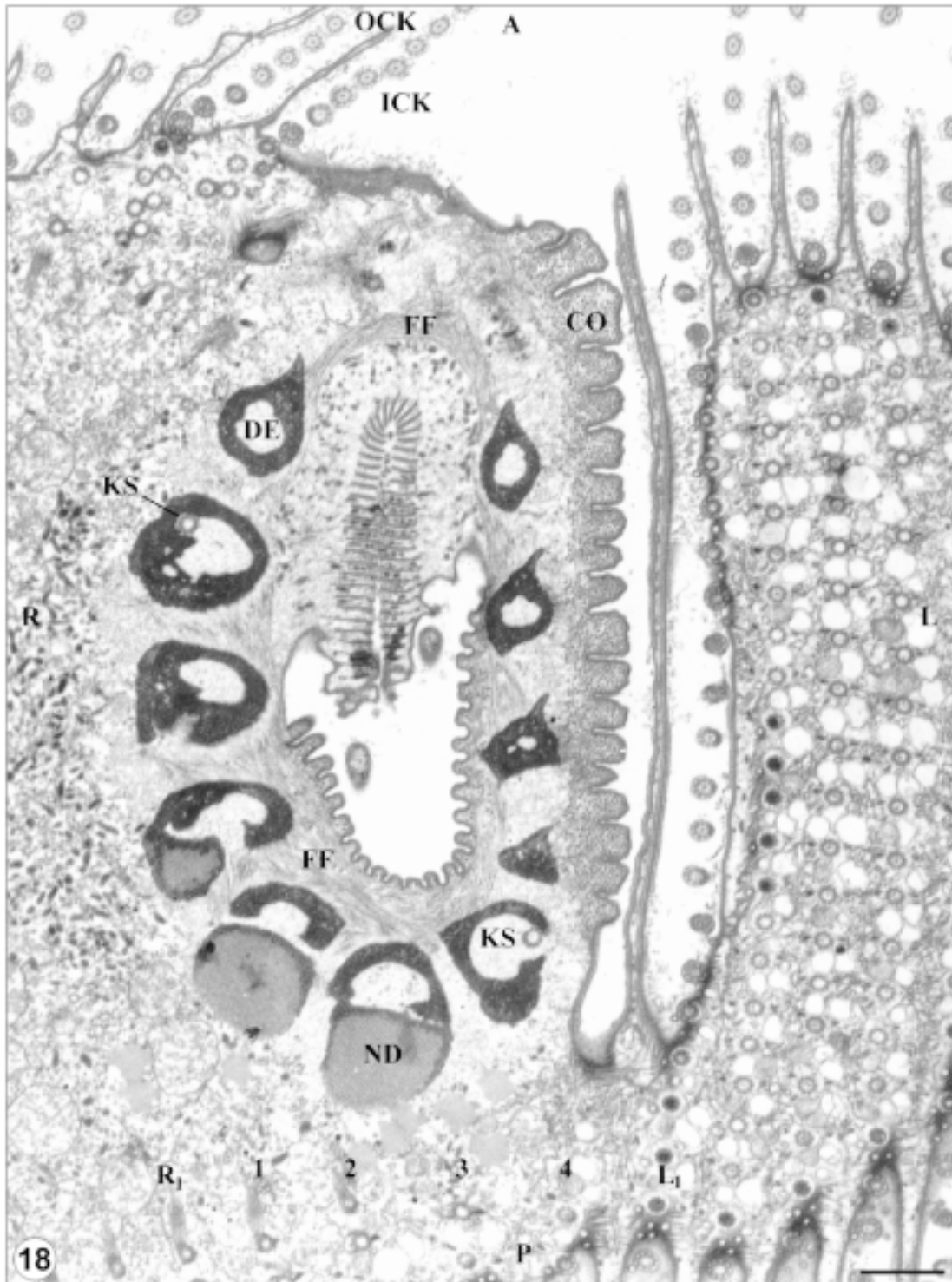


Fig. 18. Slightly oblique cross-section through the oral apparatus of *Chlamydomonas*. The dominating structures in this micrograph are the dentes (DE) on the upper end of 11 nematodesmal rods (ND). These dentes enclose a barren kinetosome (KS) each. The dentes are connected to each other and also to the corrugations of the collar (CO) by fine fibrillar material (FF). The collar has very flat pellicular alveoli. Immediately underneath the epiplasm there is an accumulation of numerous granules. The inner borderline of the collar shows a wavy demarcation around an empty looking extracellular space with two bacteria near the centre of the micrograph. The cytopharyngeal lamellae are seen in the upper part of the oval array of the cytostomal dentes. Note the dikinetids of the inner (ICK) and outer circumoral kinety (OCK). Only the outer (which is the anterior) kinetosome of each oral dikinetid has a cilium. In the somatic cortex the four postoral kineties are labelled with numbers 1 through 4. The right-most of five somatic kineties of the left somatic ciliature is labelled L_1 , while R_1 is the left-most kinety of the right somatic ciliature. Scale bar - 1 μ m

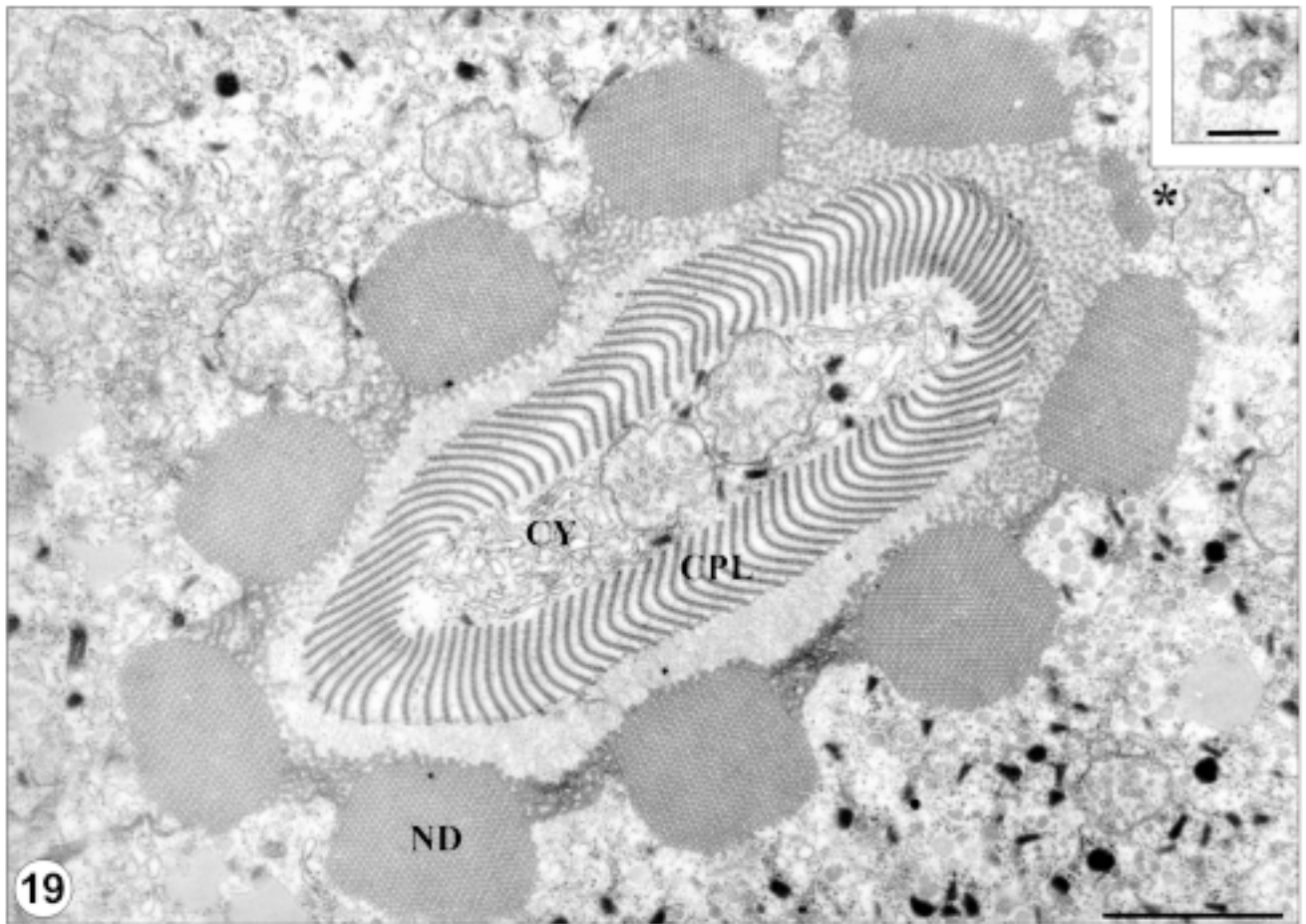


Fig. 19. An oblique section of the cytopharyngeal basket of *Chlamydomon*. In this specimen there are nine massive nematodesmata (ND) and two small ones lying adjacent to each other in the upper right corner of the micrograph (asterisk). Each rod shows a hexagonal arrangement of nematodesmal microtubules. Between the rods less regularly arranged microtubules are seen. Some 100 cytopharyngeal lamellae (CPL) form the inner part of the basket in this non-feeding specimen. The site where the cytopharynx would form, is occupied by cytoplasm (CY) in this micrograph, which may be an artefact. The inset in the upper right corner shows the two kinetosomes, which are located near the upper end of the two small rods. Scale bars - 1 μm , inset 0,5 μm

Fremiet 1950). *Chlamydomon mnemosyne* is a rather variable species, in particular with respect to the size of the cell which ranges from 30 μm in length (Dragesco and Dragesco-Kernéis 1986) to 150 μm (Dragesco 1960). The total number of somatic kineties likewise varies from 26 to 40, but all strains have four postoral kineties. Also the number of cytopharyngeal rods is variable ranging from 8 to 16, but it has to be recalled that the existence of the two thin rods at the anterior circumference of the basket may cause some uncertainty about the precise number. The strain used in this study was not a clonal culture, therefore probably showing some variations in the above mentioned parameters and probably belongs to the smaller strains.

A different *Chlamydomon* species, *C. roseus* described by Dragesco in 1966 is said to contain many reddish vacuoles. We presume that the colour of the vacuoles, most probably food vacuoles, originates from ingested cyanobacteria. It is well known that copiously fed nassulids can assume all sorts of colours due to the changes of the photopigments during digestion. Thus colour is probably not a valuable diagnostic character in specialised ciliates feeding on cyanobacteria. But since *C. roseus* has 54-58 somatic kineties it may well be a separate species (Dragesco 1966). An amiconucleate species, *C. pedarius* was described by Kaneda (1953).

There is general agreement on the overall organisation of the somatic ciliature of *C. mnemosyne* except for the

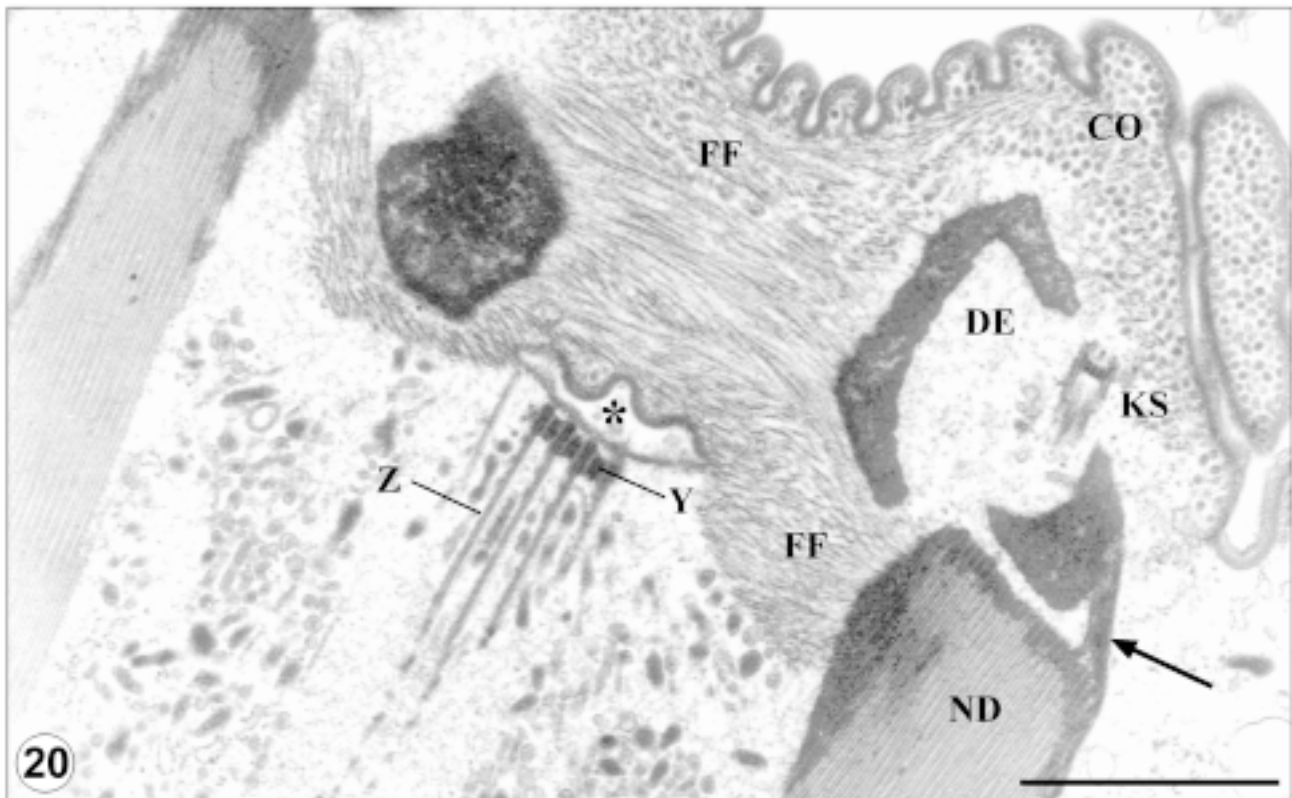


Fig. 20. Upper end of the longitudinally cut cytopharyngeal basket. Note the barren kinetosome (KS) inside the dens (DE) which covers the nematodesmal rod (ND) and is connected to it by large fibrous link (arrow). The ridges of the oral collar contain numerous granules of unknown nature, the area underneath the collar is densely packed with microfibrillar material. Two types of microtubules, labelled Z and Y constitute the cytopharyngeal lamellae. A small opening to the environment can be seen in the centre of the micrograph (asterisk). Scale bar - 1 μ m

description of the outer right kinety (Lom and Corliss 1971). In the majority of the descriptions its right lateral part is said to consist of two stretches of irregularly spaced barren kinetosomes, one stretch associated with the anterior half of the cell and one with the posterior half (see Lom and Corliss 1971). There are no fibrillar or microtubular fibers associated with these kinetosomes. It is also uncertain whether these kinetosomes have any relation to the short anterior rightmost kinety (the "cinétique droite externe") which theoretically lies on the same meridian as the right barren kinetosomes. Our EM-study shows that *C. mnemosyne* has the typical phyllopharyngid kinetid pattern (Lynn 1981). There are only minor differences compared with cyrtophorid and dysteriid ciliates. Thus *C. mnemosyne* has only a single transverse microtubule while *Chilodonella*, *Trithigmostoma* and *Brooklynella* have two (Lom and Corliss 1971, Hofmann 1987, Hofmann and Bardele 1987). Other differences do not concern the kinetid pattern directly but may be of interest nonetheless. Thus the number of triads of postciliary microtubules in the left slope of the cortical

ridges is 10-14 in *Trithigmostoma*, 8 in *Chilodonella*, up to 9 in *Chlamydodon*, and up to 17 in *Brooklynella*. Quantitative differences also exist in the number and length of the subkinetal microtubules, which are not necessarily in relation to the length of the cell. In *Trithigmostoma* and *Chilodonella* there are 6 microtubules in a sheet with 10 overlapping sheets in *Chilodonella* and up to 20 in *Trithigmostoma*, in *Brooklynella* there are 5-6 per sheet and 5 in *Chlamydodon*. In the later two species these subkinetal microtubules overlap only 3-4 ciliary territories. The fibrillar strands, which run perpendicular to the longitudinal axis of the cortical ridges seem to be a peculiarity of *Chlamydodon*. Similarly looking fibrils have been observed in *Brooklynella* (Lom and Corliss 1971; Figs. 19, 25); but since the latter are lying more distal from the cortical ridges it is uncertain whether they are homologous to the fibrils in *Chlamydodon*.

The organisation of perioral kineties is almost identical to the situation seen in *Chilodonella*, *Trithigmostoma* and *Brooklynella*. A well-developed

cytopharyngeal basket is found in all nassulid and cyrtophorid ciliates. While the Nassulida cluster with the Oligohymenophora, the Phyllopharyngea are a separate class of ciliates and their precise position with respect to the other ciliate classes is still a matter of discussion (Fleury *et al.* 1992, Schlegel and Eisler 1996, Wright *et al.* 1997). Thus in all probability the cytopharyngeal basket in both groups resulted from independent convergent evolution. But for sure, the cytopharyngeal baskets are derivatives of somatic or oral kinetosomes. The kinetosome has the potentiality to grow nematodesmal microtubules which may stay with the kinetosome or separate from it after nucleation. Secondly, postciliary microtubules which line the cytopharynx either remain attached to kinetosomes of the paroral membrane or adoral organelles or they separate from their kinetosomes as it is e.g. the case in *Chilodonella* (Hofmann 1987). The number of nematodesmal rods seems to be of minor significance, more important are their sizes as they appear in cross-sections. While in the Nassulida and the Chilodonellida all rods are of similar size, those in the Chlamyodontida are of different size. So far we have no explanation what determines the smaller size of the two anterior rods. The oral structures show a certain degree of bilateral symmetry due to the two anterior kinetosomes which are closer to each other, and the smaller cytopharyngeal rods which originate in their neighbourhood. In addition, the specific array of the corrugations of the collar likewise displays a bilateral symmetry.

The situation with microtubular lamellae within the basket is more complex. There is a maximum of three such microtubular lamellae, the cytostomal (Z), the subcytostomal (Y), and the nematodesmal (X) lamellae. The XYZ-nomenclature given in parentheses goes back to Tucker (1968). The nematodesmal lamellae originate from the nematodesmal rods, while the other two are derived from postciliary microtubules (increased in number when located inside the basket). All three lamellae are found in *Nassula* and *Furgasonia*; other nassulid ciliates, like *Pseudomicrothorax* and *Leptopharynx* have only nematodesmal lamellae (Eisler 1988). In the cyrtophorid ciliates *Chilodonella* and *Trithigmotoma* cytostomal and subcytostomal lamellae have been demonstrated. Both can also be found in *Chlamyodon* (this study). For *Brooklynella* only one set of microtubules was mentioned. They correspond to cytostomal lamellae but the micrographs let us presume that there are also subcytostomal microtubules (Lom and Corliss 1971). Some of these microtubules have dynein-like arms and

may be involved in transportation of ingested material, either indirectly through movement of phagoplasm around the ingested material or more directly through contact with the incoming membrane which surrounds the ingested food.

Elaborate dentes or capitula are known for the orders Dysteriida (Lom and Corliss 1971) and the Chlamyodontida (this study). In both *Brooklynella* and *Chlamyodon* there is a barren basal body in each dens. Though no direct proof can be given we assume that the barren basal body is somehow involved in the nucleation of the nematodesmata. Fibrillar caps on top of the nematodesmata also occur in *Chilodonella* and *Trithigmotoma*, but they lack kinetosomes within these caps. Nonetheless, the nematodesmata in these ciliates originate during stomatogenesis from basal plates of kinetosomes on the proximal end or some kinetosomes which are located in front of the future perioral segments (Hofmann 1987).

According to MacDougall (1928) the dens can be pulled back to open the mouth during feeding. Though we have not yet been able to verify this observation such movement of the dens either passive or active is not unreasonable. No other phyllopharyngid ciliate is known with such a prominent collar. Similar to the oval of the nematodesmal rods this structure has a bilateral symmetry. The collar is open at the anterior pole, interestingly at the same position where two thin nematodesmata are located. The collar was compared to a circular myoneme or a sphincter (MacDougall 1928). We have no indication that the collar itself is contractile but fine fibrillar filaments found almost everywhere in the upper oral area could well be contractile. Contractile actin filaments have been detected in the oral area of *Pseudomicrothorax dubius* by immunofluorescence (Hauser *et al.* 1980) and ATPase activity was detected in its cytopharyngeal basket (Hauser *et al.* 1980, Hauser and Hausmann 1982).

Kinetosomes associated with nematodesmal rods have gained considerable interest. Microtubule nucleating capacity is ascribed to these kinetosomes. Usually there is some electron dense material in form of a thin plate associated with the proximal end of the kinetosome and it is this plate which is said to serve as template. In prostome ciliates (*Coleps*, *Prorodon*) circumoral dikinetids are permanently associated with the proximal end of the nematodesmal rods (Huttenlauch 1987, Hiller 1993). In *Nassula* the nematodesmata grow from ciliated kinetosomes (Tucker 1970). In another nassulid ciliate, *Pseudomicrothorax*, there is one barren kineto-

some on top of each rod through the entire trophic life of the cell, but a pair of kinetosomes is associated with each rod in the proter during early stomatogenesis (Peck 1974). These paired kinetosomes are said to be homologous to stichodyads. Experimental studies have been performed with *Nassula* as model systems which show the significance of intertubular linkers for microtubule pattern formation. On the other hand, close-packing-effects as well as a microtubule-nucleating-template function of the material associated with the proximal end of the kinetosome seem to be involved (Tucker *et al.* 1975). However, nobody really knows how microtubule nucleation and patterning is performed at the molecular level.

The cross-stripped band is a totally enigmatic structure. To MacDougall (1925), the clasps of the striped band looked like cross-ties of a railroad track, and she coined the popular name of the entire structure, the "railroad track". No other ciliate is known with a similar structure and frankly spoken our investigation, except for the first description of its fine structure, has given us no hint to its function. One might speculate that the C-shaped clasps keep the lumen of the tube-like structure open as the tracheal cartilages do, but for what purpose? One might also argue that this structure is just another though very elaborate version of the cytoskeleton, perhaps with the function to stretch the dorsal surface. The ventral surface with its numerous cortical ridges and the rather rigid subkinetal microtubules does not seem to need further supportive structures. Hitherto cytoskeletal function has been ascribed to subpellicular microtubules, cortical networks of microfilaments and to the epiplasm in general. Perhaps the most complex differentiation of epiplasm was described for the nassulid ciliate *Pseudomicrothorax* made of seemingly rather ubiquitous proteins, the articulins (Huttenlauch *et al.* 1995). We have also checked whether there is any relationship between the many contractile vacuoles and the cross-stripped band, and one might ask whether the latter structure has something to do with osmoregulation in this euryhaline ciliate. But we found no such relationship. The contractile vacuoles look quite normal, and as described, each vacuole empties independently to the ventral side of the cell.

As mentioned in the Results there are numerous fibrillar strands seen in the cytoplasm of *C. mnemosyne* interconnecting almost all cellular structures. Very elaborate fiber systems have been depicted with the Protargol technique in the dysteriid ciliates (Dragesco 1967). These fibrils might belong to the cytoskeletal system as well, they certainly deserve further studies.

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Light and Scanning Electron Microscopical Study of Stomatogenesis in the Cyrtophorid Ciliate *Chlamyodon mnemosyne* Ehrenberg, 1837

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Summary. Using Fernández-Galiano silver impregnation technique and scanning electron microscopy of artificially deciliated specimens the divisional morphogenesis of the cyrtophorid ciliate *Chlamyodon mnemosyne* was investigated. Morphogenesis in this ciliate is of the merotelokinetal type with four postoral kineties and two left somatic kineties involved in the formation of the oral ciliature in the future opisthe. Like in other cyrtophorid ciliates (*Trithigmostoma*, *Chilodonella* and *Brooklynella*) the stomatogenic kinetofragments perform a complex morphogenetic migration to yield the inverted preoral kinety and the inner and outer circumoral kineties. The differentiation of an oral rosette made of 9-11 large and two smaller nematodesmal rods covered by a prominent collar of cytostomal corrugations is described. Oral reorganisation in the proter, involving dedifferentiation of the entire parental cytostome and the *de novo* formation of a new cytostome in front of the old one is best seen in deciliated dividers. Replication of kinetosomes in particular the transformation of somatic monokinetids into oral dikinetids as judged from light microscopy seems similar to the events in *Trithigmostoma*. Details observed in *Chlamyodon* are discussed in comparison with other cyrtophorid ciliates.

Key words: artificially deciliated ciliates, *Chlamyodon mnemosyne*, Cyrtophorida, morphogenesis, oral reorganisation, scanning electron microscopy.

INTRODUCTION

Chlamyodon mnemosyne Ehrenberg, 1837 is an euryhaline cyrtophorid ciliate which can easily be cultivated in 50 % sea-water with filamentous cyanobacteria as food (Kurth and Bardele 2001). Pulse-fed mass cultures provided enough cells in various stages of binary division to study divisional morphogenesis.

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Stomatogenesis in *Chlamyodon* as in all other Cyrtophorida belongs to the merotelokinetal type. As described in detail both at light and electron microscopical level for *Chilodonella cyprini* (Hofmann 1987) and *Trithigmostoma steini* (Hofmann and Bardele 1987) only a few somatic kineties are involved in the formation of the new mouth of the posterior daughter cell, the opisthe. Kinetofragments of these stomatogenic kineties which consist of monokinetids separate from the rest of the ventral somatic kineties and transform into dikinetids. Subsequently these kinetofragments assemble into three entities which perform a complex morphogenetic migration around the position of the future oral area, resulting in three inverted perioral kineties known as the inner and

outer circumoral kinety and the preoral kinety. During this morphogenetic movement parts of these kineties separate to nucleate the new nematodesmal rods of the cytopharyngeal basket and the microtubular cytopharyngeal lamellae which form the inner tube of the basket. Earlier light microscopical studies on stomatogenesis e.g. by Lom and Corliss on *Brooklynella hostilis*, an ectoparasite on gills of marine fishes (Lom and Corliss 1971), and several other dysteriid ciliates by Deroux (1994) support a fairly uniform stomatogenesis in the opisthe of cyrtophorid ciliates.

Contrary to the well-understood processes in the opisthe almost no information is available about reorganisation of the oral apparatus in the proter. It is the aim of this study to add new data here. As a prerequisite for thin-section studies currently in progress we give a description of the light microscopical aspects of stomatogenesis in *Chlamyodon mnemosyne* supplemented with SEM observations on artificially deciliated specimens, which were of great help to study the asynchronous events in the two daughter cells, the proter and the opisthe.

MATERIALS AND METHODS

The origin of cultures of *Chlamyodon mnemosyne* and the methods to grow this ciliate have been given in the preceding paper (Kurth and Bardele 2001). In order to increase the number of cells in division starved cultures were pulse-fed, but no long-term synchronisation was reached. For light microscopy bulk samples were stained with pyridinated silver carbonate according to Fernández-Galiano (1976) with the following modification. Cells were first fixed with 1 % formaldehyde and then stored in 0.6 % formaldehyde for several days in the refrigerator before the standard procedure was continued. For SEM cells were fixed in Párducz solution (Párducz 1967) and processed as described in Kurth and Bardele (2001). Prior to fixation cells were artificially deciliated by mechanical agitation for 3 min with a Pasteur pipette in a solution of 4 % ethanol in a 1:1 mixture of Eau Volvic (French mineral water from the Auvergne) and natural seawater. The deciliated cells allowed us to describe in more detail than in silver-stained preparations the asynchronous morphogenetic events in both the proter and the opisthe.

RESULTS

Prior to the description of morphogenesis we give a short summary of the general morphology of *Chlamyodon mnemosyne*. The cell measures about 70 x 45 µm. The slightly curved dorsal face is completely

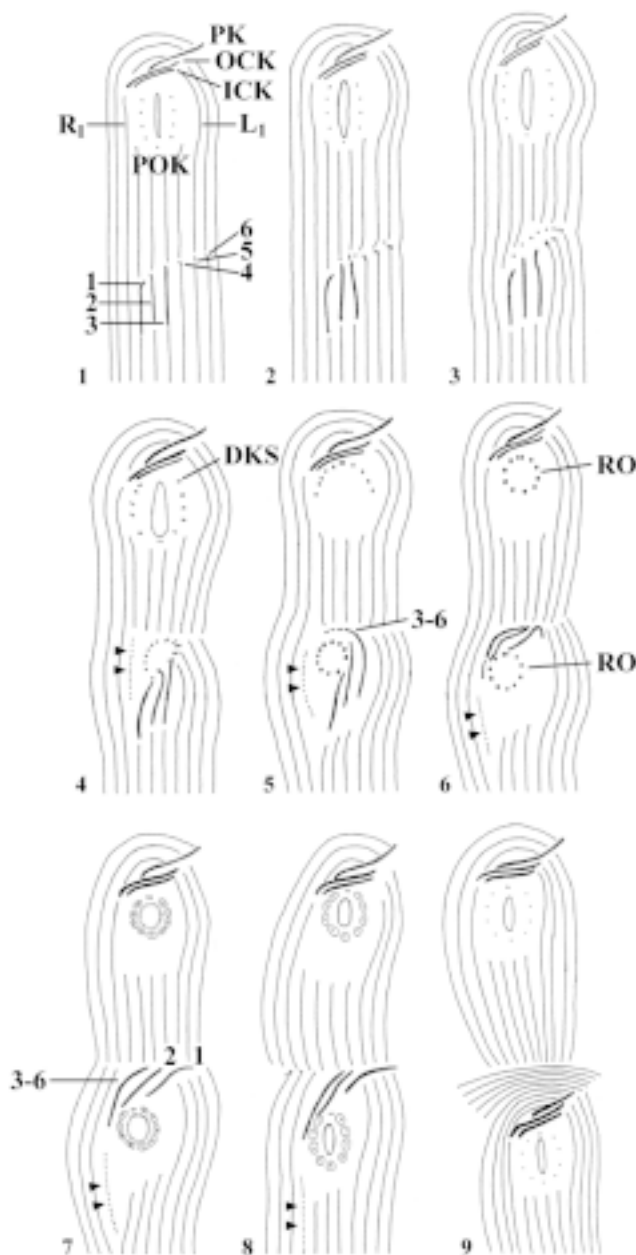
free of cilia. At the borderline between the ventral and the dorsal face of the cell there is the genus-specific "striped band" of unknown function, colloquially called "railroad track" (MacDougall 1928). The ventral surface of the cell is flat and completely ciliated except for the oral area.

The somatic ciliature on the ventral surface consists of a right and a left field of somatic kineties with four postoral kineties in between. The right somatic field consists of 12-15 kineties, their anterior ends bend over to the left anterior corner of the ventral surface and abut to the anterior ends of the left somatic kineties, 9-11 in number. The somatic kineties are made of monokinetids displaying the typical cyrtophorid kinetid pattern (Lynn and Small 1981). The most characteristic feature of the somatic kinetids are the sheets of subkinetal microtubules which overlap in an anterior direction (for details see Kurth and Bardele 2001).

The slit-shaped cytostome is located in the anterior third of the ventral surface. The cytostome is surrounded by an oval collar of solid corrugations. In front of the cytostome are three more or less straight inverted perioral kineties, the inner and outer circumoral kinety and the preoral kinety, all three made of dikinetids with only the anterior kinetosome ciliated. The cytopharyngeal basket consists of 9-12 nematodesmata and an inner ring of cytopharyngeal microtubular lamellae. The nematodesmata are capped each by a so-called dens (or capitulum) which contains a barren kinetosome.

We found it convenient to divide morphogenesis of *Chlamyodon* into nine stages (Fig. 1). This somewhat unusual way to present the summary first helps to orientate the readers and facilitates the description of details. Note that Fig. 1 shows only the middle part of the ventral face, which is the area where the important changes take place during morphogenesis. The course of stomatogenesis is not synchronous in both the future anterior and posterior daughter cell, the proter and the opisthe. In the description of the morphogenetic stages we first mention the events which take place in the opisthe. Changes in the opisthe are more complex than the changes in the proter which are restricted to the reorganisation of the parental cytopharyngeal complex.

The formation of the oral structures of the opisthe is entirely from somatic components. No constituents of the parental oral structures are involved. A special role is played by the four postoral kineties, labelled 1 through 4 in Fig. 1, also involved are single kinetosomes of the somatic kineties to the cell's left, labelled 5 and 6. Since



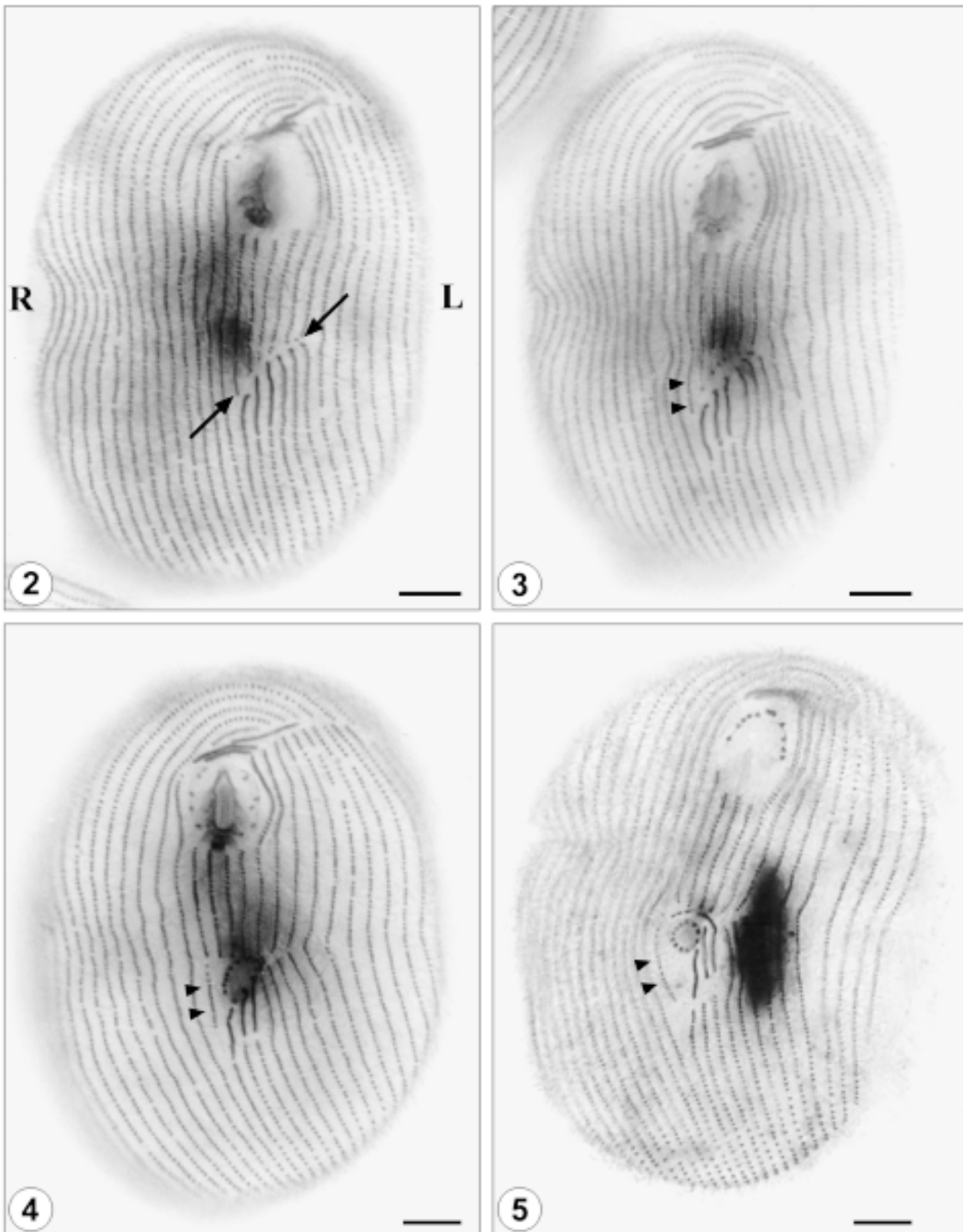
both at light microscopical and at SEM level no visible changes are seen in the proter during *Stage 1-2* the first events concern the opisthe only.

Stage 1. As the first sign of the beginning of stomatogenesis in *Chlamydodon* an oblique interruption of the straight course in the postoral kineties is seen immediately behind the middle of the ventral surface of the cell. This stage is not shown in a silver-stained specimen, it corresponds to the cell outlined in the upper left corner of Fig. 1. The three kinetofragments labelled 1-3 and the three pairs of kinetosomes shown anterior to

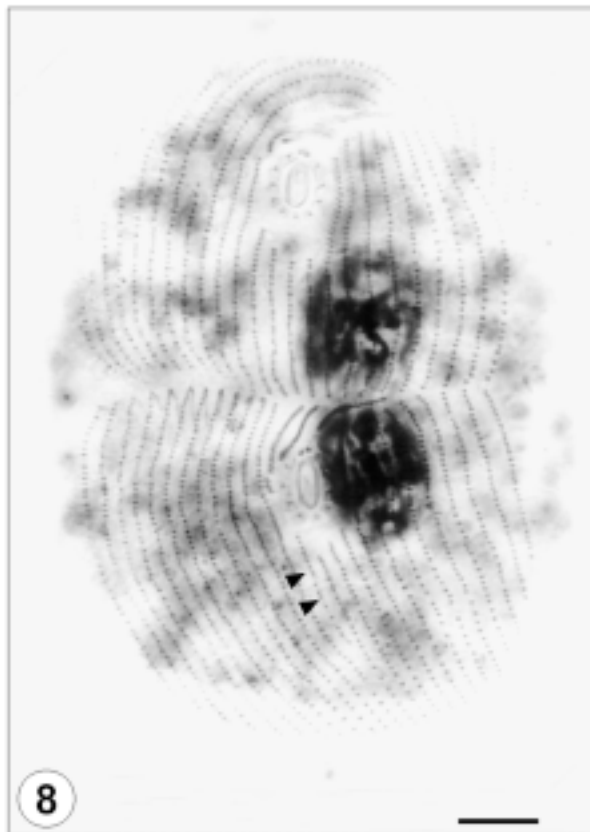
Fig. 1. Diagram of the morphogenesis in *Chlamydodon mnemosyne*. Nine stages labelled 1 through 9 are recognised. Note that only the middle part of the ventral surface is shown. In subfigure 1 the perioral ciliature consisting of the preoral kinety (PK), the outer circumoral kinety (OCK) and the inner circumoral kinety (ICK) are shown by a thicker line, since these three kineties are made of dikinetids. Also the first three segments (labelled 1-3) of the four postoral kineties are shown in thicker lines since in these segments somatic monokinetids transform into dikinetids. These segments will perform a complex morphogenetic migration and become the perioral ciliature of the opisthe (see text for details). The three kineties to the left, labelled 4-6 (or K4 - K6 in text), are also involved. Single kinetosomes or pairs of kinetosomes which lie in front of the stomatogenic fragments become more distinct. In the subsequent stages of stomatogenesis these kinetosomes form the so-called rosette which nucleates the nematodesmata of the cytopharyngeal basket of the opisthe. The cross-sectional profiles of the nematodesmata are shown as small circles round the kinetosomes of the rosette (subfigures 7-8). Actually, the rosette kinetosomes are localised in fibrous dents sitting on top of the nematodesmata. Reorganisation of the oral structures in the proter is characterised by the complete disassembly of the parental nematodesmata (not shown) by significant changes in the array of the rosette kinetosomes. Note the intermediate opening of the circle into a crescent line (subfigure 5), its subsequent closure, and re-assembly of the nematodesmata in the proter. In *Stage 1 to 3* in the proter, and in *Stage 9*, both in proter and opisthe, only few details are seen of the cytostome-cytopharynx complex due to the fact that the corrugations of the collar obscure the structures lying underneath. Further abbreviations: DKS, double kinetosome; L1, first left kinety; POK, postoral kineties; R1, first right kinety; RO, rosette. Starting from subfigure 4 through 8 a middle segment of the first postoral kinety is shown as a dotted line and labelled with two arrowheads. This "new" kinety in the opisthe compensates for the fact that the opisthe does not get a part of the leftmost parental somatic kinety (not shown) because it is too short to reach the opisthe. In some preparations pairs of kinetosomes can be seen in the cytopharyngeal region of the proter as depicted in subfigure 4

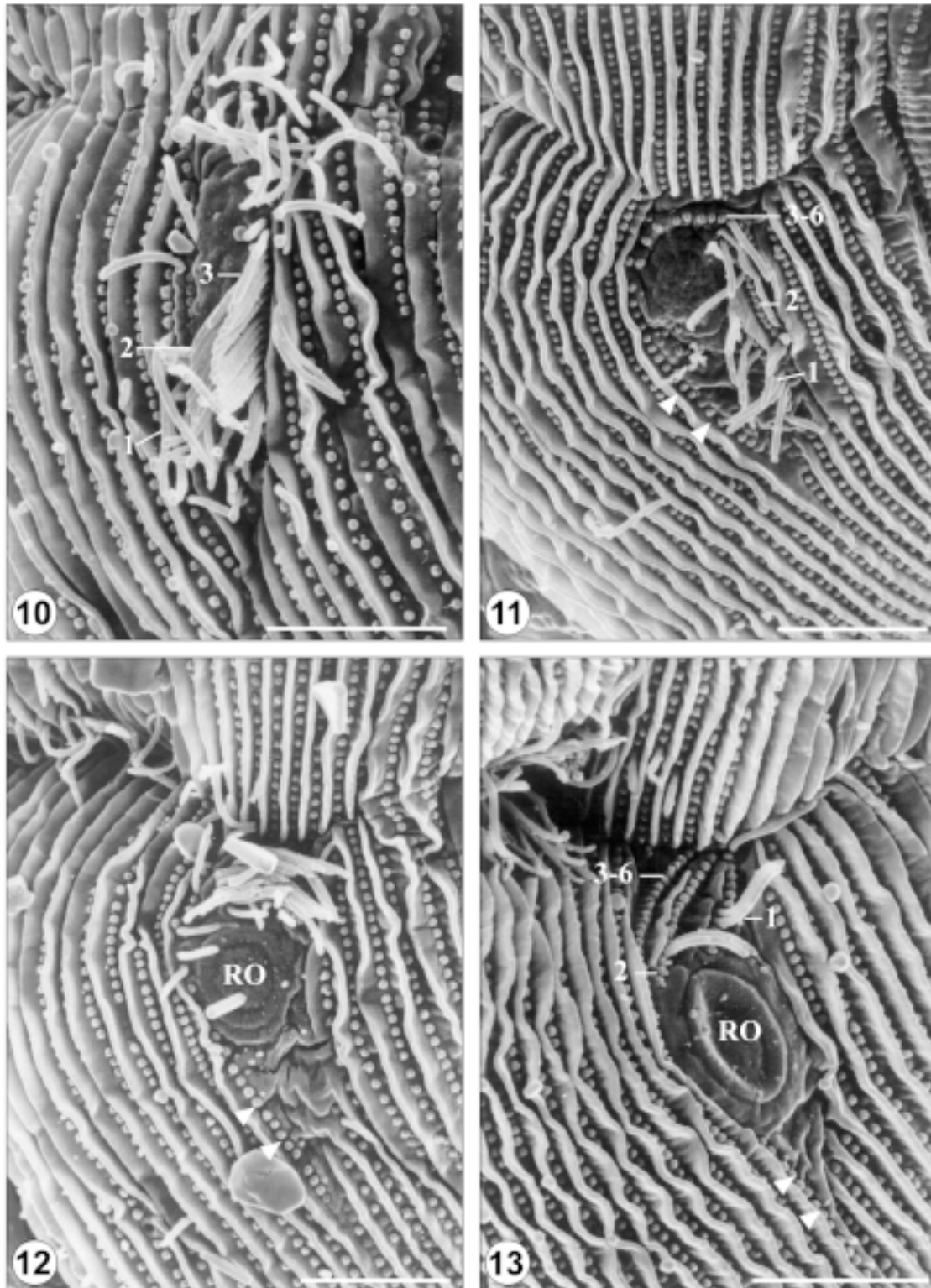
the fragments labelled 4-6 will become the three perioral kineties of the opisthe. Note that in the following eight micrographs of silver-stained cells the number of the figure is identical with the morphogenetic stage it represents, thus e.g. Fig. 2 shows *Stage 2*, and so on.

Stage 2. The oblique interruption anterior to the stomatogenic kineto-fragments (K1 through K6) is seen more clearly (Fig. 2). Since they appear thicker than the other kineties they probably have replicated their kinetosomes. During the further course of stomatogenesis the three thicker parts, called hitherto stomatogenic kinetofragments, will separate from their anterior and posterior endings. Stomatogenic kinetofragment number 1 is the first to show its anterior end bending to the left (Fig. 2). Moreover, the very anterior ends of K4 to K6 appear more dense than the rest of the kineties. At times, pairs of kinetosomes were seen in this area, as drawn in Fig. 1 (subfigures 1, 2). Finally, several argentic dots, probably representing single kinetosomes, are seen at the posterior ends of the anterior fragments of the postoral kineties in an area close to the future

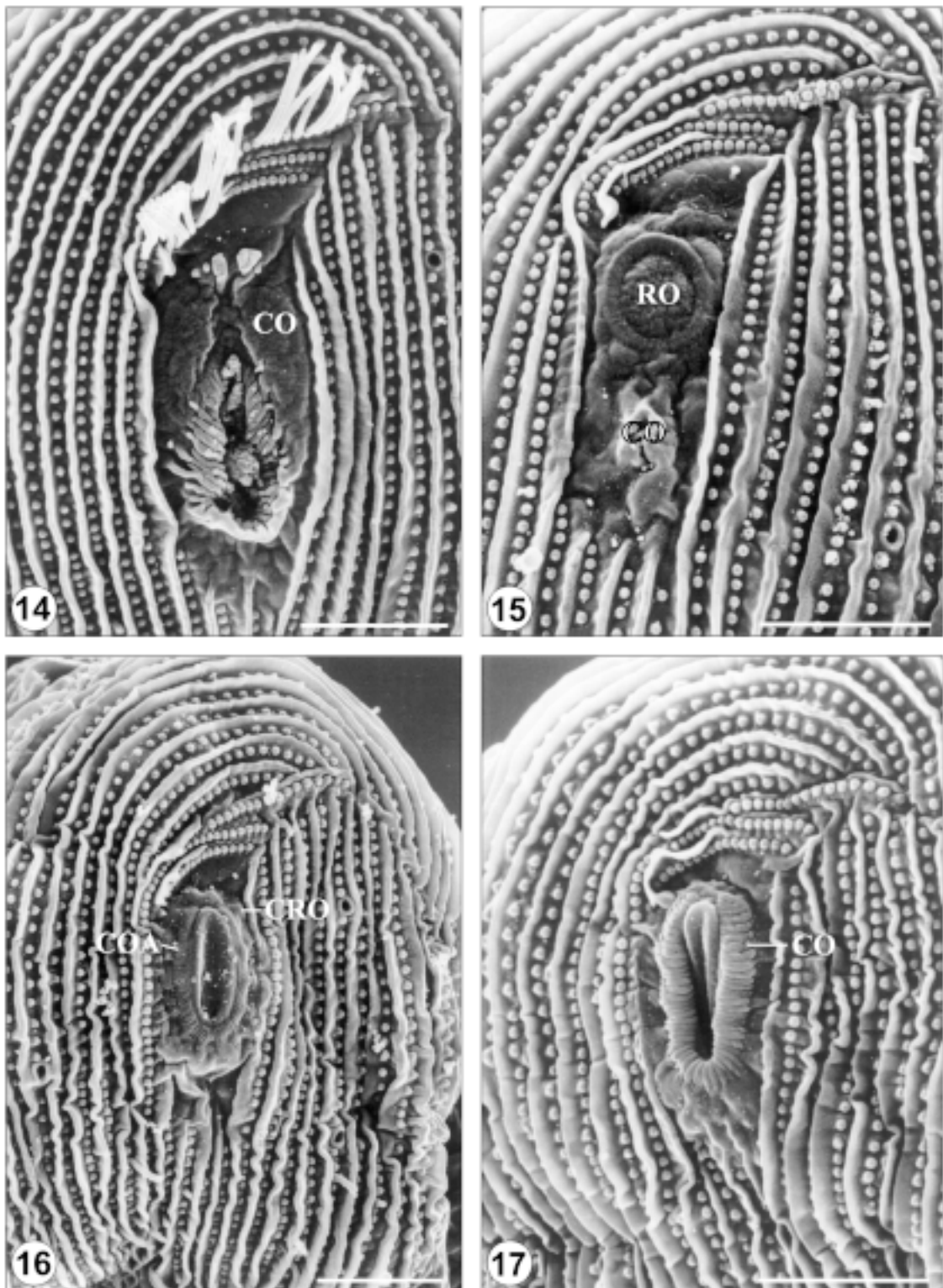


Figs. 2-9. Stage 2 through Stage 9 of morphogenesis of binary fission in *Chlamydomonas mnemosyne* stained with pyridinated silver carbonate according to Fernández-Galiano; arrows in Figure 2 indicate the oblique interruption in front of the stomatogenic kinetofragments; arrowheads indicate the "new" kinety forming the prospective first postoral kinety of the proter. For details see text. Scale bars - 10 μ m





Figs. 10-13. SEM view of artificially deciliated cells showing the morphogenetic events in the opisthe starting with *Stage 4* (10) and ending with *Stage 7* (13). For unknown reasons the perioral cilia sometimes withstand the deciliation procedure. Thus in (10) the three stomatogenic kinetofragments 1 to 3 show cilia. In (11) the compound of kinetofragment 3-6, later becoming the preoral kinety, has moved around the future rosette. Note that the perioral kineties show a single row of ciliary stubs more closely spaced than the somatic cilia. The second barren basal body of each perioral dikinetid cannot be shown with this technique. The "new" first postoral kinety of the opisthe is labelled with two white arrowheads in (11-13). In *Stage 6* (12) the oral rosette (RO) is labelled. This latter structure is seen more clearly in (13), where it shows an inner slightly higher rim and a flatter outer rim, from which the corrugations of the collar will develop. Scale bars - 5 μm



Figs. 14-17. SEM view of the reorganisation of the cytostomal area in the proter after artificial deciliation. **14** - invagination and resorption of the parental collar (CO); **15** - a new rosette (RO) has formed anterior to the old cytostomal area; **16** - differentiation of the corrugations or the collar anlage (COA) and cytopharyngeal rods (CRO); **17** - the corrugations of the collar (CO) have almost reached their final shape. Scale bars - 5 µm

posterior end of the proter. All these dots (or kinetosomes) arrange themselves in a slightly crescent line in the following stage.

Stage 3. The gap between this line of single kinetosomes and the anterior end of the stomatogenic kinetofragments widens. Another important feature is the elongation of the first anterior postoral kinetofragment by proliferation of its posterior kinetosomes in a backward direction (two arrowheads in Fig. 3). It is in *Stage 3* that for the first time changes in the proter become obvious. The oral slit, till now very small, widens gradually. The oval array of kinetosomes, which are located in the dentes heading the nematodesmata, assume a U-shaped array (Fig. 3). The central area of the cytostome region looks empty, probably due to the detaching of the cytopharyngeal basket from the prior cytostome.

Stage 4. In the future oral area of the opisthe, single kinetosomes and one or two pairs of kinetosomes begin to arrange themselves in a circular array (Fig. 1, subfigure 4 and Fig. 4). Other pairs of kinetosomes or derivatives seemingly originating from kinetofragment 4 to 6 are now positioned anterior to the stomatogenic kinetofragment 3. The anterior ends of all three major stomatogenic kinetofragments are bowed to the left side of the cell and seem to start their morphogenetic migration around the position of the future oral area of the opisthe (see also Fig. 10). The rightmost postoral kinety in the future proter has grown in length. The nematodesmal kinetosomes are duplicated resulting in pairs of kinetosomes (Fig. 1, subfigure 4) consisting of an old and a new kinetosome. (Note that the scanning electron microscopical aspects of *Stage 4* through *Stage 7* of the opisthe are illustrated in Figs. 10-13.)

Stage 5. This stage is an intermediate stage of the morphogenetic movement of the stomatogenic kinetofragments. Kinetofragment 3 is the quickest in its movement around the now circular "rosette", the nucleation site of the cytopharyngeal rods (Fig. 5). The term rosette was introduced by Lom and Corliss in their study of *Brooklynella* (Lom and Corliss 1971), later such an array of kinetosomes was also observed in *Chlamydonella*, *Lynchella* and *Chilodonella* (Deroux 1976, 1994). At the anterior end of kinetofragment 3 short streaks of argentophilic elements are seen. These are presumably the derivatives of K4 through K6 first seen in *Stage 2* and probably represent replicated kinetosomes. This interpretation is influenced by our knowledge of the corresponding events in *Trithigmostoma* and *Chilodonella* (Hofmann 1987, Hofmann and Bardele 1987). It is a derivative of K6 which leads the elephant

dance to the right around the oral rosette, followed by elements of K5 and K4, while kinetofragment 3 forms the tail. Altogether K3, K4, K5 and K6 (labelled 3-6 in Fig. 1, subfigure 5 and Fig. 11) will form the future outer circumoral kinety of the opisthe. Note that in *Stage 4* and *Stage 5* the somatic kineties which form the inner part (close to the mouth) of the somatic ciliature have assumed a slightly bowed configuration in preparation of their course in the mature opisthe (Figs. 4, 5).

In the proter the oral slit has become invisible in silver-stained specimen but more important the kinetosomes formerly associated with the dentes, and once sitting on top of the nematodesmata have assumed a semicircular linear array with one or two pairs of kinetosomes in the middle of the arc. Compared to *Stage 4* the array of kinetosomes in the proter of *Stage 5* now shows an inverted U-shape. From observation of living cells we know that in *Stage 5* the cytopharyngeal basket has separated from the cytostome, and sunken into the cytoplasm where it starts to disassemble. It is probably through the separation of the basket from the dentes that the kinetosomes can change their arrangement. But quite unexpectedly, the circle of kinetosomes obviously opened at its posterior end and not at the anterior end where two small cytopharyngeal rods were observed in thin sections (Kurth and Bardele 2001). Finally, at least in *Stage 5* the beginning of the fission furrow cuts in from the right side of the cell.

Stage 6. In this stage the oral rosette is complete. In most cases it consists of 11 kinetosomes (Fig. 6), two of them difficult to see in silver-stained preparations, but known from TEM (see inset in Fig. 19 in Kurth and Bardele 2001), lie close to each other at the anterior end of the rosette. Though circular in outline (see Fig. 12) a bilateral structure has formed, as an announcement of the bilateral symmetry of the oral collar seen in the mature cell. Very complex rearrangements of the future circumoral kineties take place in *Stage 6* and the following stages, which for lack of space cannot be documented in every detail. The migration of the future perioral kineties, when seen from the ventral side, is in a counter-clockwise fashion. The anterior ends of all three perioral kineties point to the right side of the cell and during further migration their anterior ends point to the posterior end of the cell while the posterior ends of these same kineties point in an anterior direction, thus resulting in an inverted position of the perioral kineties. In particular K1 which later will become the preoral kinety, has a distinct position in *Stage 6*, its anterior end lies anterior to the apex of the rosette while its posterior

end forms a hook (see Fig. 1, subfigure 6 and Fig. 6). Otherwise, the cell shown in Fig. 6 is somewhat untypical, the opisthe shows only two (instead of three) postoral kineties and the third being added to the right of the former.

As far as the proter is concerned the open arch of kinetosomes from *Stage 5* has now closed again to re-establish the circular array of the rosette. The changes which occur on the surface of the oral area of the proter are seen best in deciliated specimens. The oral collar of the parental mouth is invaginated beginning with the anterior part as shown in Fig. 14. Moreover, Fig. 6 (upper right) shows the short length of the leftmost somatic kinety, which consists of eight kinetids only in this particular micrograph. They all will end up in the proter which is a typical behaviour of the leftmost somatic kinety in other cyrtophorid ciliates, likewise (Deroux 1994). The solution of this problem, loss of one somatic kinety in the opisthe, comes from the observation of the behaviour of the "new" kinety which slides down past the right of the future oral area of the opisthe (arrowheads in Fig. 1, subfigures 4-8 and Fig. 11) and enters the V-shaped empty space seen on the right side of the three postoral kineties in Figs. 5, 6, 12.

Stage 7. The hook-like posterior end of K1 has straightened. Its posterior end has moved toward the left part of the fission furrow, into a zone which later becomes the anterior suture. The anterior end of K1 still lies posterior left to K2. A major characteristic of *Stage 7* is the threading of the new first postoral kinety (arrowheads in Fig. 1, subfigure 7, and Fig. 13) between R_1 and the now second postoral kinety of the opisthe.

The majority of rosette kinetosomes in both the proter and the opisthe are now surrounded by a broader halo of dense material, which may indicate the beginning assembly of the nematodesmal rods. This does not hold for the two anterior kinetosomes of the rosettes of both daughter cells, thus resulting in baskets with nine massive rods at light microscopical level. It is noteworthy that thin-sections have revealed two very thin nematodesmata associated with the anterior kinetosomes (not shown). In *Stage 7* all somatic kineties of the former parental cell show a distinct interruption at the level of the future fission zone. The posterior end of the anterior fragments as well as the anterior end of the posterior fragments seem to proliferate their kinetosomes. The oral area of the proter in SEM micrographs now shows the new rosette anterior of the former mouth region of the parental cell (Fig. 15).

Stage 8. The circular rosettes both in the proter and the opisthe have changed into oval structures. The diameter of the nematodesmata in both cells has enlarged compared to *Stage 7* (Fig. 1, subfigure 7 and 8). In the opisthe K1, the future preoral kinety of the opisthe has further moved to the left side of the cell, but its anterior end is still posterior to the posterior end of K2. The two anterior dots of the oral rosette in the opisthe are particularly dense in this stage (Fig. 8). Thin sectioning has to show whether kinetosome replication occurs at this site. Deciliated specimens of *Stage 8* cells show that in the proter the formation of the new corrugations of the oral collar has begun (Fig. 16).

Stage 9. The new preoral kinety of the opisthe has reached its definitive position in front of the outer circumoral kinety (the former K3-6) which means that K1 has moved with its anterior end ahead to the right of the cell thus moving around the posterior ends of the two other perioral kineties. The anterior part of all right somatic kineties has bowed over to the left side of the cell, abutting the preoral kinety which lies in the anterior suture (Fig. 9). The distal ends of the nematodesmal rods have become undetectable at light microscopical level in both daughter cells probably through differentiation of the corrugations of the oral collar as indicated in Fig. 17. This same figure shows that finally the central part of the former ring-shaped but later oval covering of the cytostome becomes overgrown by the oral collar.

DISCUSSION

Technical remarks

Silver impregnation techniques have a long tradition in the study of morphogenetic events in ciliates. Not all available procedures are equally suited for any particular group. For cyrtophorid ciliates the Fernández-Galiano technique gives a clearer picture of the kinetosomes compared to the Chatton-Lwoff technique (Frankel and Heckmann 1968) while the Protargol technique (Tuffrau 1967) (at least in our hands) gave no satisfying results. We got the impression that the Chatton-Lwoff technique does not stain barren basal bodies as distinctively as ciliated ones. But other than that there is as yet no rational explanation why in a certain group a particular technique is superior to another one.

The deciliation technique combined with traditional scanning electron microscopy are powerful tools to

study divisional morphogenesis. The explanation is straight forward. Ciliates with a distinct layer of epiplasm are particularly well suited for the deciliation technique with 3-4 % ethanol. Cilia break off just above the kinetosome and precisely indicate the former position of a cilium. Barren kinetosomes cannot be demonstrated with this technique as long as they are covered by epiplasm. The dense spacing of the kinetosomes in the perioral kineties compared to the somatic kineties (see e.g. Fig. 16) gives a very realistic view. In addition, we have at times observed a differential persistence of cilia undergoing resorption and re-growth, an interesting process of renewal of oral cilia, to be dealt with later. Likewise, for the illustration of cortical ridges between the kineties as well as the mapping of the openings of the contractile vacuoles the deciliation technique is of great value. This holds also for the visualisation of the dynamic processes which occur both during stomatogenesis and reorganisation of the structures associated with the cytostome proper (Figs. 10-17). Finally, not yet illustrated in this paper, it is quite obvious that certain morphogenetic changes seen in thin sections are much easier to understand if a clear three-dimensional view is at hand.

Divisional morphogenesis regularly comprises two separate events, somatogenesis (replication of the somatic ciliature) and stomatogenesis, the formation of new oral structures for the opisthe. Though in a few cases the old oral structures of the parental cell seemingly without any major alterations, remain with the proter, in an increasing number of ciliates complex reorganisation of the parental oral structures is recognised when studied at greater depth. These processes are more difficult to observe since they occur within an already differentiated structure of the proter. Some entities develop while others, close by, gradually vanish following a timetable difficult to unravel. Static pictures seen in silver-stained preparations, in SEM and likewise in thin sections need to be arranged in a correct sequence. For this procedure to be done it needs special landmarks and key events which can be recognised reliably.

The type of stomatogenesis which is realised in *Chlamydon* was formerly called "somatic-meridional" since only kinetosomes of somatic kineties are involved (Corliss 1968). Now it is called "merotelokinetal" (Bardele 1989, Foissner 1996) since not all but only a certain number of kinetofragments, which separate from the parental kineties, transform into the oral ciliature of the opisthe. Dedifferentiation and subsequent re-differ-

entiation of the oral structures in the proter are variable in degree.

Among the cyrtophorid ciliates to which *Chlamydon* belongs we have the most detailed information about divisional morphogenesis on *Chilodonella* studied most carefully by Hofmann (1987). Since the morphogenesis of *Chilodonella* has become a kind of model system for cyrtophorid ciliates important highlights of this study are mentioned again since they are the basis for further discussion: (i) the transformation of somatic kinetofragments originally made of monokinetids into dikinetids, (ii) the documentation of complex morphogenetic migration of these stomatogenic kinetofragments to the area of the future formation of the oral apparatus while at the same time these arrange themselves into the three inverted perioral kineties, (iii) the differentiation of the nematodesmal rods from some specialised kinetosomes and finally (iv) the assembly of the inner ring of cytopharyngeal lamellae from postciliary microtubules originating from somatic kinetids. Thus *Chilodonella* became one of the best understood model systems of ciliate stomatogenesis as far as the new formation of the cytostome in the opisthe is concerned. However there is little information on the reorganisation in the proter. It is this topic from which our interest in *Chlamydon* came when we had performed the first investigations on deciliated cells.

Initial studies on the morphogenesis of *C. mnemosyne* performed by Fauré-Fremiet (1950) and Kaneda (1960) led to results now prone to be corrected. Both authors describe an anarchic field of irregularly positioned kinetosomes in the area of the future opisthe's cytostome. In our material of *C. mnemosyne* we have seen no such anarchic field. Instead we have always observed a highly ordered migration of the morphogenetic kinetofragments as described for *Trithymostoma*, *Chilodonella* (Hofmann 1987, Hofmann and Bardele 1987) or *Brooklynella* (Lom and Corliss 1971). At best, the few anterior kinetosomes of K4 through K6 when they align into the tip of the future preoral kinety can give the illusion of a small anarchic field. One speculation could be that during certain steps of the Chatton-Lwoff procedure used by Fauré-Fremiet (1950) desmose-like connections between kinetosomes become weakened to yield a more disordered array of kinetosomes. On the other hand we know from preliminary thin section studies that there is a large number of osmophilic granules of an appropriate size in the oral area of proter and opisthe which are less numerous in non-diving cells. These granules could also obscure the ordered array of

kinetosomes in living or Chatton-Lwoff processed specimens.

The denser appearance of K1 through K3 in Figs. 2-4 is probably due to kinetosome duplication in these stomatogenic kineties. This interpretation is based on thin section studies currently in progress and in accordance with observations made in *Trithymostoma* and *Chilodonella* where ultrathin sectioning has shown that kinetosome proliferation is accomplished by the "transition" of monokinetids into dikinetids (Hofmann 1987) in the same space. This means that the morphogenetic kineties do not elongate but their doubled kinetosomes are spaced more densely and such display a more distinct appearance.

The migration of the morphogenetic kinetofragments in *Chlamyodon* is similar to those in *Chilodonella*, *Trithymostoma* and *Brooklynella*. The migration behaviour of K1, however, is more closely fitting to that of K1 in *Brooklynella*, where it reaches its final position relative to the other kinetofragments rather late in development. In *Chilodonella* and *Trithymostoma* the migration of K1 is retarded and it moves early around the other fragments reaching its final position relative to the other kinetofragments at middivisional stages.

In the proter, Fauré-Fremiet (1950) observed the U-shaped opening of the parental oval array of cytopharyngeal rods. This seems to correspond to our *Stages 2* and *3*; but no inverted-U-shape array (*Stage 5*) has been described in the proter by other investigators. In addition, there are some uncertainties about the reorganisation in the preoral and circumoral kineties in the proter. In *Trithymostoma* "oral" cilia become resorbed and grow out again (Hofmann and Bardele 1987). The corresponding kinetosomes stay in place. As already indicated such "reorganising" cilia seemingly withstand the deciliation procedure more often than non-reorganising cilia. A more subtle elaboration of "differential deciliation" technique with various ethanol concentrations and varied periods of mechanical agitation might be useful to document the various stages in the life history of a cilium.

Contrary to the more gradual reorganisation of the oral ciliature in *Trithymostoma* and *Chlamyodon*, in *Brooklynella* a seemingly sudden rebuilding of the oral ciliature seems to take place as judged from light microscopical observations (Lom and Corliss 1971). New kinetosomes for the oral cilia of the proter are said to originate from the anterior tips of the middle postoral somatic kineties. We have not been able to decide whether a similar process occurs in *Chlamyodon*.

Stage 4 to *Stage 5* is the most important period for this event. We have shown in Fig. 1, subfigure 4 and 5 the sudden change of a U-shaped array into an inverted-U-shaped array of kinetosomes, which could be either pairs of kinetosomes or single ones becoming covered by the material of the future dentes and thus appearing more voluminous than ordinary kinetosomes. This remains a problem which ought to be clarified by thin sectioning.

Other types of oral replacement, e.g. the reorganisation of the paroral (or undulating) membrane in *Tetrahymena* (Nelsen 1981) likewise display subtle changes, and took quite a time before they were realised as regular phenomena in divisional morphogenesis. Reorganisation of parental adoral organelles is the most difficult process to be documented. From unpublished work on the colpodid ciliate *Platyophrya* we know that single cilia within an adoral organelle become resorbed and grow out again, a process which was only seen in Cryo-SEM studies.

Another very dramatic event is the detachment of the cytopharyngeal basket in the proter (see Kaneda 1960). Its dissolution in the cytoplasm as well as the formation of the new basket could not be studied with silver impregnation or with SEM, these processes must be clarified by thin section studies. Unfortunately, in light microscopical studies, both the beginning of the detachment of the basket and likewise the first signs of its new formation are hidden by the massive corrugations of the oral collar (Kaneda 1960).

With respect to the timetable and contrary to the drawing by Fauré-Fremiet (1950) there is no synchronous development in the differentiation of oral structures of the opisthe and the proter. As seen most clearly in our Fig. 5 the opisthe is ahead of the proter. We know of no published explanation for the asynchronous development in proter and opisthe which ought to be a very general problem encountered also in any form of asymmetric cell division.

Morphogenetic events in the somatic cortex are less spectacular. The migrating fragment of the first postoral kinety (dotted line in Fig. 1, subfigures 4-8, labelled with two arrowheads) is of special significance for the completion of the full number of the somatic kineties since the leftmost somatic kinety due to its short length has ended up completely in the proter. This type of kinety compensation has also been described for *Chilodonella*, *Trithymostoma* and *Brooklynella* and is of considerable significance for lateral turnover of somatic kineties, at least for the opisthe. Though this mode of cortical shift is regarded by Deroux (1994) as a synapomorphic

character of the Cyrtophorida, lateral turnover of somatic kineties seems to be more widely distributed among ciliates. One of the most impressive examples of lateral turnover is the cortical slippage of artificially inverted kineties in *Paramecium tetraurelia* (Beisson and Sonneborn 1965). Lateral turnover is a general morphogenetic feature leading to a complete renewal of the cortex in 20 - 30 generations in normal cells of *P. tetraurelia* (Iftode and Adoutte 1991). Moreover, a longitudinal turnover of the somatic cortex of ciliates is implicated in the "clonal cylinder" model by Frankel (1989).

Neither at light microscopical level nor with the SEM we have seen any indication of the growth of the cross-striped band. In mid-divisional stages there is a more pronounced indentation on the right side of the cell than on the left side at the height of the future fission furrow. Further thin section studies have to show whether this indentation which sometimes looks like a small cavity is involved in the necessary increase of the C-shaped elements of the so-called railroad-track.

Phylogenetic remarks

In continuation of our aims to reconstruct ciliate phylogeny through comparative studies on ciliate morphogenesis we are constantly puzzled by the quite unique type of oral ciliature in cyrtophorid ciliates. At first glance there is a certain resemblance of the oral apparatus of the Cyrtophorida with the Nassulida. But this resemblance only holds for the basket. Nassulids show a paroral and an adoral ciliature, found in all ciliates with the exception of the Phyllopharyngea and the Litostomatea. While it seems most likely that the Litostomatea have lost their primary oral ciliature and substituted it through "oralisation" of somatic ciliature (Foissner and Foissner 1988), in the Phyllopharyngea a more cryptic way of "deuterostomisation" might have happened. The current oral ciliature, the preoral kinety and the circumoral kineties, display substantial differences to a paroral or an adoral ciliature. Though derived from the somatic ciliature they must have had some other kind of secondary origin together with the cytostome proper. We envision the following scenario: primitive phyllopharyngids, no longer existent, had lost their primary oral ciliature and their primary cytostome and developed suctorial tentacles, thus Suctorina being a very basal branch of the Phyllopharyngea and not on top as usually argued. Some suctorians form microtubular lamellae which are major components of their tentacles in

close neighbourhood to basal bodies (Hitchen and Butler 1973). These microtubular lamellae we regard as homologous to the cytopharyngeal lamellae in cyrtophorids which originate from postciliary microtubules of somatic kinetosomes (Bardele 1987, Hofmann 1987). As oralised kinetosomes in litostomes or kinetosomes of ophryokineties (Didier 1971) can form nematodesmata, somatic kinetosomes form cytopharyngeal rods in nassulids (Tucker 1970, Peck 1974, Eisler 1989) and by convergence in cyrtophorids (Hofmann 1987). The cytostome in cyrtophorids (and consequently also in chonotrichids) is a secondary one and the oral kineties in both are not homologous to the paroral or adoral ciliature of other ciliates. The main purpose of their kinetosomes at least during a certain period of morphogenesis is to nucleate cytopharyngeal microtubules which carry dynein-like arms to propel the incoming food. This hypothesis is in line with the suggestions by Eisler (1992) on the significance of inner kinetosomes of the paroral membrane in the nucleation of the postciliary microtubules which in all ciliates (except for the litostomes where transverse microtubules have taken their part) line the cytopharynx.

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Blepharisma intermedium Padmavathi, 1959 (Ciliophora: Heterotrichida) from Al-Hassa Inland Hypersaline Oasis in Saudi Arabia

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Summary. A medium-sized, pink heterotrich ciliate was found in hypersaline ponds in the inland Al-Hassa Oasis. The morphology and infraciliature were studied *in vivo*, and in silver carbonate and protargol impregnated cells. The organism has a slender filiform macronucleus without terminal swellings. The morphology and morphometric data agree largely with the original description of *Blepharisma intermedium* Padmavathi, 1959; however, the present organism has fewer kineties and both kinetosomes of somatic dikinetids are ciliated. The findings are discussed on the basis of a summary made from available data on other *Blepharisma* species with a filiform macronucleus.

Key words: *Blepharisma intermedium*, hypersaline ponds, infraciliature, inland oasis, protargol impregnation.

INTRODUCTION

The Al-Hassa Oasis is one of the largest in the world. It is situated some 50 km inland from the Arabian Gulf coast, West of the vast sand desert of Al Jafurah (25° 30' N; 49° 40' E). This oasis is an inland sabkha with some similarities to the coastal sabkhas of the Arabian Gulf (Johnson *et al.* 1978). There are numerous wells and artesian springs with abundant fresh water, which makes the oasis an important producer of dates and other agricultural products. The ground water

level of the oasis is particularly shallow, which causes mixing of fresh water with the salty sediments, forming scattered ponds of various salinity levels (4-18 ‰). The raised salinity of the ponds may be due to dissolution of salts from soil surfaces (AL-Rasheid 1997). Salinity of the ponds decreases southwards, where the number of springy wells is increases, along with increasing vegetation. Due to high temperature and lack of rain during the long summer season (the mean water temperature is 15° C during January, the coldest month, and 35° C during July, the hottest month of the year), several of the shallow fresh and brackish water ponds turn saline and/or hypersaline (70-160 ‰). These ponds are surrounded by the salt-tolerant tallreed (*Phragmites*) and mangroves (*Avicennia*). The ciliate fauna of the oasis has been studied by AL-Rasheid (1997) who reported

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37 species of typical marine ciliates including the two well known saline-tolerant species, *Fabrea salina* and *Condylostoma reichi*.

A medium-sized, pink *Blepharisma* sp. with filiform macronuclei has been found in hypersaline ponds in the Saudi Arabian inland oasis Al-Hassa. Members of the genus *Blepharisma* are widespread and have been reported from fresh, brackish and sea water as well as from soils of many parts of the world (see for example Borror 1963; Isquith *et al.* 1965; Kattar 1965; Nilsson 1967; Dragesco 1970; Larsen 1982, 1983, 1992; Larsen and Nilsson 1983, 1988; Dragesco and Dragesco-Kernéis 1986, 1991; Foissner 1989; Foissner and O'Donoghue 1990; Aescht and Foissner 1998; AL-Rasheid 1999). A few species have been found in extreme hypersaline habitats, such as *B. halophila* (Ruinen 1938, Post *et al.* 1983), *B. dileptus* and *B. tardum* (Kahl 1928) and *Blepharisma* sp. (Wilbert 1995). The taxonomy of the genus *Blepharisma* has undergone several taxonomical revisions starting with Kahl (1932) then Bhandary (1962), Hirshfield *et al.* (1965, 1973) and finally Repak *et al.* (1977).

The aim of the present work is to describe a population believed to be of *Blepharisma intermedium* found in Saudi Arabia and to give some information regarding the taxonomy of blepharismas with filiform macronuclei.

MATERIALS AND METHODS

The organism was found in hypersaline ponds (100-160 ‰ salinity) North of the Oasis on several occasions during the summer and winter seasons of 1999 and 2000. Freshly collected sediment and water samples were studied on site. Several specimens of the ciliate organisms were collected, studied *in vivo* then in protargol and silver carbonate-stained preparations (Foissner 1991). Several short and long-term cultivation methods were conducted, but with no success as the cultures declined and died out rapidly. Specimen preparations of protargol and silver carbonate impregnated cells have been deposited in the Museum of Zoology Department, College of Science, King Saud University, Riyadh, Saudi Arabia.

OBSERVATIONS

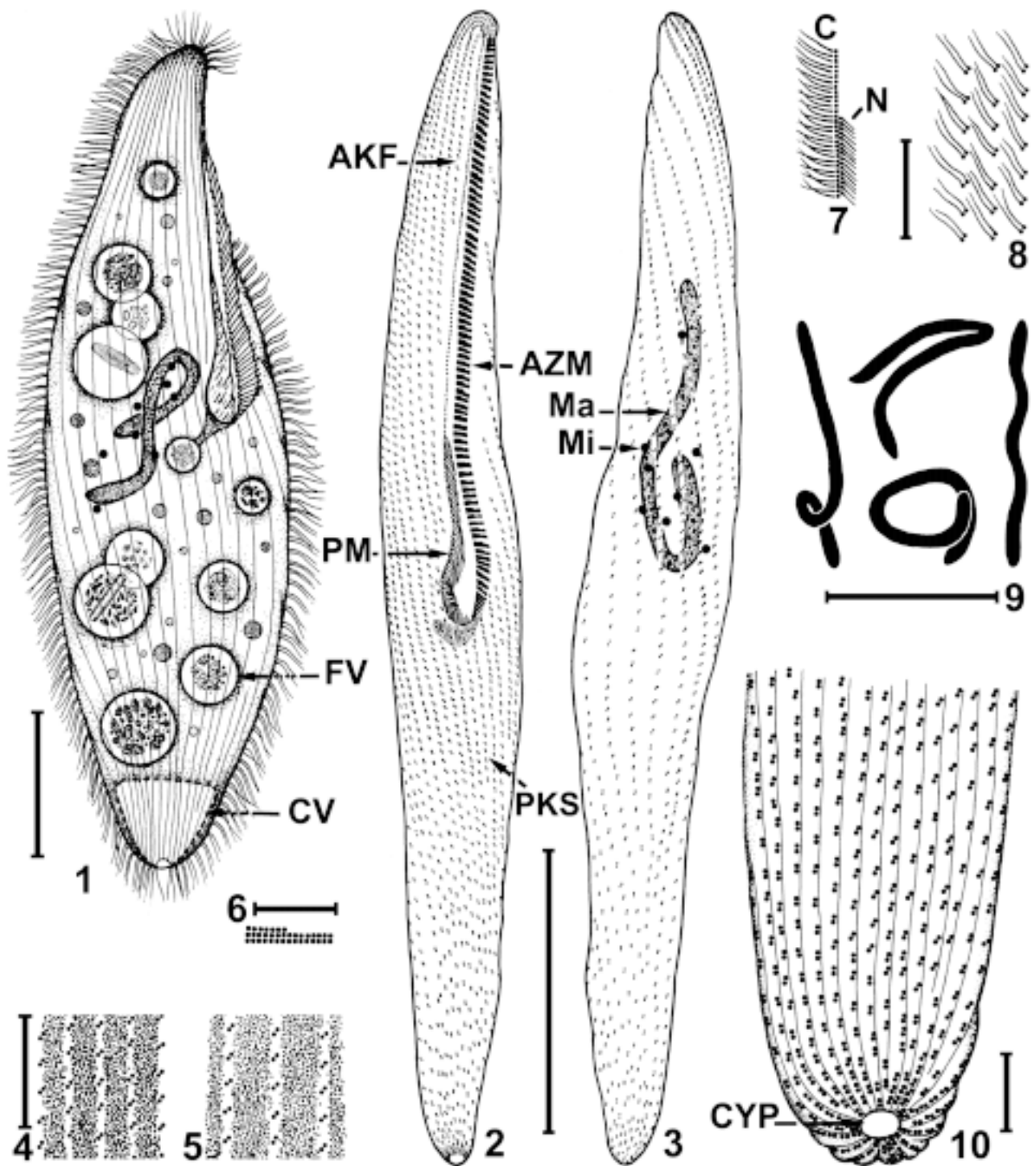
The Saudi Arabian organism is slender, rounded anteriorly and posteriorly, and has a size of 239-359 x 39-52 µm (*in vivo* ca 250 - 400 x 44 - 60 µm) (Figs. 1, 11; Table 1). The pellicle is flexible with numerous dark pink subpellicular granules (0.4-0.5 µm in diameter) arranged in rows of approximately 8-12 granules across

between the ciliary rows of kineties (Figs. 4, 5, 13, 14). Food vacuoles may be abundant, filled with bacteria, algae and diatoms (Figs. 11, 12). The contractile vacuole is found terminally (Fig. 1). The oral groove is long and narrow starting slightly below the anterior pole and stretching posteriorly to about the middle of the body (Figs. 2, 17). The entire left side of the groove is furnished with an adoral zone of membranelles (Fig. 15) in which each membranelle base consists of rows of basal bodies, two long and one short (Fig. 6). The right side is occupied by a paroral membrane, which ends posteriorly at the cytostome. The anterior third of the paroral membrane consists of a line of closely spaced basal bodies, while the remaining part consists of zigzagging dikinetids having only the right basal body ciliated (Fig. 15). Single long fibers (nematodesmata) originate from the left basal bodies in the posterior third of the paroral membrane and extend over the buccal cavity and enter the cytopharynx as oral ribs (Fig. 7). There are 25 (21-27) somatic kineties; on the right side of the organism the rows of cilia run parallel to the oral groove, while on the left side the rows run obliquely (Figs. 2, 3, 13, 14; Table 1). The kineties consist of paired basal bodies and both are ciliated (Fig. 8). The somatic cilia are about 6-9 µm long. The kineties stop short just before the posterior end leaving a bare cytophyge free of cilia and subpellicular granules (Fig. 10). The single filiform macronucleus is without terminal swellings (Figs. 1, 3) and occurs in many twisted configurations (Fig. 9). The 5-9 spherical micronuclei, each about 2 µm in diameter, are located close to the macronucleus (Figs. 3, 16; Table 1).

Cultivation was unsuccessful, so it was not possible to study cell division, yet a few early and late postdividers were found naturally in several samples collected on different occasions. Examples are recorded in Figs. 18, 19 for future studies.

DISCUSSION

In order to identify the present Saudi Arabian blepharisma taxonomically, a summary (Table 2) was made of the available data from the literature on *Blepharisma* spp. with filiform macronuclei. The present Arabian organism matches in some aspects *Blepharisma intermedium* Padmavathi (1959), e.g. in the general size of the organism, in shape of the filiform macronucleus, and in some other morphometric features (see Table 2). However, the number of somatic kineties is lower (21-27) in the Arabian blepharisma than the 42-64



Figs. 1-10. *Blepharisma intermedium* from life (1), after protargol impregnation (2, 3, 6-10) and after silver carbonate impregnation (4, 5). 1 - right lateral view of a compressed organism showing the general appearance, many food vacuoles and the terminal contractile vacuole; 2, 3 - infraciliature of right and left sides showing adoral zone of membranelles, paroral membrane, macronucleus and micronuclei; 4, 5 - details of pellicle with subpellicular cortical granules of right and left sides; 6 - detail of kinetosome arranged in adoral membranelle; 7 - detail of paroral membrane; 8 - detail of somatic infraciliature showing that both kinetosomes in a pair are ciliated; 9 - macronucleus variations; 10 - somatic infraciliature of the posterior end showing that the cytopygic opening is free of kinetosomes. AKF - anterior kinety fragment, AZM - adoral zone of membranelle, C - cilia, CV - contractile vacuole, CYP - cytopygic, FV - food vacuole, Ma - macronucleus, Mi - micronucleus, N - nematodesma, PM - paroral membrane, PKS - shortened postoral kinety. Scale bars - 75 μ m (Figs. 1-3, 9), 15 μ m (Figs. 4, 5, 7, 8, 10) and 5 μ m (Fig. 6)

Table 1. Morphometric characteristics of *Blepharisma intermedium* (Data based on randomly selected protargol-impregnated specimens). Measurements are in μm

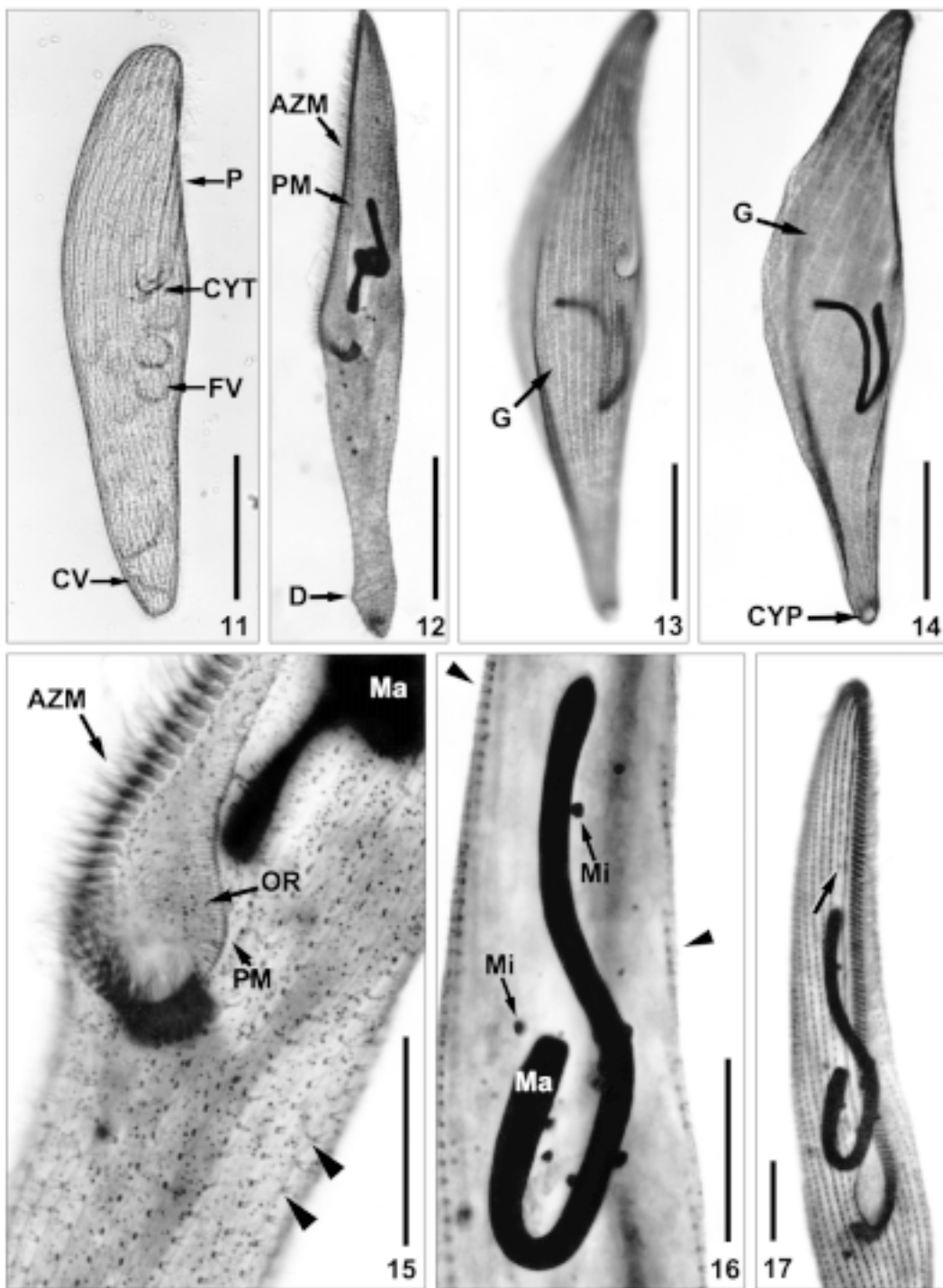
Character	\bar{x}	M	SD	SE	CV	Min	Max	n
Body, length	306.3	314	43.1	3.6	14.1	239	359	10
Body, maximum width	44.8	45	4.3	1.4	9.6	39	52	10
Adoral zone of membranelles, length	144	151	16.8	5.3	11.7	119	171	10
Adoral zone of membranelles, width	5.0	5.1	0.2	0.1	4.0	4.5	5.2	20
Adoral membranelles, number	86.4	87.5	6.8	2.2	7.9	76	98	10
Paroral membrane, length	71.2	73.5	10.9	3.4	15.5	54	83	10
Macronucleus, length	131	130	12.9	4.1	9.8	114	156	10
Macronucleus, diameter	3.8	3.9	0.2	0.1	5.3	3.2	4.1	30
Micronuclei, diameter	2.3	2.3	0.3	0.1	13.0	1.8	2.7	30
Micronuclei, number	7.1	7.0	1.3	0.2	18.3	5	9	10
Somatic kineties, number postoral	25	26	1.8	0.6	7.2	21	27	10

CV - coefficient of variation in %, M - median, Max - maximum, Min - minimum, n - number of individuals investigated, SD - standard deviation, SE - standard error of the mean, \bar{x} - arithmetic mean

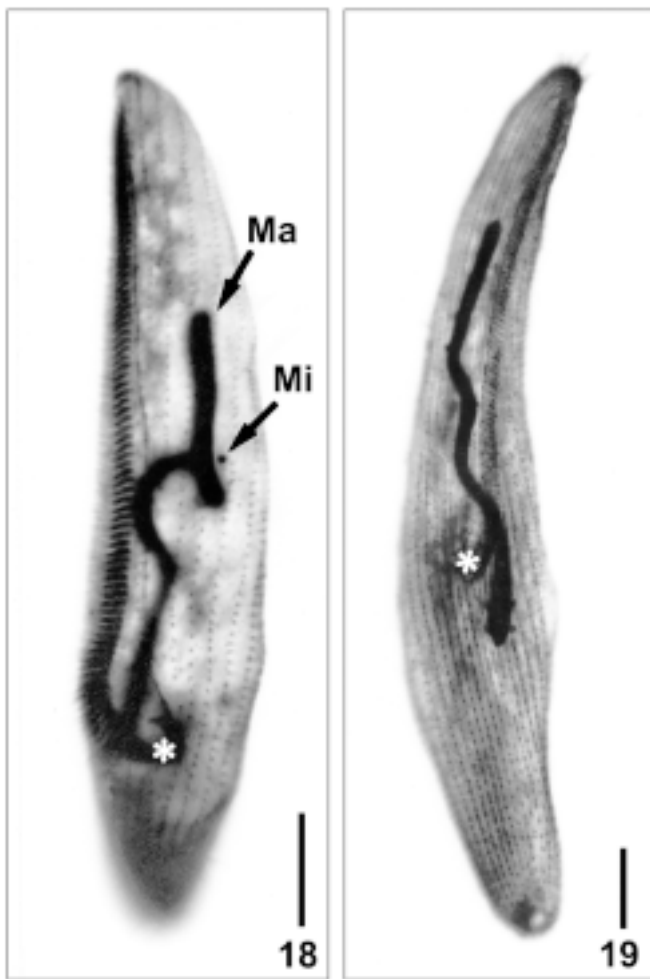
Table 2. Comparison of some reported characteristics of *Blepharisma* with filiform macronucleus

Species Author	<i>B. japonicum</i> Suzuki 1954	<i>B. japonicum</i> Nilsson 1967	<i>B. japonicum</i> Dragesco 1970	<i>B. japonicum</i> Dragesco & Dragesco- Kernéis 1986	<i>B. stoltei</i> Isquith 1966**	<i>B. briviformis</i> Isquith 1966**	<i>B. intermedium</i> Padmavathi 1959	<i>B. intermedium</i> present study
Locality	Japan	Uganda	Cameroon	Uganda	Japan	Japan	India	Saudi Arabia
Length μm	150-500	250-700	400-650	225-400	145-225	120-195	200-350	239-359
Width μm	62-215	90-260*	100-200	80-150	45-85	35-90	45-100	39-52
Peristome length μm	75-250	75-350	150-230	100-185	70-125	50-85	70-140	119-171
Somatic kineties number	50-70	60-100	54	76	50	36-54	42-64	21-27
AZM number	91	90-178*	80	112	72	42-57	82	76-98
PM length μm	30-50	50-230	50-110	25-50	24-60	15-35	25-50	54-83
PM type	prominent	prominent	prominent	prominent	not prominent	not prominent	not prominent	not prominent
Terminal swelling of macronucleus	conspicuous	conspicuous	inconspicuous	conspicuous	conspicuous	inconspicuous	inconspicuous	inconspicuous
Micronuclei diameter	1.2	1	1.8-2.8	2	1.5-2	?	1.97	1.8-2.7
Micronuclei number	2-22	15-25	13-26	15-25	4-18	2-7	6-30	5-9
Subpellicular granules color	deep red	deep red	red	reddish	pale pink	?	dark pink	dark pink
Subpellicular granules number	6-10	5-8	?	?	3-6	?	?	8-12

* unpublished data, ** see Hirshfield *et al.* (1973)



Figs. 11-17. Light micrographs of *Blepharisma intermedium* from life (11), after protargol impregnation (12, 15-17) and after silver carbonate impregnation (13, 14). 11 - right lateral view of a slightly compressed organism showing the general appearance; 12 - left lateral view showing the macronucleus, somatic kineties and a food vacuole containing a diatom (D); 13, 14 - right lateral views at different focus showing the arrangements of the longitudinal and oblique subpellicular granules of the right and left sides (the cells are swollen due to the silver carbonate technique); 15 - part of Fig. 12 at higher magnification showing the adoral zone of membranelles, macronucleus, the zigzagging part of the paroral membrane and the oral ribs. Arrowheads indicating that both paired kinetosomes of the dikinetids are ciliated; 16 - the filiform macronucleus with inconspicuous terminal swelling and the micronuclei. Arrowheads indicating that both paired kinetosomes of the dikinetids are ciliated; 17 - ventral view showing the anterior kinety fragment and other parts of the peristome. AZM - adoral zone of membranelles, CV - contractile vacuole, CYP - cytophyge, CYT - cytostome, D - diatom, FV - food vacuole, G - subpellicular granules, Ma - macronucleus, Mi - micronucleus, OR - oral ribs, P - peristome, PM - paroral membrane. Scale bars - 75 μ m (Figs. 11 - 14), 25 μ m (Figs. 15, 16) and 50 μ m (Fig. 17)



Figs. 18-19. Light micrographs of *Blepharisma intermedium* after protargol impregnation. Early (18) and late (19) postdividers. Cytostome positions are marked by asterisks. Ma - macronucleus, Mi - micronucleus. Scale bars - 25 μ m

kineties reported originally by Padmavathi (1959) for *B. intermedium*. In revisions of the genus *Blepharisma* (Bhandary 1962; Hirshfield *et al.* 1965, 1973), the number of somatic kineties in *B. intermedium* is not mentioned. An unusual low number of 25-35 kineties was also found by Sawyer (1977) in *B. japonicum* Suzuki, so this feature may be unimportant (see Table 2).

A striking feature of the Arabian ciliate is, however, that both kinetosomes of the somatic pairs are ciliated. This is an unusual feature in *Blepharisma* where most descriptions on the different species state that only one kinetosome of the somatic pairs is ciliated. To the best of our knowledge, the only other blepharisma reported to have both kinetosomes of somatic pairs ciliated, is *B. parasalinarium* (Dragesco 1996). This organism

does not have a filiform macronucleus but about 60 small irregular, spheroid macronuclei which rules out any close relationship to the present Arabian organism.

The colour of the ciliate is not an accurate taxonomical feature, as it is well known that the *Blepharisma* pigment bleaches on exposure to light. The dark pink colour of the present organism may be explained by the heavy vegetation shading the saline ponds where it was found.

In spite the fact that the somatic pairs of kinetosomes are both ciliated, an unusual feature, we conclude that the present organism with its filiform macronucleus is an Arabian strain of the freshwater *Blepharisma intermedium* Padmavathi (1959).

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Isoospora bronhocelae (Apicomplexa: Eimeriidae), a New Coccidian Parasite from the Green Crested Lizard (*Bronhocela cristatella*) from Malaysia

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Summary. A new species of *Isoospora* is described from the feces of the green crested lizard, *Bronhocela cristatella* from Malaysia. Oocysts of *Isoospora bronhocelae* sp. n. were found in 7/48 (14.6%) green crested lizards. Sporulated oocysts were subspherical or ovoidal, 25.2 x 22.3 (20-28 x 19-24) μm , with a bilayered wall; the outer wall marked with internal, perpendicular striations and a heavily pitted surface. The average shape index is 1.13 (1-1.22). No micropyle or oocyst residuum are present but the oocysts contain one polar granule composed 1-2 globules stuck together or a splinter-shaped mass. Sporocysts are ovoidal, 14.8 x 9.9 (13-16 x 9-10) μm , average shape index is 1.5 with a smooth, single-layered wall, and composed of a broad, dome-like Stieda body and a large fan-like substieda body. The sporocyst residuum is composed of coarse granules clustered in an amorphous mass and lying among the sporozoites. The sporozoites are vermiform with a large oblong, centrally located nucleus and circumscribed with parallel striations running the length of the sporozoite except at the location of the nucleus and no visible refractile bodies.

Key words: coccidia, *Isoospora bronhocelae* sp. n.

INTRODUCTION

The green crested lizard, *Bronhocela cristatella* (Kuhl, 1820) are medium length (\approx 432 mm), slender, insectivorous, arboreal lizards with long tails (Diong and Lim 1998). They range from the province of Kanchanaburi in Thailand and the southwestern Philippines in the north, through Peninsular Malaysia, Borneo, Indonesia, and associated islands, to New Guinea in the southeast. Previously, no coccidian parasites have been

reported in *B. cristatella* but this study describes a new isosporan species in the green crested lizard.

MATERIALS AND METHODS

During 4 lizard-collecting expeditions between October 1999 and February 2000, fecal samples were obtained from 48 *B. cristatella* from the foothills of the Cameron Highlands near Tanah Rata in Peninsular Malaysia. The samples were sent to the first author's laboratory for examination. Procedures for preserving fecal material and for measuring and photographing oocysts were described by McQuistion and Wilson (1989). All measurements are presented in micrometers (μm) with size ranges in parentheses following the means. Oocysts were approximately one month old when examined, measured, and photographed.

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RESULTS

Seven of the 48 lizards were passing coccidian oocysts. Upon sporulation, the oocysts appeared similar

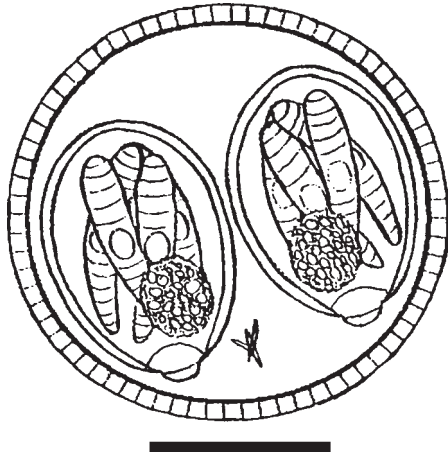


Fig. 1. Composite line drawing of sporulated oocyst of *Isospora bronhocelae* sp. n. from *Bronchocela cristatella*. Scale bar - 10 µm

in morphological characteristics and were found to represent a previously unreported species, which is described below.

***Isospora bronhocelae* sp. n. (Figs. 1-3)**

Description of oocysts: subspherical to ovoidal, colorless, 25.2 x 22.3 (20-28 x 19-24)(N=42) double walled

(≈1.6), outer wall with internal, perpendicular striations and a heavily pitted surface; shape index (length/width) 1.13 (1-1.23). No micropyle or oocyst residuum but containing a small, polar granule composed of 2-3 globose granules stuck together or 2-3 splinter-like granules stuck together. Sporocyst ovoidal, 14.8 x 9.9 (13-16 x 9-10) (N=19) with a smooth, thin wall; shape index 1.5 (1.4-1.6). The Stieda body is broad, dome-like with a conspicuous, large fan-like substieda body located directly below the Stieda body. Sporocyst residuum present, consisting of coarse granules in a subspherical cluster. Sporozoites are vermiform, lacking visible refractile bodies and circumscribed with parallel concentric lines along its entire length except at the ovoid, centrally located nucleus.

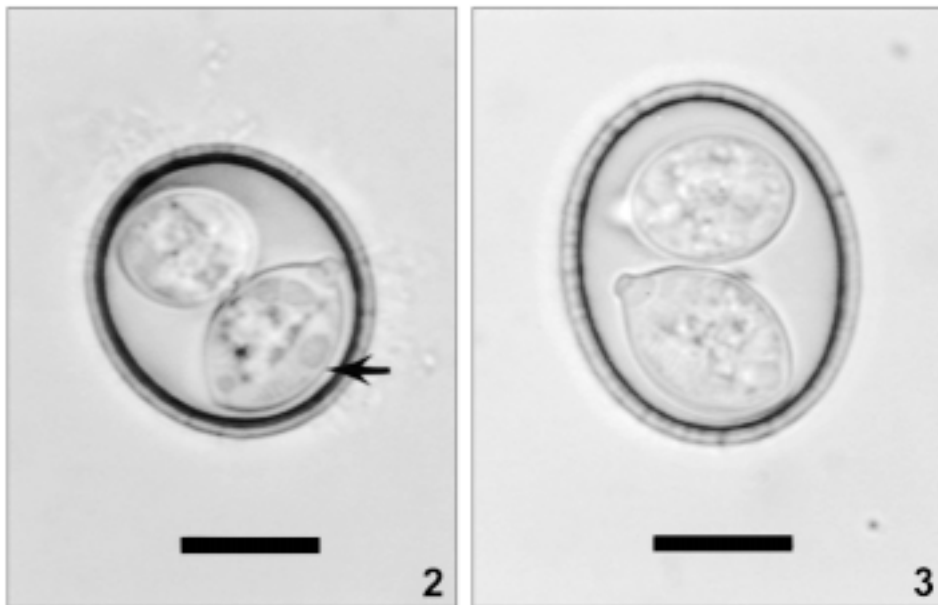
Type-host: *Bronchocela cristatella* Kuhl, 1820 “Green Crested Lizard” (Squamata: Agamidae), C. H. Diong, Raffles Biodiversity Research Centre, National University of Singapore, Republic of Singapore, ZRC.2.4815.

Type specimens: Phototypes and buffered formalin preserved syntypes of *Isospora bronhocelae* sp. n. have been deposited in the U.S. National Parasite Collection in Beltsville, Maryland as USNPC No. 90622.

Type location: Cameron Highlands near Tanah Rata in Peninsular Malaysia.

Prevalence: 7/48 (14.6%) were infected with *Isospora bronhocelae* sp. n.

Sporulation time: unknown; oocysts were partially sporulated when received at the laboratory and became



Figs. 2, 3. Photomicrographs of sporulated oocysts of *Isospora bronhocelae* sp. n. **2** - typical oocyst showing striated outer oocyst wall. Note the lateral view of the lower sporocyst in the oocyst with a sporozoite located along its lower wall showing a prominent nucleus and concentric lines along its length (see arrow). **3** - more elongated oocyst of *Isospora bronhocelae*. Note fan-shaped substieda body and striated outer oocyst wall. Scale bars - 10 µm

Table 1. Isosporan species in agamid lizard hosts sympatric with *Bronhocela cristatella*

Isosporan species	<i>I. bronhocelae</i>	<i>I. lacertae</i>	<i>I. gonocephali</i>	<i>I. choochotei</i>	<i>I. caryophila</i>
Type host	<i>Bronhocela cristatella</i>	<i>Calotes versicolor</i>	<i>Gonocephalus grandis</i>	<i>Calotes mystaceus</i>	<i>Gonocephalus grandis</i>
Oocyst length/width	25.2 x 22.3	28.1 x 26.5	22.3 x 18.7	30.5 x 29.3	23.5 x 21.9
Oocyst l/w ratio	1.13	1.06	1.20	1-1.1	1.16
Oocyst wall	bilayered, striated outer layer, pitted surface	bilayered, striated outer layer, smooth surface	bilayered, striated outer layer, pitted surface	single-layered	single-layered
Polar granules	1	none	1	none	1
Sporocyst l/w	14.8 x 9.9	14.6 x 10.3	13.5 x 9.2	16.5 x 11.2	13.2 x 8.2
Sporocyst l/w ratio	1.50	1.42	1.50	1.11-1.66	1.61
Sporocyst residuum	coarse granules, amorphous shape	coarse granules, subspherical cluster	coarse granules, amorphous cluster	present	scattered about sporocyst
Stieda body	dome-like	dome-like	dome-like	present	very small
Substieda body	large, fan-like	boxy, squarish	large, fan-like	none visible	none
Sporozoite shape	vermiform	vermiform	sausage-shaped	no description	elongated
Sporozoite features	concentric grooves along entire length	incomplete concentric lines confined to anterior end	no concentric lines	no description	no description
Refractile bodies	none	none	1	2	none
Reference	McQuiston, <i>et al.</i> , present report	Saum <i>et al.</i> , 1997	Maupin <i>et al.</i> , 1998	Finkelman & Paperna, 1994	Rogier & Colley, 1976

fully sporulated after exposed to air for several days prior to examination.

Site of infection: unknown, oocysts found in feces.

Etymology: the specific epithet, *bronhocelae*, is the Latin, genitive form of the host genus name.

Remarks: 22 lizards were passing *Strongyluris calotis* eggs in their feces. Four lizards were passing both *S. calotis* eggs and *I. bronhocelae* oocysts in their feces concurrently.

DISCUSSION

The species *Bronhocela cristatella* is the most widely distributed agamid lizard in the genus and is

superficially similar to members in the genus *Calotes*, *Pseudocalotes*, and *Dendragama*. Indeed, *Bronhocela cristatella* was placed in the genus *Calotes* periodically since it was originally described in 1820. Based on Wagner tree algorithm, Moody (1980) however, showed that *Bronhocela* is phylogenetically closer to *Dendragama* and *Gonocephalus* than to *Calotes* and *Pseudocalotes*. Diong and Lim (1998) conducted a detailed review of *B. cristatella* and demonstrated its validity as the type species in the *Bronhocela* genus.

The similarity in morphological characteristics of *Isospora bronhocelae* to *I. lacertae* in *Calotes versicolor* (Saum *et al.*, 1997) and *I. gonocephali* in *Gonocephalus grandis* (Maupin *et al.*, 1998) (see Table 1) seem to reflect the close phylogeny of

Bronhocela cristatella to its sympatric hosts. Aside from oocyst size and length/width ratios, all three isosporan species have a bilayered oocyst wall with the outer layer striated, similar sporocyst residuum, and shape of the Stieda bodies. The primary differences center around the morphological features of the sporozoite, the surface of the oocyst wall, and the presence of a polar granule.

The sporozoites of *I. bronhocelae* are vermiform-shaped with no refractile bodies and parallel, concentric grooves or lines encompassing the entire length of the sporozoite except at the centrally located nucleus. *I. lacertae* sporozoites have incomplete concentric lines confined to the anterior end, the oocyst has no polar granule, and the substieda body is dissimilar to sporocysts of *I. bronhocelae* and *I. gonocephali*. Sporozoites of *I. gonocephali* are sausage-shaped, possess a refractile body and have no concentric lines compared to *I. bronhocelae* and *I. lacertae*.

Oocysts of *I. choochotei* and *I. caryophila* are very different from *I. bronhocelae*. The oocysts walls are smooth and single-layered and the sporocysts have no substieda bodies. The sporozoite descriptions are sketchy but specifically report two refractile bodies on *I. choochotei* sporozoites. These characteristics along with several others specific to the species, sets them apart from *I. bronhocelae*, *I. gonocephali*, and *I. lacertae*.

Acknowledgments. Thanks are due to Ms. Mary Ellen Martin for assistance in naming the parasite.

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*Stylonychia ammermanni** sp. n., a New Oxytrichid (Ciliophora: Hypotrichida) Ciliate from the River Yamuna, Delhi, India

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Summary. *Stylonychia ammermanni* sp. n. is widely distributed in the backwaters of the Yamuna river of Delhi region. The cell measures about 135 x 50µm and the vegetative ciliature consists of an adoral zone of membranelles, two parallel undulating membranes, 18 frontal-ventral transverse cirri, one row each of right and left marginal cirri, four dorsal kineties, two dorsomarginal rows and three caudal cirri. The new species belongs to the *S. mytilus-lemnae* complex as judged by the morphology and division morphogenesis. A comparison of various morphometric characters (size, shape and cirral pattern) with the other two described species of the complex reveals that *S. ammermanni* sp. n. is distinct from them.

Key words: ciliate, morphogenesis, morphometry, *Stylonychia ammermanni* sp. n.

Abbreviations used: AZM - adoral zone of membranelles, CC - caudal cirri, DK - dorsal kinety, DM - dorsomarginal, DMP - dorsomarginal primordium, DP - dorsal primordium, FVT - frontal ventral transverse, LMC - left marginal cirri, MP - marginal primordium, OP - oral primordium, RMC - right marginal cirri, UM - undulating membrane

INTRODUCTION

Stylonychia is a widely distributed and well investigated member of the family Oxytrichidae (Subfamily Oxytrichinae; Berger and Foissner 1997). The genus was originally described by Ehrenberg (1830) and later Kahl (1935) recognized the taxonomic integrity of eleven species. Subsequent systematic revisions have been

carried out by Borror (1972) and Hemberger (1982). In a recent monograph on oxytrichids, Berger (1999) has done an exhaustive review of the systematic status of *Stylonychia* validating eleven species in his scheme of classification. Presently, we report the occurrence of an undescribed species, *Stylonychia ammermanni* sp. n. belonging to the *S. mytilus-lemnae* complex (Wirnsberger *et al.* 1986).

MATERIALS AND METHODS

Stylonychia ammermanni sp. n. was isolated from the backwaters of river Yamuna flowing through the Delhi region (28°34'N, 76°07'E). Isolated cells were acclimatized to the laboratory conditions and then grown at 23 ± 1°C with axenically cultured *Chlorogonium elongatum* (Ammermann *et al.* 1974) as the food organism.

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* The species is being named after Prof. Dieter Ammermann in honour of his pioneering contributions to the knowledge of the biology of *Stylonychia*

Morphometric characterization was done on randomly selected non-dividing cells after staining with the modified protargol method (Kamra and Sapat 1990). The terminology employed was that of Wallengren (1900), Borror (1972), Hemberger (1982) and Martin (1982).

Most of the cells exhibiting better details have orientation with their ventral sides facing downwards so that the AZM appears on viewers left.

RESULTS

Stylonychia ammermanni sp. n. (Fig. 1) feeds voraciously on the alga *Chlorogonium elongatum* and healthy cells are very motile. With its rigid body and broad anterior and narrow posterior ends it shows morphological similarities to the *S. mytilus-lemnae* complex. However, *S. ammermanni* sp. n. appears comparatively flattened as it lacks the peristomial bulge. This feature distinguishes it from the other two related species viz. *S. mytilus* and *S. lemnae* (Fig. 2). On an average *S. ammermanni* sp. n. measures 135 x 50 µm with length to width ratio of 2.5:1. The generation time under laboratory conditions is 11±0.5 hrs. Encystment and excystment are occasional in the laboratory as well as in wild cultures. Each cell possesses two macronuclei and two to four micronuclei. The right and left marginal cirral rows (RMC and LMC) are straight and posteriorly well separated. Morphometric characterization of the species is shown in Table 1.

Stylonychia ammermanni sp. n. (Fig. 1) has a large peristome with the adoral zone of membranelles (AZM) extending slightly more than fifty percent of the body length. Two parallel undulating membranes (UMs) are situated on the right wall of the peristome which appear crossed in stained preparations due to flattening. Of the 18 FVT cirri, the eight frontals (F_{1-8}) are disposed characteristically. The posterior two pairs ($F_{5,6}$ and $F_{7,8}$) are separated from the anterior hypertrophied frontals (F_{1-4}) and also from each other by distinct gaps. Posterior frontals ($F_{5,6}$) are in linear orientation and parallel to the RMC. The posteriormost pair ($F_{7,8}$) is separated apart from ($F_{5,6}$) and is situated near the cytostome. The transverse cirri (T_{1-5}) are placed in two groups of three and two cirri. Among the five ventrals two are close to the oral region and are termed the left (V_1) and right (V_2) postoral ventral cirri, while the other three (V_{3-5}) are more posteriad. Marginal cirri are evenly spaced in one row each near the right and left margins of the cell.

These right and left marginal cirral rows (RMC and LMC) are widely separated posteriorly.

Dorsally, *S. ammermanni* sp. n. shows the presence of six dorsal rows (DK_{1-4} and $DM_{1,2}$) and three caudal cirri. Three of the dorsal kineties (DK_{1-3}) are curved apically. The first dorsomarginal (DM_1) terminates at the posterior quarter region of the cell and the DM_2 before the mid region. There are three caudal cirri (CC_{1-3}), one each at the end of dorsal kineties $DK_{1,2,4}$ and are equally spaced (Fig.1). The right caudal cirrus (CC_3) is positioned between, terminal 2-3 cirri of the RMC row.

The morphogenetic pattern during division (Figs. 3-6) is essentially similar to that in *S. lemnae* and *S. mytilus* (Wirnsberger *et al.* 1986).

A small group of basal bodies evolves very close to the uppermost transverse cirrus (T_1). The kinetosomes proliferate linearly to form an anarchic field, the oral primordium (OP). A new AZM for the opisthe (posterior daughter cell) is formed from the OP. The parental AZM is retained for the proter (anterior daughter cell).

The FVT cirri for the two daughter cells develop from two sets of six cirral primordia (I-VI) each. In the proter, the parental UMs function as primordium I. The other primordia originate from the OP (primordium II), F_8 (III), F_7 (IV) and right postoral ventral cirrus (V and VI). In the opisthe, three primordia (I-III) originate in conjunction with the OP and the other three (IV-VI) from the right postoral ventral cirrus.

The origin of the two sets of six primordia involves three parental cirri (2 frontals and 1 ventral) and they differentiate into 18 FVT cirri by the cleavage pattern 1, 3, 3, 3, 4, 4.

The marginal and dorsal ciliature are formed in a manner similar to that described for other *Stylonychia* species (Hemberger 1982; Wirnsberger *et al.* 1985, 1986; Berger and Foissner 1997).

In the proter, marginal primordia are formed at the anterior ends of the marginal rows by reorganization of 2-3 parental marginal cirri. In the opisthe, marginal primordia originate similarly slightly below the prospective division furrow.

Dorsal kineties DK_{1-3} are generated by intrakinetal proliferation of kinetosomes in parental kineties DK_{1-3} . The kinety DK_4 originates by unequal fragmentation of the third dorsal primordium (DP_3). Dorsomarginals ($DM_{1,2}$) are generated at the anterior end of the new right marginal rows which shift to the

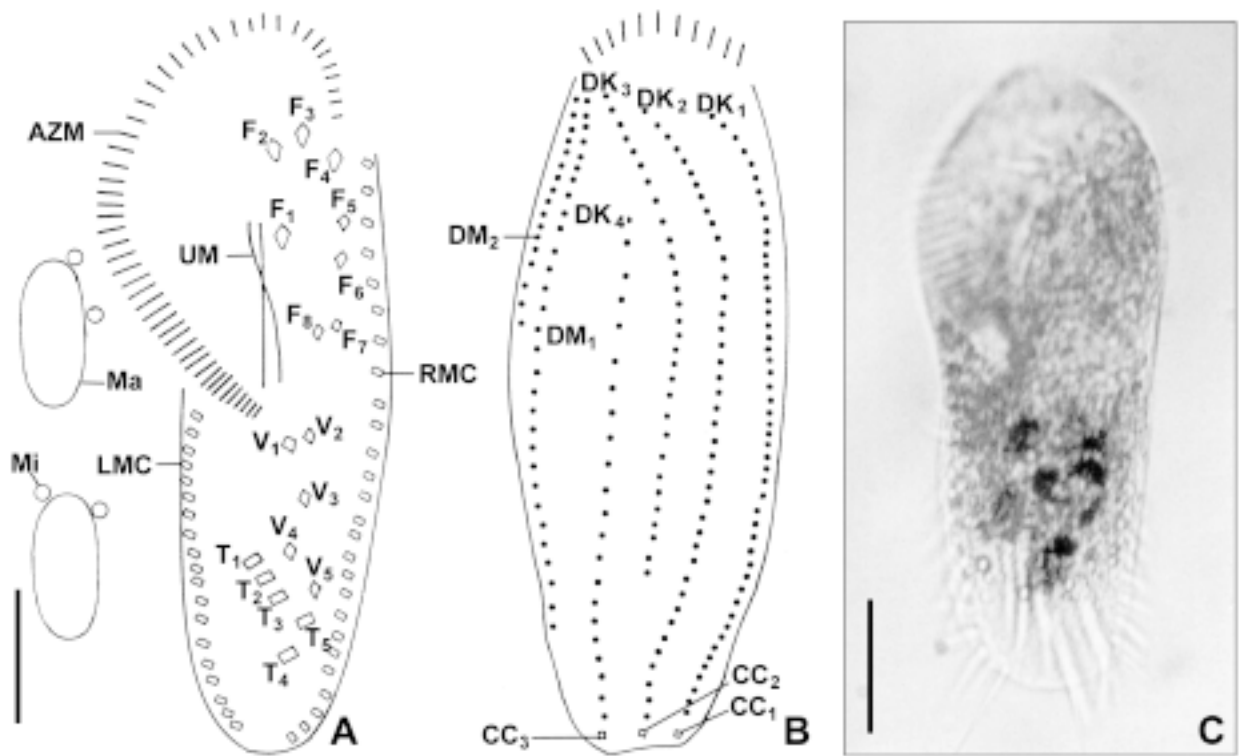


Fig. 1. Line diagrams of protargol impregnated cells and photomicrograph of *S. ammermanni* sp. n. **A** - nuclear apparatus and ventral ciliature. **B** - dorsal ciliature. **C** - live cell. AZM - adoral zone of membranelles, CC₁₋₃ - caudal cirri, DK₁₋₄ - dorsal kineties, DM_{1,2} - dorsomarginal rows, F₁₋₈ - frontal cirri, LMC - left marginal cirri, Ma - macronucleus, Mi - micronucleus, RMC - right marginal cirri, T₁₋₅ - transverse cirri, UM - undulating membranes, V₁₋₅ - ventral cirri. Scale bars - **A, B** - 25 μ m, **C** - 30 μ m

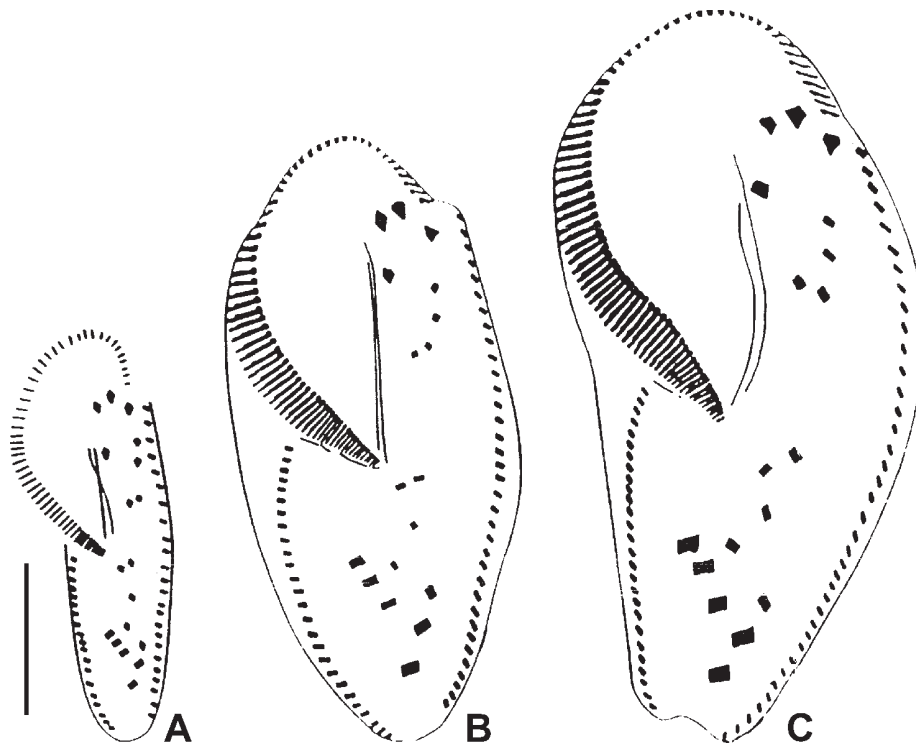


Fig. 2. Line diagrams of protargol impregnated cells of: **A** - *S. ammermanni* sp. n., **B** - *S. lemnae* and **C** - *S. mytilus* showing the ventral ciliature (Figures **B** and **C** have been modified from Wirsberger *et al.* 1986). The differences in the position of the posterior frontal cirri (F₅₋₈). Scale bar - 47 μ m

Table 1. Morphometric characterization of *Stylonychia ammermanni* sp. n. (Data from protargol impregnated cells. All measurements are in μm)

Character	Mean	Minimum	Maximum	SD	CV	n
Body length	133.98	125.65	146.48	5.00	3.73	25
Body width	52.12	47.25	57.75	3.01	5.78	25
Body length/Body width	2.58	2.33	2.97	0.15	5.94	25
Macronuclear, number	2.00	2.00	2.00	0.00	0.00	25
Macronuclear, length	25.27	24.33	26.08	0.61	2.41	12
Macronuclear, width	10.33	9.98	10.68	0.53	5.72	12
Micronuclear, number	2.00	2	4	-	-	25
Micronuclear diameter	2.62	2.45	2.97	0.20	7.91	12
Adoral membranelles, number	51.43	46	55	2.24	4.35	30
Adoral length	70.80	65.10	80.50	3.95	5.58	25
Adoral length/Body length	0.53	0.48	0.61	0.03	5.05	25
Frontal cirri, number	8.00	8	8	0.00	0.00	25
Ventral cirri, number	5.00	5	5	0.00	0.00	25
Transverse cirri, number	5.00	5	5	0.00	0.00	25
Left marginal cirri, number	17.17	13	18	1.12	6.51	30
Right marginal cirri, number	24.20	22	27	1.45	5.98	30
Dorsal kineties (DK), number	4.00	4	4	0.00	0.00	25
Dorsomarginal (DM), number	2.00	2	2	0.00	0.00	25
Dorsal bristles, number in						
DK ₁	52.67	44	58	4.19	7.95	12
DK ₂	40.58	36	45	3.20	7.89	12
DK ₃	28.83	24	33	2.66	9.22	12
DK ₄	20.83	19	25	1.99	9.56	12
DM ₁	29.33	26	32	2.10	7.17	12
DM ₂	17.00	15	20	1.86	10.93	12
Dorsal bristles, total number	189.25	176	200	8.01	4.23	12
Caudal cirri, number	3.00	3	3	0.00	0.00	25

Abbreviations: CV - coefficient of variance, SD - standard deviation, n - number

Table 2. Morphometric comparison of *S. ammermanni* sp. n. with *S. mytilus* and *S. lemnae*. Measurements are in μm

Characters	<i>S. ammermanni</i> sp. n. (Present investigation)	<i>S. mytilus</i> (German population, Wirnsberger <i>et al.</i> 1986)	<i>S. lemnae</i> (German population, Wirnsberger <i>et al.</i> 1986)
Appearance	No bulge in the peristomial region	Bulge in the peristomial region	Bulge in the post-peristomial region
Body length	130	227	188
Body width	50	110	82
Macronuclear, size	25 x 10	37 x 11	27 x 11
Micronuclear, size	2.6	3.6	4
Adoral length	$\geq 50\%$ of body length	$\geq 50\%$ of body length	$\leq 50\%$ of body length
AM, number	50	71	63
Posterior frontal cirri (F _{5,8})	Arranged in two pairs; posterior pair close to cytostome	Arranged in a 'L' shaped manner	Arranged in a 'J' shaped manner
Left marginal cirri (LMC)	Straight	Anterior 2-3 cirri curved inward	Anterior 2-3 cirri curved inward
LMC, number	17	27	25
RMC, number	24	38	37
Dorsal kineties	Bent apically	Curved apically	Bent apically
Conjugation	Occasional	Frequent	Frequent
Encystment	Occasional	Frequent	Frequent

Abbreviations: AM - adoral membranelles, RMC - right marginal cirri

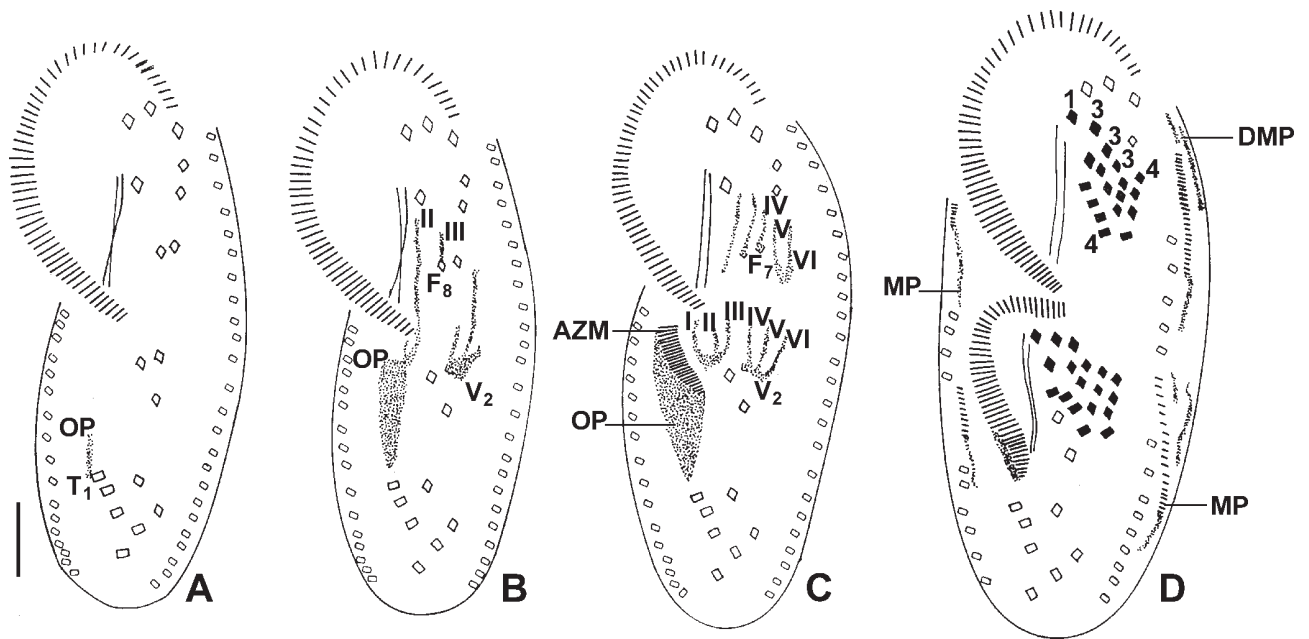


Fig. 3. Line diagrams of protargol impregnated cells of *S. ammermanni* sp. n. showing division morphogenesis on the ventral surface. **A** - origin of oral primordium (OP) by proliferation of kinetosomes near transverse cirrus T_1 . **B** - expansion of kinetosomes in OP to form an anarchic field. Anterior movement of kinetosomes from OP to form primordium II of proter. F_8 disintegrates to form primordium III of proter. Anterior movement of kinetosomes of right post oral ventral (V_2) to form primordia V and VI of proter. **C** - differentiation of membranelles (AZM) starts at the right anterior border within the OP. Origin of primordia: I-III of opisthe from OP; IV-VI of opisthe from V_2 ; IV of proter from F_7 ; V and VI of proter from V_2 . **D** - cirri differentiate in 1, 3, 3, 3, 4, 4 pattern in FVT primordia. (The old cirri are depicted only by contour, whereas the new ones are filled in). Formation of marginal primordia (MP) and dorsomarginal primordia (DMP). Scale bar - 20 μ m

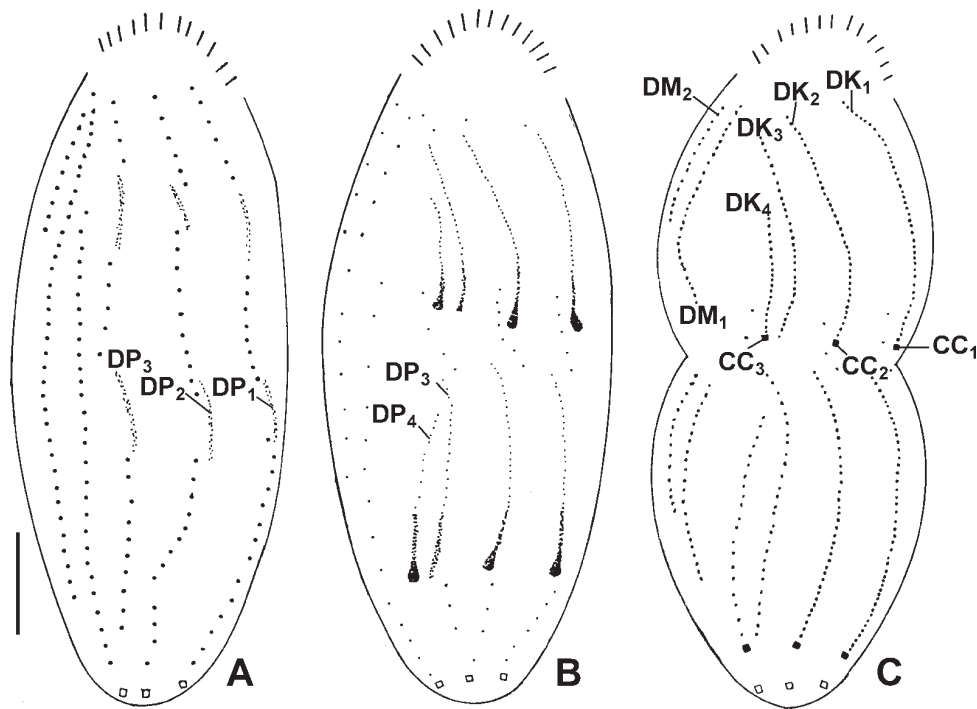


Fig. 4. Line diagrams of protargol impregnated cells of *S. ammermanni* sp. n. showing division morphogenesis on the dorsal surface. **A** - formation of dorsal primordia (DP_{1-3}) within the parental kineties. **B** - unequal split in DP_3 to form DP_4 . **C** - formation of 3 caudal cirri (CC_{1-3}) one each at the ends of $DK_{1,2,4}$. Newly formed dorsomarginal ($DM_{1,2}$) rows shifted from the ventral surface. Scale bar - 20 μ m

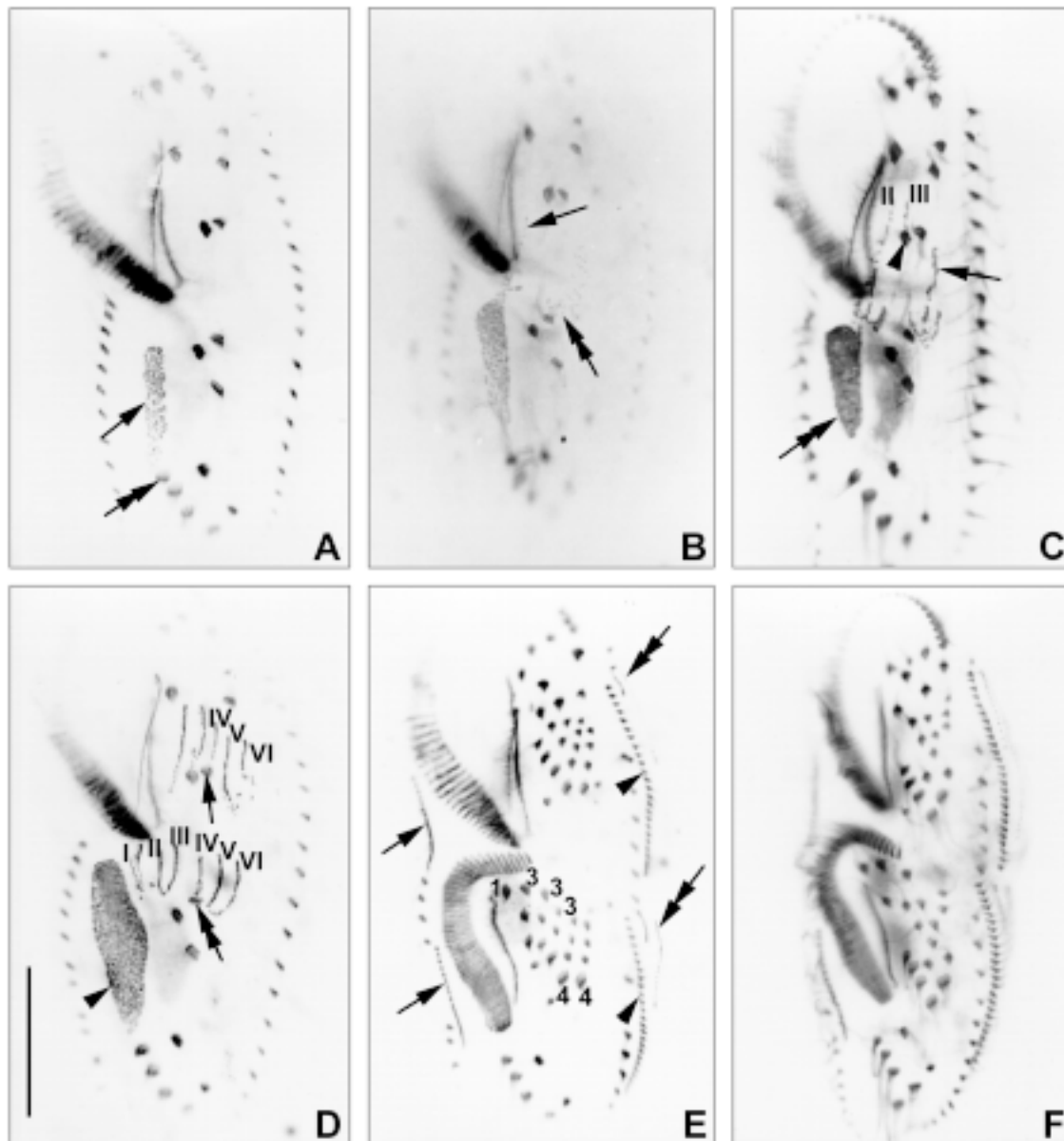


Fig. 5. Photomicrographs of protargol impregnated cells of *S. ammermanni* sp. n. showing division morphogenesis on the ventral surface. **A** - linear proliferation of oral primordium (arrow) close to uppermost transverse cirrus T_1 (double arrow). **B** - anteriad movement of kinetosomes (arrow) from oral primordium and disaggregation of right postoral ventral cirrus V_1 (double arrow). **C** - anteriad movement of kinetosomes (arrow) from V_2 . Origin of primordia: II of proter from OP (double arrow); III of proter from F_8 (arrow head) **D** - origin of primordia: IV of proter from F_7 (double arrow); V and VI of proter from V_2 (double arrow); I-III of opisthe from OP (arrow head); IV-VI of opisthe from V_2 . **E** - differentiation pattern of 1, 3, 3, 3, 4, 4. Within row formation of left (arrows) and right (arrow heads) marginal primordia. Dorsomarginal primordia (double arrows) formed near right marginal primordia. **F** - cell in cytokinesis. Scale bar - 35 μ m

dorsal surface during cytokinesis. Caudal cirri are formed by proliferation of kinetosomes at the posterior ends of the new $DK_{1,2,4}$ in each daughter cell.

Type species are deposited at the Biology Centre of the Upper Austrian Museum no. 2000/155 in the collection of "Evertebrate varia."

DISCUSSION

Diagnosis of *Stylonychia ammermanni* sp. n.

Member of the *Stylonychia mytilus-lemnae* complex lacking peristomial bulge. Size in protargol preparations about 135 x 50 μ m with parallel borders and rounded

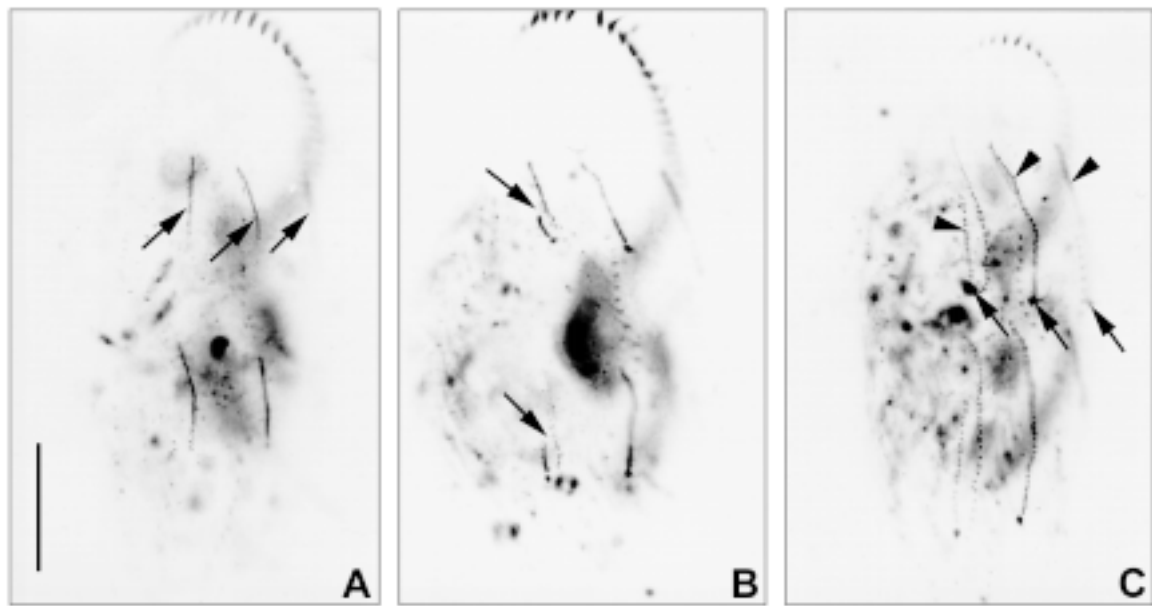


Fig. 6. Photomicrographs of protargol impregnated cells of *S. ammermanni* sp. n. showing division morphogenesis on the dorsal surface. **A** - within row formation of dorsal primordia (arrows). **B** - unequal split of DP_3 (arrows). **C** - proliferation of caudal cirri (arrows) at the ends of dorsal kineties ($DK_{1,2,4}$; arrowheads). Scale bar - 30 μ m

ends. Nuclear apparatus consisting of 2 ellipsoidal macronuclear nodes and 2-4 spherical micronuclei. Posterior frontals ($F_{5,6}$) in linear orientation and almost parallel to the RMC. Posteriormost pair of frontals ($F_{7,8}$) separated from $F_{5,6}$ by a distinct gap and is near to the cytostome. Adoral zone 50-60% of the body length, with about 50 adoral membranelles. Left and right marginal cirral rows with around 17 and 24 cirri respectively. Six dorsal kineties with about 190 dorsal bristles.

Wirnsberger *et al.* (1986) divided the members of the genus *Stylonychia* into two complexes viz. *S. mytilus-lemnae* complex and *S. pustulata-vorax* complex on the basis of certain morphological and morphogenetic criteria. Application of these criteria as detailed below shows that *S. ammermanni* sp. n. belongs to the *S. mytilus-lemnae* complex. The characters shared by *S. ammermanni* sp. n. with this complex are as follows:

- (1) The cell body is rigid with a broad and large peristome.
- (2) The marginal cirral rows are straight and are posteriorly open.
- (3) Dorsal kineties (DK_{1-3}) are apically bent.
- (4) The dorsomarginal rows are long (DM_1 being a complete row).

(5) The right caudal cirrus is present between, terminal 2-3 cirri of the RMC row.

(6) Formation of new FVT ciliature for the two daughter cells involves three parental cirri (2 frontals and 1 ventral).

(7) Second FVT primordium of the proter originates from the OP. Fifth and sixth originate from V_2 .

(8) Fourth FVT primordium of the opisthe originates from the right postoral ventral cirrus (V_2).

S. ammermanni sp. n. is clearly distinguishable from *S. mytilus* and *S. lemnae* by its size, shape, cirral pattern, absence of peristomial bulge (Fig. 2), and micronuclear size (Table 2). It is reproductively isolated from these two species as no conjugation could be induced among them in the laboratory. Comparative features of *S. ammermanni* sp. n. with the closely related members of the *S. mytilus-lemnae* complex are shown in Table 2.

Studies on the isozyme pattern, RAPD characterization and internal transcribed sequence analysis of rDNA (Jacob 1996; the species is mentioned as *S. indica*) also suggests a clear separation of *S. ammermanni* sp. n. from *S. mytilus* and *S. lemnae*.

Stylonychia ammermanni sp. n. shows certain morphometric similarities to *S. putrina* reported from

Yaounde, Africa (Dragesco and Njiné 1971, Dragesco and Dragesco-Kernéis 1986). However, in the absence of any available details of dorsal ciliature and the morphogenetic pattern, it is not possible to ascertain the synonymy of the two species.

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INSTRUCTIONS FOR AUTHORS

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