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## Biodiversity and Biocomplexity of the Protists and an Overview of Their Significant Roles in Maintenance of Our Biosphere

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**Summary.** The ubiquitous and very numerous protists, eukaryotic organisms mostly unicellular in structure and small in size, play numerous roles of importance that are often neglected or overlooked in biodiversity, biocomplexity, and conservation considerations of life on planet Earth. The present review article reminds readers, who hopefully include general biologists and other scientists as well as persons engaged directly in research activities that primarily involve solely protists (e.g. diverse studies on species belonging mainly to many individual groups of algae or protozoa), of such roles whether they be in areas directly or indirectly beneficial to human comfort and survival or in ones deleterious to our health and welfare. Matters become complex when multiple interactions are part of the overall picture (e.g. in food webs or in symbiotic relationships). Clearly, our far from complete knowledge of protistan taxonomic and phylogenetic interrelationships, as well as of their ecology, physiology, biochemistry, and molecular and evolutionary biology, hinders rapid progress in better understanding of their multiple roles in sustaining today's biosphere.

**Key words:** algae, complexity, conservation, diversity, lower fungi, protists, protozoa.

### INTRODUCTION

It may not be generally realized that protists - eukaryotic organisms embracing what are classically known as the protozoa, the algae, and the so-called lower fungi - are far more speciose than the viruses and the prokaryotes (essentially the bacteria *sensu lato*), the two latter groups so widely hailed as the most important (or even only!) microorganisms seriously affecting the lives of the more visible (so-called higher)

organisms, the plants and the animals of the world. Many such tiny microbes (i.e. bacteria) are indeed highly important to the well-being of the latter, including humans. But there is also an immense assemblage of *protistan* microorganisms (representing dozens of phyla, scores of classes, hundreds of orders) that taxonomically far outnumbers the totals of known species of viruses and prokaryotes and that likewise has a major impact on us and on the sustainability of our world (see very brief preliminary note, a letter to the Editor of *BioScience*, by Corliss 2001).

The primary purpose of this article is to highlight some of the characteristics, actions, and interactions of the protists *sensu lato* that often seem to have been neglected in addressing the pressing problems of biodiversity,

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biocomplexity, and conservation, issues now in the forefront of public attention as well as representing topics of widespread biological research efforts. Scores of major publications and reviews dealing with such subjects have appeared since the stimulating pioneering essays of E.O. Wilson (e.g. see his 1992 book and references to his early papers therein), but they give scant mention, if any, to the significance of our predominantly unicellular lower eukaryotes.

A second aim of this overview presentation, persistently urged by protistological colleagues who have seen my preliminary note (cited above), is to offer an annotated guide (space limitations unfortunately require it to be a rather brief one) to the pertinent growing literature on protists in such highly diverse fields as systematics, ecology, parasitology, morphology, cell biology, evolutionary biology, and even paleontology. I have also tried to keep in mind the importance of *not* neglecting either of two seemingly disparate areas, the one involving essentially ubiquitous free-living forms (so numerous everywhere in ecological niches surrounding us) and the other devoted to symbiotic and especially parasitic species (e.g. the causative agents of malarias, trypanosomiasis, leishmaniasis, and the like).

## TAXONOMICALLY, WHAT ARE THE PROTISTS?

Because taxonomy has often been heralded as the core of biodiversity, by Wilson and other biologists, we must pay some attention to it here, although space restrictions forbid detailed treatment. Arguments will long continue among classificational specialists, cladists, and molecularly oriented evolutionary biologists over the exact boundaries of both long and more recently recognized assemblages of protists (see especially discussions in Sleigh 1989; Karpov 1990; Margulis *et al.* 1990; O'Kelly 1993; Cavalier-Smith 1993, 1998a,b, 2000a, 2002; Corliss 1994a, 1998, 2000a; Blackwell and Powell 1995, 2001; Hausmann and Hülsmann 1996; Margulis 1996; Norton *et al.* 1996; Andersen 1998; Barnes 1998; Coombs *et al.* 1998; Vickerman 1998; Patterson 1999, 2002; Alimov 2000; Atkins *et al.* 2000; Johnson 2000; Leadbeater and Green 2000; Ben Ali *et al.* 2001; Simpson and Patterson 2001; Lee *et al.* 2002).

For most of our needs here, the protozoa - or protozoan protists - at the level of a kingdom PROTOZOA (written throughout this paper, when employed as a taxonomic name, in all capital letters to highlight its

supraphylum rank; this is also done for the other four kingdoms of organisms very briefly referred to below), may be considered as comprising the majority (but not all!) of those groups embraced by the classically long-familiar vernacular names (after Kudo 1966) of amoebae (rhizopods *s. l.* and the actinopods, many of both groups known only as fossil forms, plus the heliozoa); flagellates (diverse zooflagellates, including also the choanozoa and the opalinids, plus some so-called phytoflagellates); sporozoa *s. l.* (all symbiotic or parasitic, embracing the telosporidians, acnidosporidians, and cnidosporidians: the last composed of the microsporidians + myxosporidians); and ciliates (holotrichs, spirotrichs, peritrichs, suctorians, *in toto* the most diverse and speciose of all in extant forms: Corliss 1979, de Puytorac 1994, Lynn and Small 2002). There are numerous known species of these largely microscopic unicellular forms, many times the number recognized for bacteria and viruses; and their populations in nature exceed by several orders of magnitude those of all taxa of multicellular organisms combined.

Originally a taxonomic subcategory of the animals, as a *phylum* Protozoa, some former "protozoan" taxa in the above list appear today in other than just the relatively newly recognized formal *kingdom* PROTOZOA (refined and reduced in size, less paraphyletic in composition and thus more meaningful: Cavalier-Smith 1993, Corliss 1994a). For example, some zoosporic protists, a few slime molds *s. l.*, the opalinid infusorians, and various "phytoflagellates" (but not dinoflagellates and euglenoids) have been relocated to positions in the CHROMISTA (see below). The remarkable *Volvax* (Kirk 1998) is to be found with the chlorophytes in the PLANTAE. The microsporidians are in the FUNGI (Canning 1998, Cavalier-Smith 1998a) and the myxosporidians in the ANIMALIA (Siddall *et al.* 1995, Anderson 1998, Kent *et al.* 2001). Yet there are still (Corliss 2000a) some 83,000 protists embraced by the newly defined kingdom PROTOZOA, including extinct forms (e.g. among foraminiferans and radiolarians) known from fossil material; and probably many more are awaiting discovery.

The algae or algal protists have long been considered informally mainly as embracing pigmented (plus some non-pigmented and/or amoeboid) species classically assigned to such groups (often at the rank of class) as the cyanophytes (the blue-greens, but now recognized as prokaryotes), euglenoids, dinoflagellates, cryptophytes, chlorophytes (the greens), chrysophytes (the golden or yellow-greens), heterokonts (or chromophytes,

chromobionts), haptomonads (prymnesiophytes), phaeophytes (or fucophytes, the browns), rhodophytes (the reds), and charophytes (stoneworts, and now including the desmids), plus a few other small groups: see Andersen (1992, 1996). Such protists, never contained in a single top-level taxon of their own (having always been attached to a taxonomic subcategory of plants), are distributed among multiple kingdoms now, with many allocated to the kingdom CHROMISTA. [To be noted: The popular “stramenopiles” of Patterson (1989, 2002) and other phylogenetic cladists (e.g. Leipe *et al.* 1996) are basically the refined/redefined heterokonts of traditional phycologists and as such are thus mostly identical to the major portion of Cavalier-Smith’s (1981, 1986, 1989, 1998a, 2002) assemblage of the chromists. Therefore, in my opinion, the name “Stramenopila” of some authors is essentially a synonym of CHROMISTA. But see the recent scholarly reviews by Medlin *et al.* (1997) and Blackwell and Powell (2000).]

Mostly because of the incredible numbers of the chrysophyte diatoms (extant and extinct), the algal protists outnumber by several tens of thousands of species even the large assemblage of protozoan forms discussed briefly above. The chromists, today circumscribed as an independent kingdom, include about 107,000 species, but with >90% being diatoms. The groups now transferred (back) to the PLANTAE (essentially the greens, the reds, and the stoneworts *s. l.*) contain an additional 21,000 species; and those (the euglenoids *s. l.* and dinoflagellates) assigned to our PROTOZOA number some 6,000 species (Cavalier-Smith 1998a, Corliss 2000a).

The so-called lower fungi, conventionally a small group in the kingdom FUNGI (although they, like the algae, long represented just a taxonomic subcategory of plants), have in the past embraced the myxomycetes *s. l.*, the labyrinthulids *s. l.*, the oomycetes, the hyphochytrids, and the chytrids. The groups vernacularly just named and together totaling some 3,000 species are today quite widely separated, with only the chytrids, 900 species, remaining in the FUNGI proper; although to them, also as “lower fungi” of a sort, have now been added some 800 species of microsporidians, removed from their classical protozoan status.

The grand total of described-to-date protists, no matter how classified, reaches at least 213,000 species distributed among about three dozen phyla (see Table 2 of Corliss 2000a) belonging to the five eukaryotic kingdoms - the PROTOZOA, the CHROMISTA, the PLANTAE, the FUNGI, the ANIMALIA - recognized by Cavalier-Smith (1998a, 2002) and the author (Corliss

1998, 2000a). These kingdoms are not universally accepted, although their hierarchical ranked structures are convenient (“user-friendly”) for non-protistologically oriented biologists, for students, and for other scientists, as well as for indicating their relationships to groups of past conventional schemes of classification still in wide usage around the world.

The principal opponents to the serviceable taxonomic framework or Linnean System used in this review (one allegedly accepting paraphyletic and occasionally polyphyletic assemblages and susceptible to some subjectivity) are the cladists (champions of Phylogenetic Systematics), led mainly by Patterson (1994, 1999, 2002), who favor division of all organisms into *clades*, phylogenetic lines defined objectively by synapomorphies, innovative or unique derived characters or features (subjectively chosen?) held in common - or if absent, presumably secondarily lost - by all species comprising a given unit (Wiley 1981). The main problem to this alternative approach, as I see it, is the complete incompatibility of hierarchically arranged, named higher taxa with a system of clades, sister clades, etc. (Mayr 1990, 1997; Corliss 1994a, 1998, 2000a). Can the two approaches ever be integrated? Patterson (2002) himself confesses, “Unfortunately, we still lack the plans [to create a lasting systematic edifice] which will come in the form of a robust and probably molecular understanding of evolutionary relationships among [protozoan] taxa...”

I feel obliged to repeat my statement made eight years ago on pages of this journal (Corliss 1994a), “We are frustratingly trapped between existing classifications of protists that are recognized to be faulty and some future scheme (hopefully closer to the ideal natural system long awaited) not yet available.” Thus, also *still* true, “The current status of the megasystematics of the protozoa and their nearby relatives is unsettled, in a state of flux” (Corliss 1998). Ideal solutions seem to depend on revelations yet to appear.

As stated above, today I find it acceptable to consider the species of protists as distributable among all five kingdoms of the supraprotozoan (empire or domain) Eukaryota. The once attractive idea of an isolated/separate kingdom for the protists alone (called “PROTISTA” or “PROTOCTISTA”: see especially Whittaker 1969, Margulis 1974, Whittaker and Margulis 1978, Margulis *et al.* 1990, Margulis and Schwartz 1998) has been widely abandoned by research workers in protistology, pedagogically convenient and popular though it was (and often still is!): see Sogin *et al.* (1996), Bardele (1997), Ragan (1997), Andersen (1998), Cavalier-Smith

(1998a, 2002), Corliss (1998, 2000a), Lipscomb *et al.* (1998), Schlegel (1998), and other relevant earlier papers, including Rothschild (1989) on nomenclature for such a group.

For a little further clarification of the taxonomic matter of (my acceptance of) the appearance of protists in all five eukaryotic kingdoms, let me briefly make an additional comment or two here. Although *all* members of the kingdoms PROTOZOA and CHROMISTA may be considered as true protists, the protists or superficially protist-like forms often associated with the *other* kingdoms (FUNGI, PLANTAE, ANIMALIA, where mostly *multicellular* species predominate) fall into *three* broad categories. This point can be best illustrated using the fungi *sensu lato* as an example, but it is essentially true for the plant and animal assemblages as well. (1) There are true *fungal* protists or *protistan fungi* (e.g. chytrids and microsporidians, the latter formerly considered to be unique protozoa). (2) There are *fungus-like* protists (e.g. members of Cavalier-Smith's chromistan phylum Pseudofungi). (3) There are *protist-like* fungi (e.g. *Pneumocystis* and some species of *Saccharomyces*, two true fungal genera *not*, incidentally, closely related to one another).

These distinctions need to be kept in mind. One could add here that the ubiquitous protists even have superficial "look-alikes" (pseudo-protists) among the *prokaryotes*, too (e.g. species of the cyanobacteria, still often known as "the blue-green algae" and more frequently studied by phycologists than by bacteriologists).

### Numbers and kinds of species of protists

As a kind of footnote to our taxonomic discussions on the preceding pages, I should briefly mention the "species problem" in protistology (see especially Andersen 2000), but it is a vast subject mostly beyond pertinence to the main topics of this paper. Taxonomists and ecologists, however, are keenly interested in the proper identification of the diverse forms involved in their researches, and arguments of ubiquity versus endemism, not trivial matters, depend in part on concepts of the nature of a species at the level of the lower eukaryotes. Such controversial problems are not made any easier by either the legitimate concerns of nomenclaturists (worried about synonymies, etc.) or the sobering predictions of census- or inventory-takers who estimate that protistan species-yet-to-be-described are probably at least two or three times greater than those named and cata-

logued to date. Finally, add to this the present furor over newly proposed codes of biological nomenclature, documents that purport to bring a measure of order out of the continuing chaos related to some of the just-listed woes (and also extending taxonomically upward well above the level of species).

Selected major works on those matters (above) are the following. For the "species problem" overall: Hammond (1995), Norton *et al.* (1996), Claridge *et al.* (1997), Howard and Berlocher (1998), Corliss (2000a), May (2000), Wheeler and Meier (2000). For ubiquity *versus* endemism: Finlay *et al.* (1996, 1998, 2001), Foissner (1997, 1998, 2000a,b), Finlay (1998), Finlay and Fenchel (1999), Foissner *et al.* (1999), Esteban *et al.* (2000, 2001), Finlay (2002). For discussions over changes of or in the codes of nomenclature: Hawksworth (1992), Patterson and Larsen (1992), Corliss (1993, 1994b, 1995, 2000a), Hawksworth *et al.* (1994), Blackwell and Powell (1999), Forey (2001), de Queiroz (2001).

### GENERAL CHARACTERISTICS OF PROTISTS

The principal structural, physiological, and ecological features characteristic of protistan species overall separate them from other major assemblages of micro- and macroorganisms. But the "constellation of characters" principle (Corliss 1976) needs to be applied here. There is no single derived feature (a synapomorphy, if you will) conveniently setting them apart. If there were, then we should have justification for recognizing a unique kingdom here (e.g. "PROTISTA"), but the very fact that we do not have such an isolated character has been and is still the main reason for rejecting the Margulis proposal, first made years ago (Margulis 1974), of her artificial "kingdom Protoctista" (see above). Recall also that many protists are, simultaneously, *both* single, independent, complex cells *and* functionally complete, whole organisms: these terms are not mutually exclusive (Corliss 1989a). But no multicelled-multitissued organism can lay claim to exhibition of such a dual condition of life.

Thus we should consider briefly major morphological, ecological, physiological, behavioral, and evolutionary characteristics of protists, some of which indeed - alone or in combination - serve as unique features separating individual protistan phyla (and/or lower taxa) one from another and from members of still other phyla comprising the five kingdoms of eukaryotic organisms accepted by the writer. Space does not permit an exhaustive listing

of citations, but a limited number to the most recent and/or comprehensive publications appear in each of the following subsections.

### Main morphological/structural features

Primarily because of continuous development of improvements in methods of microscopy and cytology and the relatively recent advent of molecular approaches, we can today analyze and amass data on scores of protistan characteristics of great value in comparative morphological and taxonomic studies. Such features range from body shapes (all kinds) and sizes (usual range 2–2000  $\mu\text{m}$ , with notable exceptions to several meters in length) and exhibited symmetries (bilateral, radial, etc.) to nuclear numbers and nuclear characteristics (centrioles, spindle fibers, telomeres, etc.), kinds of endo- or exocytoskeletal structures, extrusive organelles (of diverse sorts), stalks (contractile with myonemes or non-contractile) and diverse other attachment or adhesive accoutrements, pigments, cortical alveoli, and contractile vacuoles to numerous kinds of specific cytoplasmic inclusions [e.g. endoplasmic reticulum, ribosomes, lysosomes, mitochondria/chondriosomes (with lamellar, discoid, vesicular, or tubular cristae), hydrogenosomes, Golgi bodies/dictyosomes, peroxisomes, plastids (e.g. chloroplasts, typically with stacked thylakoids and pyrenoid bodies), cyanelles, eyespots/stigmata, and carbohydrate/lipid/protein food reserves].

Even a brief list must be extended to include locomotory and feeding apparatuses, often involving pseudopodia of various kinds, flagella (some with mastigonemes), cilia and their subparts (including basal bodies/kinetosomes) and their compound derivatives (membranelles, cirri, etc.). Some protists have a highly structured oral apparatus (which may include sucking tentacles) for particulate food ingestion, while others are able to obtain nourishment by absorption directly through the cell membrane, with and without the use of exoenzymes. Still others have the machinery to engage in photosynthesis, an activity often essential for their very survival. Numerous species secrete or otherwise construct enveloping walls, sheaths, scales, thecae, loricae, tests, or shells, sometimes with “exit” pores and often of complex chemical and/or structural composition. Many such forms are easily fossilized (e.g. among dinoflagellates, foraminiferans, radiolarians, diatoms, ciliates). Cysts and/or spores, serving as dispersal or protective forms, are not uncommon in full life cycles of numerous species

from a large number of different taxa of protists (Corliss 2000b).

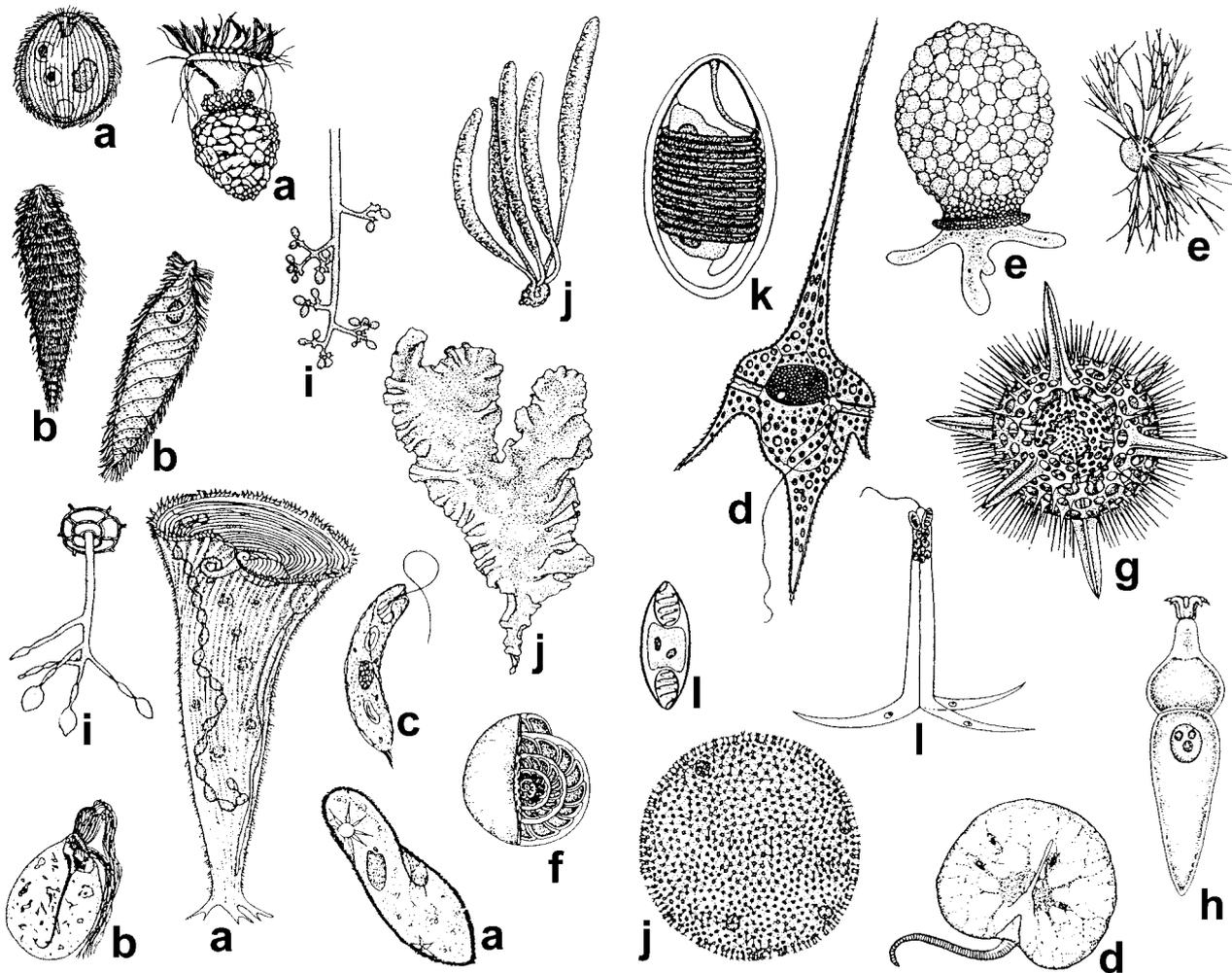
Organizationally, cells of protists may remain independent or be grouped together in chains, filaments, plasmodia, coenobia, or colonies (gregaloid, discoid, spheroid, arboroid/dendroid) of various kinds and sizes and for varying periods of time under specific environmental circumstances. Some colonial species (e.g. the peritrich ciliate *Ophrydium*: see Duval and Margulis 1995) are members of a consortium formed by sharing their own microcosm temporarily or permanently with other organisms. Other protists are truly multicellular, at least at some stage, but they seldom form more than one somatic tissue. Polymorphism is common in many protistan life cycles.

Such elaborations of cytoarchitectural diversity and complexity as those mentioned above - and the list is far from exhaustive - are, *in toto*, unique among all living things on Earth. Recall that they occur within - or are produced by - basically a *single cell* (see Figs 1-3).

Most of the structures listed are familiar to protistologists. But for the benefit of other readers and for details and examples of specific protistan taxa involved, the following publications by specialists are offered, works that also serve as excellent sources of numerous individual papers from both the recent and the older - often still indispensable in protistology - research literature: Grell (1973), Corliss (1979), Grassé (1984), Irvine and John (1984), Bold and Wynne (1985), Lee *et al.* (1985, 2002), Kristiansen and Andersen (1986), de Puytorac *et al.* (1987), Green *et al.* (1989), Sleigh (1989), Margulis *et al.* (1990), Harrison and Corliss (1991), Melkonian *et al.* (1991), Patterson and Larsen (1991), Perkins (1991), Hori (1993a,b, 1994), Müller (1993), Wetherbee *et al.* (1994), van den Hoek *et al.* (1995), Alexopoulos *et al.* (1996), Hausmann and Bradbury (1996), Hausmann and Hülsmann (1996), Lee (1999).

### Ecological characteristics

Protists are cosmopolitan in overall distribution, although obviously symbiotic forms are limited to ranges of their hosts. In similar habitats or niches, many of the same species may be found in abundance worldwide. Most protozoa play roles as phagotrophs (particulate consumers), many algae as phototrophs (primary producers), most fungi as saprotrophs (decomposers). But there is considerable overlapping in nutritional modes



**Fig. 1.** Diversity among protists, as shown in figures (not drawn to scale) of selected representatives from the five eukaryotic kingdoms. Protozoan protists: **a** - Four examples of common free-living ciliates. **b** - Three symbiotic colorless flagellates. **c** - One photosynthetic euglenoid. **d** - Two free-living (upper figure freshwater, lower marine) dinoflagellates. **e** - Two testaceous amoebae, with differing pseudopodia. **f** - One foraminiferan (a nummulite shell). **g** - One radiolarian, showing complexity of skeleton. **h** - One cephaline gregarine sporozoon, with epimerite. Chromistan protists: **i** - Two saprozoic pseudofungi. Plant protists: **j** - Two marine algae (second known as “sea lettuce”) and (lowest figure) one freshwater volvocine coenobium. Fungal protists: **k** - One microsporidian (spore, *greatly* enlarged). Animal protists: **l** - Two myxosporidians (a bipolar spore and a trivalved epispore). [All 23 figures modified from ones used in Corliss (1991) which, in turn, had been redrawn from multiple, widely scattered, sources in the protistological literature of the past century.]

among protists overall, and it is clear that the protistan role in ecosystem regulation stems from their diversity in ways of feeding as well as in body size and location (of aquatic forms) in the water column.

Free-living species have a very broad distribution, as mentioned above. They live as planktonic or benthic forms in oceans, seas, rocky intertidal pools, hydrothermal vents, tropical reefs, estuaries, fjords, bays and harbors, lakes, rivers, streams, creeks, subterranean waters, caves, thermal springs, briny pools, lagoons, bogs and marshes, freshwater ponds, rain puddles, swimming

pools, bird baths, ice and snow, desert sands, sewage treatment plants, reservoirs, beach sands and intertidal mud flats, sediments, moss, forest litter (and on bark and leaves of trees and bushes), arable soils, and so on. Bonafide endemic forms may not be as common as generally thought in the past, but more studies need to be carried out on this continuing controversial topic, one of major importance (see below). Note that habitats may include niches manifesting extreme ranges in temperature, oxygen, water and mineral content, salinity, pH, atmospheric pressure, radiation, etc. (see the informa-



**Fig. 2.** Scanning electron microscope image of a giant cannibalistic protozoan, a hypotrichous ciliate (*Onychodromus quadricornutus*), caught in act of devouring one of its small congeners (latter still visible on floor of gaping oral opening of the hunter). The predator has also sprouted spurs on its rear dorsal surface (a phenotypic change as defense/protection from still larger carnivores in its habitat) in response to a chemical signal emitted from its foes. [SEM kindly provided to the author (with permission to use at any time), some years ago, by protistological colleague Barry Wicklow, Saint Anselm College, Manchester, NH, USA.]

tive and stimulating review by Rothschild and Mancinelli 2001).

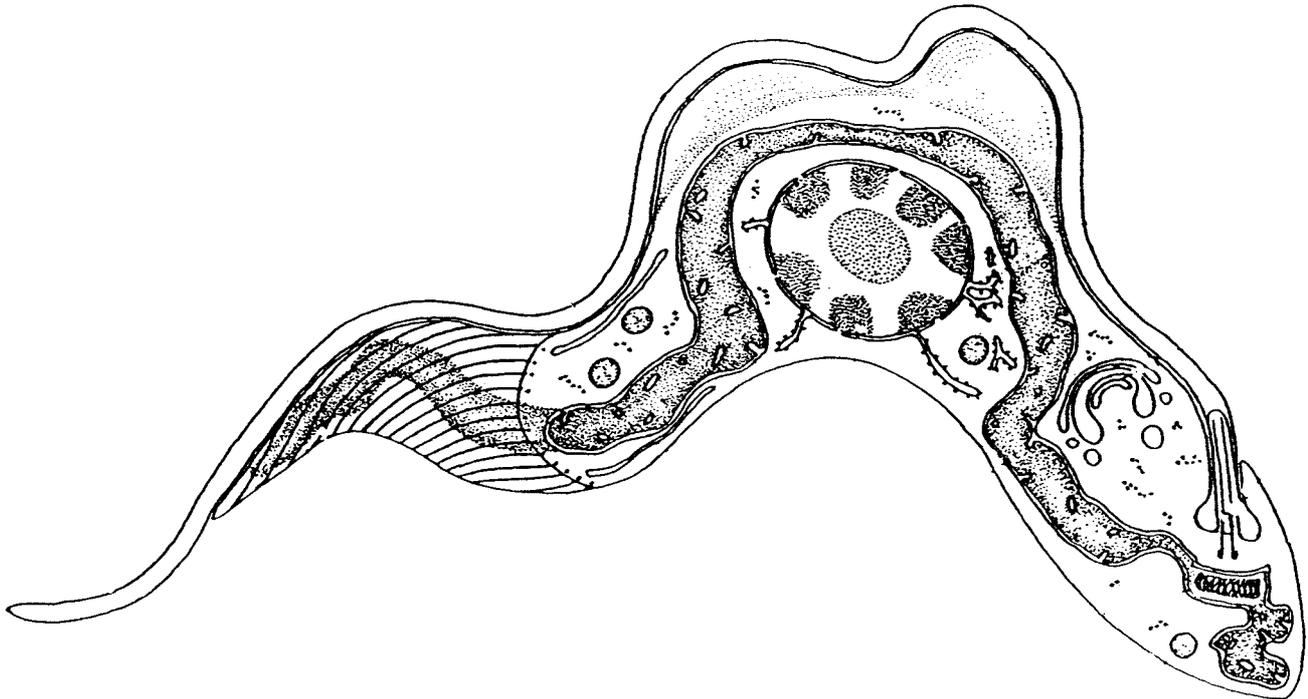
Symbiotic species (benign or pathogenic) are common in or on sponges, bryozoa, all kinds of arthropods, worms of diverse taxonomic sorts, members of all higher phyla and classes of invertebrates and vertebrates, wild or domesticated, including humans among the many mammals infected. Plants of all kinds also serve as hosts, everywhere. Free-living *or* symbiotic protists may themselves house ecto- or endosymbionts, viruses, bacteria, and/or other protists or even micrometazoa. One of the most fascinating relationships is that of protists (free-living in anaerobic habitats or parasitic in diverse hosts) that are physiologically dependent on methanogenic archaean bacteria dwelling within their cytoplasm as hydrogenosomes (of some sort).

There is abundant literature on the ecology of protists. Many references cited above (under morphology) contain information also relevant here. Some additional works on mostly free-living species: Poljansky and Cheissin (1965), Bamforth (1985), Dragesco and Dragesco-Kernéis (1986), Fenchel (1987), Foissner (1987, 1991), Anderson (1988), Patterson *et al.* (1989), Page (1991), Patterson and Larsen (1991), Sleight (1991),

Andersen (1992), Laybourn-Parry (1992), Vickerman (1992), Darbyshire (1994), John (1994), Fenchel and Finlay (1995), Sandgren *et al.* (1995), Finlay and Fenchel (1996, 1999), Finlay and Esteban (1998), Lee and Patterson (1998), Boltovskoy (1999), Wetzel (2001a). On mostly symbiotic forms: Kreier and Baker (1991-1995), Krylov (1993-1994), Ashford and Crewe (1998), Marquardt *et al.* (2000), Zimmer (2000). And on symbionts of protists: Jeon (1983), Hovasse (1984a,b), Preer and Preer (1984), Reisser (1986), Wang and Wang (1991), Görtz (1996, 2001), Hackstein *et al.* (2001, 2002).

#### **Physiological, biochemical, and behavioral traits**

The protists overall exhibit a great number of functions, reactions, tropisms, taxes, circadian and tidal rhythms, modes of food-gathering, means of locomotion, internal cycles, polymorphism, morphogenetics of fission, en- and excystation, and so on, sometimes unique, sometimes mimicking general known properties or reactions of some of their descendants, the multicelled/multitissued organisms of our biosphere. Nuclei have two main functions, replication of genetic material and release of information to the biosynthetic machinery of



**Fig. 3.** Composite semidiagrammatic drawing from transmission electron micrographs of a trypanosome (*Trypanosoma rhodesiense*) highly virulent in humans. In the intermediate bloodstream form portrayed may be seen a diversity of (unlabeled) ultrastructural features characteristic of these protists (see review of their fine structure in Vickerman *et al.* 1991). The vector is the tsetse fly. [Figure modified from a drawing kindly provided to the author many years ago (with permission to use at any time) by protistological colleague Keith Vickerman, Glasgow University, UK.]

the cell. Exhibition of specialized adaptations to particular environments or habitats is widespread. Study of trophic dynamics of protists today involves sophisticated approaches at cellular, biotechnical, and molecular levels. In the case of symbionts or parasites, accommodating to changes in hosts' reactions is likely more common than yet recognized.

Numerous protists seem to be limited to asexual modes of reproduction, by binary or multiple fission (e.g. palintomy) or by budding. This does allow stability of an established genotype, at least for some generations. With respect to sexuality (whether cross- or self-fertilization is involved), diversity of patterns within or among major protistan groups has been claimed to dwarf that of the combined multicellular members of the kingdoms of plants, animals, and fungi (Dini and Corliss 2001). Stages of senescence and rejuvenescence, as well as "natural" death, have long been recognized (since the most insightful studies of Maupas in the late 1800s) in aging populations of some ciliated protozoan lines (Simon and Nanney 1979, Smith-Sonneborn 1981, Bell 1988, Dini and Nyberg 1993), although immortality is still consid-

ered a general feature of most species of protists. Temporary dormancy, in resting or transfer stages of the life cycle - so essential to viral and prokaryotic forms - is also common among many protistan groups (Henis 1987, Corliss 2000b).

Biochemical investigations on protists (Coombs and North 1991) continue apace: consult any protistological journal or scores of other specialized biological outlets that are published every month. Molecular studies are the current rage: see Raikov (1989) for a review of their early application to research on the nuclear genome of diverse protists, and papers in the symposium chaired by Orias (2000) for the state of the art today in genomic sequencing in protists. But works in both of these domains, biochemistry and molecular biology, are generally - except for their application to protistan systematics and phylogeny - well beyond coverage in the present overview.

In ethological research, the ciliates have probably been subjected to more study than any other major taxon of protists (Miyake 1996, Ricci 1996, Ricci and Erra 2001). In modern times, researches on their sometimes

highly complex pheromone- or gamone-induced characteristics run from the early perceptive observations by Grell (1951) on pairs engaged in a prenuptial dance of the hypotrich *Stylonychia* to the signal-induced defensive phenotypic changes in other hypotrichs described by Wicklow (1997) (see Fig. 2). Equally amazing are the reactions of some epibiotic/phoretic ciliates (e.g. among peritrich, chonotrich, and apostome groups) to invertebrate hosts' molting cycle hormones. The protists are induced to temporarily vacate their spots of attachment to the host's exoskeleton, which becomes unsuitable when shed; and then their motile stages - in due time - swim and reattach to the reconstituted host body (Corliss 1979).

### Evolutionary and phylogenetic considerations

Some groups of the largely unicellular protists have surely served as transition forms (what I have called "gap-bridgers") from the structurally simpler bacterial prokaryotes to the complex multicelled-multitissued eukaryotes represented today by most contemporary species of plants, fungi, and animals (Corliss 1987, 1989b; Bardele 1997; Hausmann and Hülsmann 1999). During the last quarter of the past century, research on the phenomenon of eukaryogenesis has led many biologists to recognition of a distinct interdisciplinary field of protistology (Corliss 1986), an exciting and growing area exploiting fully what I have labeled "the protist perspective" (Margulis *et al.* 1990; Patterson and Larsen 1991; Patterson 1994, 1999; Cavalier-Smith 1995; Kristiansen and Cronberg 1996; Andersen 1998; Leadbeater and Green 2000).

In cases of many invertebrate and vertebrate animals and various plant groups, study of fossil lines has led to an understanding of evolution and evolutionary processes in those organisms. But in general this has not been possible for assemblages of protists, due largely to the relative simplicity of their remains - often limited to merely external coverings (tests, loricae, impregnated thecae, etc.) or silicifiable or calcifiable external or internal scales, spicules, spines, etc. Nevertheless, >100,000 protist species have been described solely as extinct forms (e.g. 89% of the foraminiferans, 65% of the radiolarians, 70% of haptomonads, 60% of diatoms, 55% of the dinoflagellates, 25% of prasinophytes, 15% of chrysophytes, 15% of rhodophytes, 2.5% of charophytes, and 2.5% of ciliates: Corliss 2000a), so they can hardly be ignored from consideration when one is illustrating protistan biodiversity and its usefulness in

the biosphere. Major references to literature on extinct protists are included in these compendia: Tappan (1980), Haynes (1981), Anderson (1983), Taylor (1987a), Round *et al.* (1990), Fensome *et al.* (1993), Lipps (1993), Green and Leadbeater (1994); and individual papers continue to appear in many appropriate journals.

Multiauthored considerations of evolution in diverse groups of mostly extant protists have recently appeared (e.g. Coombs *et al.* 1998, Katz 1999, Fast *et al.* 2002); but Cavalier-Smith (1990) has attempted to integrate the living and fossil evidence in his essay on "microorganism megaevolution," and Knoll (1992) has taken a geological perspective in recognizing a "terminal crown" group of eukaryotes in which he places the bulk of the protists. Barr (1992), Alexopoulos *et al.* (1996), and Beakes (1998) have presented mycologists' views of relationships among eukaryotic kingdoms; and Seravin (2001) has introduced the concept of "counter-directional morphological evolution" in his construction of a megasystem for protists and other eukaryotes. Pawlowski and Holzmann (2002) have published an up to date review on the molecular phylogeny of extant foraminiferans.

Highly visible in today's literature, whether the group under examination is at the lowest (strain, species) or highest (class, phylum, kingdom) taxonomic level, are molecular phylogenetic/evolutionary trees and cladograms - dramatic illustrations of protist biodiversity. Such dendrograms have been constructed employing most often the small subunit rRNA genes but also protein coding genes (see historical overviews in Sogin and Hinkle 1997, Schlegel 1998, Baldauf *et al.* 2000, Melkonian 2001, Cavalier-Smith 2002). All such approaches using molecular markers are said to have unique weaknesses as well as strengths (Edlind 1998, Lipscomb *et al.* 1998, Philippe and Adoutte 1998, Van de Peer *et al.* 2000). Yet, with eventual refinements, which Vickerman (1998) has cautiously implied are on the way, molecular-phylogenetic approaches should serve a major role in solving many of our currently vexatious problems in understanding the evolution of protists.

### ROLES OF PROTISTS IN NATURE AND IN RESEARCH

Having demonstrated the taxonomic diversity and structural and functional complexity of protists (and the need to increase our knowledge in such areas), we now turn our attention to their actions and reactions with

respect to each other, to bacterial populations, and especially to the higher eukaryotes with which they share the biosphere. The pivotal roles played by protists in ecosystem functions, for example, are often simply not appreciated, as recently emphasized in a brief but significant note by Wetzel (2001b: a letter to the Editor of *BioScience* commenting on Corliss 2001).

It will generally be convenient to largely separate consideration of free-living protists (photosynthetic and non-photosynthetic) from that of symbiotic (especially parasitic) forms, although there is overlapping in functions 'twixt the two groups. And certainly members of such strictly artificial assemblages are not always separable on taxonomic grounds. Incidentally, of aid to experimental studies of protists of all kinds are the international culture collections of free-living and parasitic species, both of actively living populations of carefully identified, sometimes "pure" (axenic), strains and/or of cryopreserved material revivable on request. The availability of these authenticated stocks to researchers everywhere provides an indispensable source of highly reliable experimental material, model cells, if you will. For latest information on four major collections, see Nerad (1993), Starr and Zeikus (1993), Andersen *et al.* (1997), and Catalogue of the UKNCC (2001). Andersen (1996) and Norton *et al.* (1996) have supplied a highly useful list of the names and addresses of 73 culture collections around the world that maintain strains of algal protists mostly through perpetual subculturing of living stocks. See, too, Richmond's (1986) useful handbook on microalgal mass culturing techniques, Lee and Soldo's (1992) multiauthored protocols in protozoology, Simon and Nerad's (1996) review, and Colwell's (1975) early collection of workers' papers stressing the value of the accessibility of refined collections in all areas of biological research during that time's strongly emerging Era of Molecular Biology.

Type slide collections, of fixed and stained material, are also helpful to experimentalists, and to taxonomists and ecologists as well. Often type specimens are kept in personal collections, although major museums and herbaria in various countries are expected to house official material available internationally for comparative studies and identification purposes on proper request (e.g. see Cole 1994).

### **Roles and functions of free-living forms**

Free-living (in the broadest sense) species, representing at least 85% of all non-fossil protists (therefore

>86,000 species, according to my counts and estimates: Corliss 2000a), occur in majority numbers in more than two-thirds of the major phyla. Representatives of such groups may exhibit, with overlapping, all the principal kinds of nutrition: ingestive, absorptive, photosynthetic.

Pigmented algal protists, if we include the prokaryotic blue-greens here, are said to provide 40% of global photosynthesis (carbon fixation and oxygen production) on Earth and nearly 100% of the primary production in marine systems (Andersen 1998). More poorly known is the contribution of algal photosynthesis in soils, forests, and tundra, but it is surely a major factor in life there as well (Ettl and Gaertner 1995).

Non-photosynthetic protists, particularly as represented by freely swimming flagellates and ciliates, are the most important consumers of bacteria in both aquatic and terrestrial ecosystems, having major functions in organic carbon utilization and nutrient cycling in general (Foissner 1987, Finlay and Esteban 1998, Wetzel 2001a). The ciliates, the most abundant phagotrophs in the biosphere (Finlay *et al.* 1998), exhibit high feeding rates on not only the bacterial populations but also on algae and other protists and even on the particulate detritus (the "snow" of lakes and oceans that includes fecal material from metazoa: Silver *et al.* 1984, Grossart and Simon 1993). The flagellates, typically smaller but much more abundant than ciliates and often mixotrophic in their nutrition, occupy both planktonic and benthic levels and feed on other microorganisms while also fixing carbon photosynthetically. According to figures in Wetzel (2001a,b), such flagellates are a dominant mortality factor for the bacterioplankton and frequently remove 20-60% of the algal picoplankton as well.

Protists are actively involved in essential food chains and webs, and thus in the well-known "microbial loop" of modern ecologists; as a result, they affect the health and very survival of a multiplicity of organisms including, ultimately, the carnivorous and herbivorous mammals (including humans) at the top of the nutritional chain. Flagellates and ciliates (less often amoebae), species typically <100µm in length, serve as both major decomposers and mediators of nutrient recycling in ecosystems (Patterson and Larsen 1991; Reid *et al.* 1991; Wetzel 2001a,b). Besides the open water systems of ponds and lakes and oceans, the many sewage disposal, waterworks, aquacultural, and soils operations in the world also benefit by this often perhaps little known protistan control of bacterial populations (Sieburth 1979, Capriulo 1990, Curds 1992, Arndt 1993, Foissner 1994, Sherr and

Sherr 1994, Anderson 1996, Foissner and Berger 1996, Moriarty 1997, Andersen 1998, Finlay and Maberly 2000, Ettl 2001).

Rhizopod, foraminiferan, and testaceous amoebae are involved in soil, fresh-water, and marine environments, too (Feest 1987, Foissner 1987, Arndt 1993, Ekelund and Rønn 1994, Wanner 1999, Finlay *et al.* 2001, Anderson 2002, Smith and Coupe 2002), but often in less well understood ways than are ciliates and flagellates. For the marine ecosystems especially, the revelations of Grell on the “plasmoidal protists of the sea” should be consulted (e.g. Grell 1985, 1995; Grell *et al.* 1990; Grell and Schüller 1991).

Some protists (free-living or symbiotic forms), like their metaphytan and metazoan descendants, are invasive species (Elton 2000), often causing a significant loss of biodiversity among native organisms inhabiting the locales invaded and thus upsetting the delicate balance of nature established there. A very recent example is to be found among species of the plant protist *Caulerpa*, a tropical green algal seaweed discovered just a year or two ago flourishing abundantly in a marine lagoon near San Diego, CA. Rather drastic control methods, intended to serve as a worldwide model, have been put in place to eradicate the fearsome intruder there (Withgott 2002).

The direct economic importance of free-living protists should also be briefly mentioned. Their involvements as food and industrial and medical products are many. Some 500 species of algae (processed or unprocessed, mainly seaweeds) are devoured by humans directly, with still other algal products appearing in commercially prepared foods (e.g. the gelatin in puddings, ice cream, etc.). And in modern mariculture, finfish and shellfish are routinely fed on phytoplankton. In industry, algal hydrocolloids are widely used; and recall that diatomite, fossilized diatom frustules, are the abrasive bases in many scrubbing and cleaning compounds, including toothpaste (Andersen 1996, 1998; Lembi and Waaland 1988). *Preersatz* blackboard chalk was composed mostly of tiny foraminiferan shells, mined directly from natural deposits. This brings to mind T.H. Huxley’s famous address, “On a Piece of Chalk,” delivered in 1868: now see the lecture/paper by J.J. Lee (1993) for *his* updated version on the same topic.

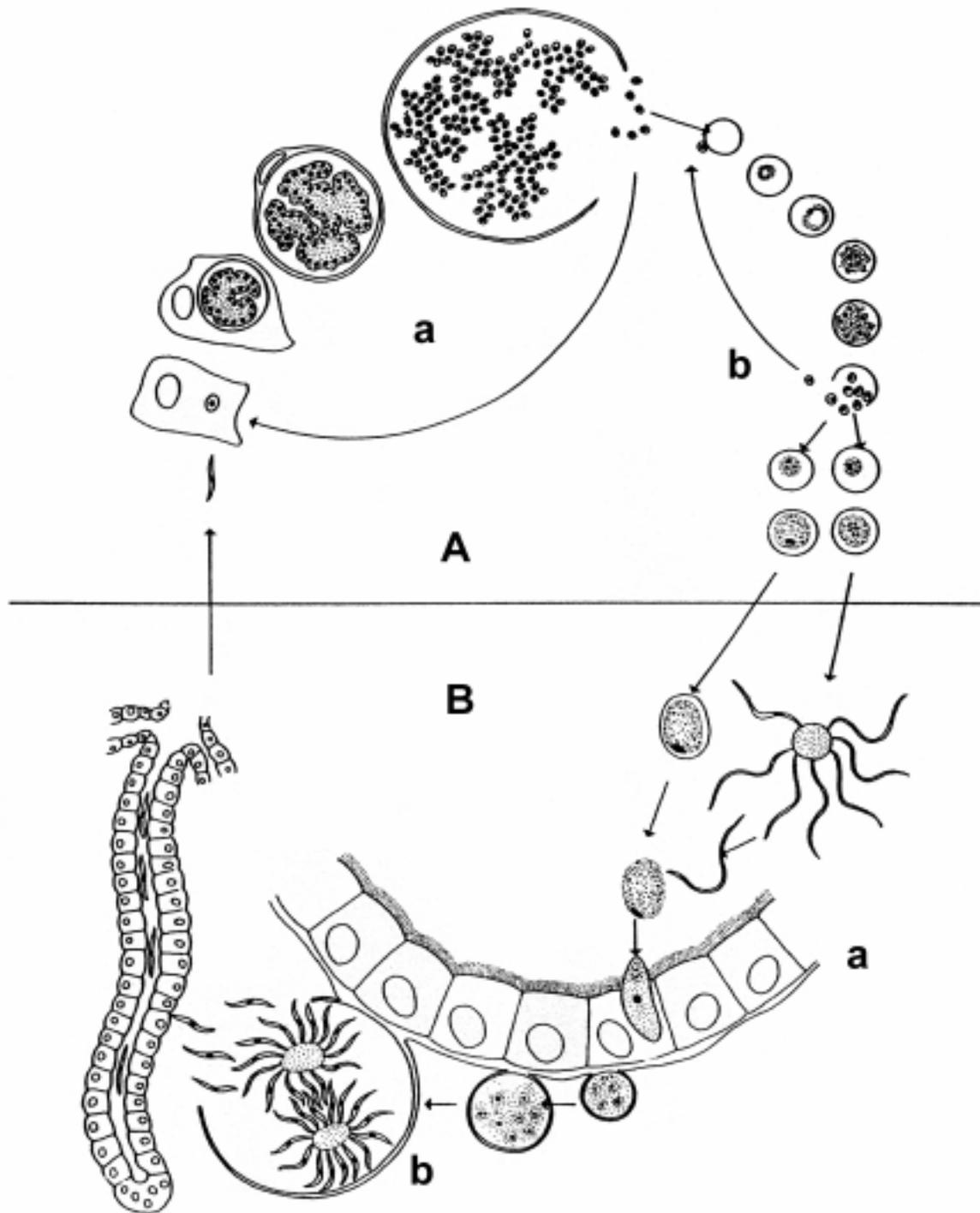
The helpful role of protists in soils, especially in restricting over-populations of bacteria and fungi and recycling nutrients, should not be overlooked, nor the

parts they play in biomonitoring and bioremediation of polluted waters and shores everywhere (Foissner *et al.* 1991-1995; Cairns *et al.* 1992; Wetzel 2001a). Habitat loss is caused mostly by human activities that result in widespread depauperization (often total loss) of species diversity, so conservation of “good” microorganisms is imperative if we are to maintain a beneficial balance in this world between nature and human beings.

Medically, algal agars and agaroses (phycocolloids) are widely used, especially in culture media and in specialized instruments (e.g. for gel electrophoresis, affinity chromatography, etc.). Human heart disease is reduced when certain fatty acids are included in the diet: these are available in/from marine fish, but their origin is in the phytoplankton (especially chrysophyte algal protists) consumed by the fish (Lembi and Waaland 1988). A large number of free-living protists, especially ciliates such as *Tetrahymena* (Frankel 2000) and a variety of pigmented and non-pigmented flagellates, are grown in axenic culture (see above); these organisms are particularly valuable in medical and biomedical research projects, both of an applied and experimental nature. They serve as perfect eukaryotic micro-size “guinea pigs” in a host of investigations ranging from toxicity studies to drug testing and beyond. They also have been used as assay organisms and pharmacological tools of value (Hutner *et al.* 1972), and as ideal microorganisms in modern molecular and genetic studies of diverse sorts (Gall 1986, Simon 1992, Asai and Forney 2000). Several growth factors (= vitamins) have owed their discovery long ago to researches on cultured strains of *Tetrahymena* and of the parasitic flagellate *Trichomonas* (see references in Hutner *et al.* 1972).

Exciting is the report, just now published in *Science* and in news outlets around the world, about a *very* recent decision of the National Human Genomic Research Institute of the U.S.A. The NHGRI has released an announcement that next on its Priority List of actions that will be taken is deciphering the genome of selected members of six taxonomically widely separated groups of organisms. Among these top chosen few are some protists: the ciliate *Tetrahymena thermophila* and several fungal or fungus-like protistan species.

The value of use of fossil protists in the petroleum industry needs to be noted. Carefully identified species of foraminiferans can be used to determine the ages of the strata penetrated by the exploring drills, thus serving as a guide to the potential richness of discovered oil and



**Fig. 4.** Two-host life cycle of a sporozoan parasite, the malarial species *Plasmodium vivax* (pathogenic in humans), illustrating (semidiagrammatically, with unlabeled structural details but with all stages drawn roughly to the same scale) the complexities often found in host-protist relationships. **A** - Stages in the human host: **a** - Repeatable cycle in liver parenchyma cells; **b** - repeatable cycle in erythrocytes of the circulating bloodstream. **B** - Stages in the female anopheline mosquito vector, which has ingested macro- and microgametocytes along with the blood meal from her human victim: **a** - Production of macro- and microgametes in mosquito digestive tract and their fusion, followed by penetration of gut wall by motile zygote (ookinete); **b** - multiplication of sporozoites in the growing oocyst, rupture of oocyst membrane releasing sporozoites into hemolymph of mosquito body cavity, with their eventual migration to and penetration of the salivary glands, thus ready for inoculation through the mosquito proboscis into a fresh human host. [Figure modified from a drawing kindly provided to the author many years ago (with permission to use at any time), by protistological colleague Keith Vickerman, Glasgow University, UK.]

gas deposits (Cushman 1948). These commercially prized products - indispensable in an industrial world - are themselves derived from the breakdown, in aeons past, of fossilized photosynthetic protists (Andersen 1998).

### Roles and functions of symbiotic forms

Because there is some confusion in the literature, I briefly offer here my own use or definitions of major terms for the principal kinds of relatively intimate partnerships of taxonomically distinct organisms, in this case, species of protists and their living hosts (other protists or, more commonly, higher eukaryotes). Symbiosis: a general overall term for such an association to any degree of closeness. Mutualism: an association of two organisms from which both the symbiont and the host derive a measure of benefit. Commensalism: an association in which only one partner, the symbiont, derives substantial benefit. Parasitism: usually a very intimate association in which the (ecto- or endo-) symbiont derives benefit at the expense of, but not necessarily fatal to, the host. If the host is also living a symbiotic existence, the phenomenon is known as hyperparasitism. Phoresis: in which one partner, the symphoriont or phoront, is carried about - and often dispersed - by the host without necessarily any (other) beneficial or destructive effect on either partner. Note that stalked protists, in particular, can also attach to substrates that are non-motile, even totally inanimate; the latter are not considered hosts, of course.

All such relationships described above may be temporary or permanent (depending on the organism/situation), obligate or facultative, and/or limited to certain stages in the life cycle of either partner. Diverse means of getting from an individual host to another, preferably a fresh one, are exhibited among parasitic protists: contamination of food or habitat, often by cysts or spores (very common), use of active (see Figs. 3, 4) or passive vectors (with or without multiplication therein), sexual intercourse, blood transfusions (and use of non-sterilized needles by drug-users), carnivory (including cannibalism), and transovarial or placental transfer.

Incidentally, symbioses may involve consortia of multiple organisms as well as often being confined to pairs, as mentioned briefly above under colony formation. Windsor (1997, 2000a) has called such an assembly a "biocartel," considering it a crucial unit in both ecology and evolution of all forms of life, a view reached independently but reminiscent of that of Margulis

(e.g. Margulis 1993, 1998; Margulis and Fester 1991).

Symbiotic species of protists, which reach a number of at least 14,000 (thus nearly 15% of all extant species) according to my counts (Corliss 2000a), occur throughout several phyla. The symbiotic membership of nine of these is practically 100%: the phyla Metamonada, Parabasala, and Sporozoa (syn. Apicomplexa) of the kingdom PROTOZOA; Opalinata, Labyrinthomorpha, and Pseudofungi of the CHROMISTA; Chytridiomycota (chytrids) and Microsporidia of the FUNGI; and Myxozoa (myxosporidians) of the ANIMALIA.

Symbiotic, especially parasitic, protists continually wreak havoc on humans' alleged control of the world's environment. Annually there are tremendous losses of farm produce, ranging from poultry and livestock to fruit orchards and grain and vegetable crops. In the latter cases, recall the massive epidemics (and their far-reaching consequences) of potato and grape blights in Ireland and France in the middle to late 19<sup>th</sup> century. Wildlife - both animal and plant - is also affected, of course. Economically, hundreds of millions of dollars are lost yearly in the U. S. A. alone through only partially controllable diseases of multicellular organisms, such as farmed birds, mammals, and plants (Levine 1973, 1985; Marquardt *et al.* 2000). Similar losses are encountered in the finfish and shellfish industries from the harmful actions of still other parasitic species (Perkins 1987, Lom and Dyková 1992, Hoffman 1999). And near-shore plants (e.g. eel grasses) and algae are often depopulated through destructive actions of fungal and fungus-like protists (e.g. chytrids and oomycetes).

Coccidian sporozoa (particularly species of the widespread genus *Eimeria*: Levine 1988, Allen and Fetterer 2002) and a number of flagellates (e.g. *Histomonas*) are among the main culprits (from the human point of view) in cases of poultry diseases. Myxosporidians, animal (cnidarian) protists often with two hosts (fish and an invertebrate: a larval chironomid "blood worm" or a bryozoon or some marine oligochaete annelid), are responsible for epidemics among marine and freshwater salmonid (and a few other) fishes around the world, with disastrous outbreaks occurring in netpen and maricultured commercial populations (Kent *et al.* 2001).

Blooms of (especially) algal protists are destructive in many aquatic ecosystems (Chrétiennot-Dinet 2001). Their occurrence when polluted lakes and coastal shores become eutrophic has long been viewed with dismay:

eutrophication usually reduces species biodiversity by the choking out of other life forms sharing the biotope (Norton *et al.* 1996, Wetzel 2001a). But their involvement in elimination of shellfish and finfish populations in the seas is compounded by their infection or contamination (via their toxins: note the “red tide” outbreaks of principally dinoflagellate species in many locations around the globe) of such hosts which, on consumption, can sometimes bring death to the non-alert human consumer (Taylor 1987a, Hallegraeff 1993).

However, by no means are all symbiotic associations involving protists of a negative nature. While the better known mutualistic and phoretic examples are at least mildly beneficial, other - often more intimate - associations are even more helpful to the host. For example, the commensalistic protozoa thriving in body cavities and digestive tracts of many an animal, from insects to mammals (including humans), may be indispensable to a normal healthy life of their hosts. Besides often supplying essential growth factors, their control of potential overgrowth by other microorganisms, notably bacteria, is a highly beneficial activity. As known for nearly three-quarters of a century (Cleveland *et al.* 1934), termites and wood-feeding roaches require a population of protozoan protists to aid in digestion of the cellulose ingested by the host. In the case of ruminants (Hungate 1975), the associated protozoa go even further than just ingesting/digesting plant tissues; they also control the deleterious (if too large in population) prokaryotes by their grazing action (Bonhomme-Florentin 1994, Hobson and Stewart 1997, Russell and Rychlik 2001). Other cases involving non-pigmented protists could be cited.

Symbiotic photosynthetic algae are widespread in organisms extending from other protists through various invertebrate groups of animals, not to mention their serving as the all-important phycobiont moiety with certain fungi to give us lichens. The associations of fungal or fungus-like protists with roots of trees and other plants (including many cultivated legumes) are indispensable to the hosts' survival. In the intimate associations of algae with corals, the protists' photosynthetic activities are of primary importance to the whole marine reef community. In all such beneficial cases described above, plus the roles of endosymbionts in organismic evolution overall, discussion of the vast topics involved would require much more space than is possible here; but see the following: Margulis (1981, 1993, 1996), Goff (1983), Jeon (1983), Lee and Corliss (1985), Taylor (1987b), Margulis and Fester (1991), Sapp (1994),

Melkonian (1996), Hackstein (1997), Ossipov *et al.* (1997), Cavalier-Smith (2000b, 2002), Corliss (2000c), Inagaki *et al.* (2000), Margulis *et al.* (2000), Windsor (2000a), Zhang *et al.* (2000).

### Protozoan parasites of human beings

Although a few algal protists may be directly or indirectly involved in causing occasional medical problems for humans (including protothecosis, a human skin lesion disease caused by the green alga *Prototheca*: see Jassby 1988), the most serious disease-causers are found among the protozoan protists. Some 70-75 species are “at home” in various parts of the human body, ranging from skin, teeth, nostrils, and eyes to favored more sheltered sites such as the digestive tract and associated structures, the circulatory system, sex organs, and brain tissue (Krylov 1993-1994; Ashford and Crewe 1998; Marquardt *et al.* 2000; Zimmer 2000). Naturally, all of such an impressive number are never found in a single person at the same time.

Some of these symbiotic protist species are much more common or more widely distributed than others; some are considerably more virulent than others; a few may ordinarily be relatively harmless commensals or even mutualistic forms; and a few others only opportunistic or occasional parasites. Nevertheless, I would guess that the high total of implicated species given above will come as an unpleasant surprise to most readers. Also, many people may not appreciate the impact of certain parasitic protozoa on the geopolitical history of the world. Consider the premature death (age 33), from malaria, of Alexander the Great, putative/poised conqueror of all known parts of Europe, Africa, and Asia in the 4<sup>th</sup> century B. C.

The periodically issued “death tables” of the World Health Organization (e.g. see WHO Report 2000) continue to show several protozoa high on the list as causative agents of some of the most serious human diseases in the world. Following the AIDS virus (causing nearly 2,700,000 deaths in 1999; and 25.3 million people were HIV-positive, with 4.7 million of the infections new, in the year 2000: UNAIDS/WHO 2000) and the bacterium responsible for tuberculosis (*ca* 1,700,000 fatalities in 1999), comes malaria (Fig. 4), caused by species of the sporozoan genus *Plasmodium* (Garnham 1966, an enduring classic in the field; Sherman 1998): well over one million deaths are recorded by WHO for the year 1999. Some 300-500 million people are affected by malarial world-wide, weakening many of those not killed

to the extent that they are susceptible to often fatal infections by various other parasites. Mainly because the blood-dwelling plasmodia involved (including those of the most deadly species, *P. falciparum*) have become resistant to drugs currently available and thus far no effective vaccine exists, the toll from malaria is alarmingly on the rise (Honigsbaum 2002). In fact, the spread of drug resistance in recent years by many pathogenic microorganisms is a looming crisis for the entire human race (Hunter *et al.* 1995).

For our struggle against the highly pathogenic *P. falciparum*, Hoffman (2000, 2001) is optimistic that we may finally be approaching an era in which we can hope to develop effective malaria vaccines. We have been aided greatly by our growing knowledge of the sequences of the genomes of *Homo sapiens* (the host), *P. falciparum* (the parasite), and *Anopheles gambiae* (the vector), by our industrious application of modern techniques of proteomics, molecular immunology, and vaccinology, and, finally, by our ability today to integrate the millions of resulting pieces of computer-generated data through the use of bioinformatics.

Hosts overall do not “stand still” while parasites evolve within their bodies: they, too, are constantly evolving, often developing counter-measures to offset gains made by their sometimes long-ago invaders (Kawecki 1998). At the same time, the members of the consortium or biocartel can co-evolve - or become extinct (Windsor 1996, 2000b), thus upsetting the balance of nature - as a unit, once again demonstrating the complexity of associations involving symbiotic protists.

Other tropical diseases of humans, for example, those caused by species of the flagellated protozoan genera *Trypanosoma* (see Fig. 3) and *Leishmania* (African sleeping sickness, Chagas’ disease, and visceral and cutaneous leishmaniases: see Hoare 1972; Lumsden and Evans 1976, 1979; Warren 1993), are also responsible for tens of thousands of deaths annually. Like malaria, insect vectors (tsetse flies, triatomid bugs, and sandflies here, rather than mosquitoes) transmit the parasitic organisms, both blood and tissue invaders. Effective vaccines, once again, are currently unavailable and other approaches to reducing human pain and fatalities from such afflictions are constantly being sought (Dumas *et al.* 1999). In addition, these trypanosomatids and other major protozoan parasites (including *Sarcocystis*, *Toxoplasma*, and *Trichomonas*) are participants in zoonoses (Hoare 1962), that is, diseases common to both humans and other mammals with the latter, often with

less debilitating infections, serving as reservoir hosts in the wild.

What WHO identifies, loosely, as “diarrheal diseases” cause over two million deaths per annum. One of the causal agents heavily involved is the species *Entamoeba histolytica*, along with other amoebae, and outbreaks are not limited to tropical areas of the world. Protists such as *Blastocystis*, *Balantidium*, *Dientamoeba*, *Giardia*, and *Trichomonas* could be listed here, too. With respect to deaths from the pandemic AIDS (see above), one should recall that compromising of the host immune system by the presence of HIV makes the body more susceptible to all sorts of infections by various *other* organisms, including bacteria, the protist-like fungus *Pneumocystis*, and several opportunistic protozoan parasites (including species of amoebae, flagellates, and sporozoa). Thus, the ultimate death of some persons with AIDS may have been caused by the activities of such *additional* pathogens, the discovered presence of which has been on the increase ever since the early recognition of the AIDS virus some 20 years ago (Kaplan *et al.* 2000).

## CONCLUDING REMARKS

The biodiversity and biocomplexity of presently recognized species of the protists appear to be much greater than is generally appreciated by many biologists, let alone the laity. Their intimate and numerous interactions with other organisms, including humans, make imperative our becoming better informed about the multiple roles of these microorganisms in nature. Already many times more speciose than the bacteria and viruses combined and arguably equally ubiquitous, their effects—both beneficial and deleterious - on our biosphere cry out for considerable further investigation.

Thus, in today’s timely quest for a sustainable world (Reaka-Kulda *et al.* 1997, Raven and Williams 2000), it behooves us to learn more about the indispensable *eukaryotic* microorganisms that have been, are, and will continue to be an integral part of life, as we should mostly like to conserve it (Wilson and Perlman 2000), on our presently endangered planet.

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## *Cyclospora cayetanensis* and Intestinal Parasitic Profile in Stool Samples in Lagos, Nigeria

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**Summary.** Diarrhoea remains a leading cause of morbidity and mortality in the developing world and accounting for over 50 million deaths globally. The involvement of *Cyclospora cayetanensis* in diarrhoea cases globally has been documented, though with little from the Africa continent. This study was thus carried out to investigate the possible existence of this emerging pathogen and the parasitic profile in stool samples in the city of Lagos, Nigeria. A total of 1109 stool samples (216 of which came from diarrhoeal patients) were screened microscopically for intestinal parasites. Parasites were detected in just 16% (177). *Cyclospora cayetanensis* was detected from 11 stool samples: 10 diarrhoeal stools and one non-diarrhoeic stool. All stool samples positive for this emerging pathogen, were from patients aged 22 to 45½ years, seven of which were seropositive for HIV ( $p > 0.05$ ). Result thus revealed for the first time in this state and country, the existence of *Cyclospora cayetanensis* and its possible involvement in diarrhoea cases. It also identified *Cryptosporidium parvum* as the most prevalent intestinal parasite in the state, a strong departure from previous reports. There is the need however for a more detailed study, in order to determine the exact extent of *C. cayetanensis* involvement in diarrhoea cases and the effect of seasons and cultural feeding practices on its epidemiology for effective management of diarrhoeal cases.

**Key words:** *Cyclospora cayetanensis*, diarrhoea, intestinal parasite, Lagos, Nigeria, stool.

### INTRODUCTION

Diarrhoea remains one of the most important health problems globally and a leading cause of morbidity and mortality in children, especially in developing countries (WHO 1998). It accounts for over 50 million deaths (in all ages) worldwide and is ranked 3rd among diseases responsible for human mortality globally (WHO 1998).

This disease condition remains a major problem in the developing countries due mainly to poverty, characterized by the absence of potable drinking water, proper sanitary habits, absence of good faecal disposal system, poor hygienic practices by the impoverished citizens and over crowding (WHO 1998).

Agents associated with diarrhoea cut across the major types of microorganisms: bacteria, viruses and protozoa. Bacterial agents mostly associated with diarrhoea are species of *Salmonella*, *Shigella*, *Campylobacter*, *Escherichia coli*, *Aeromonas*, *Plesiomonas* etc., while viral agents like Norwalk, and adenoviruses have been predominately associated with

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diarrhoea in infants and children (Alabi *et al.* 1998). The association of protozoa pathogens with diarrhoea has been on the increase (with the discovery of the *Cyclospora* in the mid and late 1990s), following the advent of the human immunodeficiency virus (HIV) infection and the acquired immunodeficiency syndrome (AIDS) pandemic. Traditional diarrhoeal protozoa agents are *Giardia lamblia*, *Entamoeba histolytica* and *Balantidium coli* and lately, coccidia agents like *Isospora belli*, *Cryptosporidium parvum*, microsporidia and the newest one; *Cyclospora cayetanensis*. (Clarke and McIntyre 1996a, Marshall *et al.* 1997, Terra *et al.* 1998, Allison *et al.* 1999, Fryauff *et al.* 1999, Wilson 1999).

The involvement of protozoan agents in humans can be traced back to the 19th century and these protozoa group constitute the highest group of parasites known to be associated with diarrhoea condition. Originally, the coccidia parasites are known to be pathogenic mainly to some species among the lower animals, insects, birds and non-human primates (Beneson 1995, Marshall *et al.* 1997, Fryauff *et al.* 1999).

Today, a newer coccidian *C. cayetanensis* has now been added to the list of protozoa agents that can induce diarrhoea. Prior to 1979, when the first human case of cyclosporiasis was reported (Ashford 1979), only four genera of the coccidia were known to infect humans and these are *Cryptosporidium*, *Isospora*, *Toxoplasma* and *Sarcocystis* (Clarke and McIntyre 1996b). *C. cayetanensis* is capable of causing prolonged gastrointestinal disorder (GIT). This is characterized by persistent and intermittent watery diarrhoea accompanied by weight loss and other symptoms in both immunocompetent and immunocompromised individuals irrespective of sex and age globally (Logar *et al.* 1997, Lepes 1998, Nassef *et al.* 1998, Terra *et al.* 1998, Fryauff *et al.* 1999, Mead *et al.* 1999). Prior to 1994 when its current name was given by Ortega *et al.* (1994), this pathogen was known by different names; *Cyanobacterium* (blue-green algae)-like bodies; *Cryptosporidium*-like bodies, coccidian-like bodies (CLBs) or even a species of *Isospora* or *Eimeria* genera (Ashford 1979; Long *et al.* 1990, 1991; CDCP 1991a,b; McIntyre and Lyons 1992, Ortega *et al.* 1993).

Route for its transmission is still not very clear, but the oral-faecal route remains the most implicated, while its association with GIT infection or diarrhoea, remains proven with the continuous increase in the number of reported cases and outbreaks of cyclosporiasis globally (Jelinek *et al.* 1997, Marshall *et al.* 1997, Steiner *et*

*al.* 1997, CDCP 1998, Ortega *et al.* 1998, Sterling and Ortega 1999).

In the developed countries like the USA and the UK, where a lot of documentation exists, there is a fair idea of the profile of parasitic pathogens. In USA, it is now a common practice for stool samples submitted to the laboratory, to be first screened for the presence of the commonest pathogenic parasites and when these are absent, the screening for newer agents like *C. cayetanensis* is done (Garcia-Lopez *et al.* 2000).

Three countries have so far been documented to report cases of *C. cayetanensis* infection from the continent of Africa. These are Morocco, South Africa and Egypt (Markus and Frean 1993, Nassef *et al.* 1998, Ortega *et al.* 1998). I am unaware of any reported case in Nigeria; neither has there been any attempt to investigate the possible presence of this emerging diarrhoeal pathogen in Lagos State. Reason can be attributed to the fact, as revealed from an earlier study (Alakpa *et al.* 1999), that physicians and health workers in the state and country were completely unaware of the existence of this pathogen. Also, in Nigeria, numerous studies into the prevalence of parasitic organisms have been conducted in recent years; however, there exists little or no published study specifically aimed at determining the parasitic profile of stool samples submitted to some laboratories in Lagos.

This study was thus carried out to determine the parasitic profile of stool samples in Lagos, with the main focus of investigating for the existence of "newer" parasitic agent(s) especially *C. cayetanensis* and its involvement in diarrhoeal cases.

## MATERIALS AND METHODS

This study was conducted in Lagos metropolis, the most commercialized and cosmopolitan city in Nigeria and situated in the southern part of the country with over 5million individuals. The Yaba Central Laboratory; Nigerian Army Base Hospital, Yaba; General Hospital, Lagos; The Lagos University Teaching Hospital: Pediatric Unit and the Microbiology laboratory; Jimsan hospital, Ijora-Badiya and two private laboratories, constitute centers for sample collection.

This was a cross sectional laboratory based study commenced in March 1998 till April 2000 with the collection of all stool specimens submitted to the Microbiology and Parasitology department of the Health Institutions/laboratories mentioned above. Of the 1109 stool samples collected, only 216 (19.5%) were from confirmed diarrhoea cases.

Stool samples were collected from 55 normal healthy individuals with no prior illness (gastrointestinal/diarrhoeal), 3 weeks to the time the stool samples were collected, to serve as negative control, while

positive *Cyclospora cayetanensis* organism sent by Dr. Stuart Clarke of the Scottish Reference Laboratory, Glasgow, UK, served as positive control.

**Collection of samples**

All stool samples submitted to the Microbiology and Parasitology departments of the above mentioned health institutions within the period of the study were collected in waterproof screwed capped plastic containers and transferred to the laboratory for processing. Bio-data of the patients, whose stools were collected, were obtained from sample request forms and some by means of a questionnaire. Data like the age, sex and reason for visiting the hospital/stool being examined were obtained.

**Stool processing**

Stool samples were processed immediately for parasitology and bacteriology. Parasitology: Samples were first concentrated employing the formal-saline sedimentation method. Smears were stained by the modified Kinyoun carbol-fuchsin staining procedure (Visvesvara *et al.* 1997) and examined at x 400 magnification using a Nikon light microscope equipped with an eye-piece micrometer calibrated using a stage micrometer, to determine the size of the oocysts.

Loop size of stool samples were made into suspension with saline, a portion smeared for Gram staining, and other seeded onto 3 bacteriological agar media: MacConkey agar; Deoxycholate Citrate agar (DCA) and Thiosulphate Citrate Bile Salt agar (TCBS), so as to determine the presence of any pathogenic bacteria agent. Inoculated plates were incubated at 37°C for 24 h. Colonies morphology determined, followed by Gram staining and sugar fermentation tests. Culture of pure isolated on Kligler Iron agar was also done.

Identification of *C. cayetanensis* oocysts was based on the size of the oocysts (8-10µm in diameter); the shape being oval/round with well-defined wall, with consistent staining colour variably from pink/red to colourless and comparing it with the positive control. Confirmation of positive stool samples was done at the Scottish Reference Laboratory in Glasgow, UK. Stools positive for *Cyclospora* oocysts, were crosschecked with results from bacteriology initial examinations.

Results were analyzed with the Epi-Info 6.4 version and “STAT” software. The chi-square and student-t tests used to determine the statistical significance.

**RESULTS**

**General intestinal profile**

From the 1109 total stool samples analyzed, parasite ova/cysts and oocysts were detected in 177 (16.0%). Frequency distribution showed that 111 (62.7%) of the 177 had protozoa cysts or oocysts, while 66 (37.3%) had helminthic parasites. *Cryptosporidium parvum* was the most detected pathogenic parasite (Table 1).

In terms of general prevalence, of the 1109 stools screened *C. parvum* was the most prevalent pathogenic intestinal parasite in Lagos (3.1%). This was closely followed by *Ascaris* sp. (2.6%), *Giardia* sp. (2.5%), *C. cayetanensis* (0.9%), *E. histolytica* (0.3%) and Hookworm (0.1%). Non-pathogenic *E. coli* had a prevalence of (3.1%) and *T. trichiura* (2.9%) (Table 2).

Few parasitic agents were detected in children less than 6 years, however among the age group 6-10years, the only pathogenic agent found was *G. lamblia* and non-pathogenic *E. coli*. In the age group 11-20 years, *A. lumbricoides*, *E. coli* and *T. trichiura* were detected. Majority of those aged 21 and above had *Cryptosporidium* oocysts in their stools. There was however no significant association between age and the detection of parasitic agents ( $p > 0.05$ ). Females were found to have more parasitic organisms in their stools than the males; this was not statistically significant ( $p > 0.05$ ). A close observation revealed that most of the parasites were from diarrhoea patients with the exception of *A. lumbricoides*. Results also showed that 41 of the 177 (23.2%) had multiple infections, indicating the possibility that mono-infection could be predominating in our environment. There was a significant association between nature of infection with age group and sex,

**Table 1.** Prevalence of intestinal parasites, based on diarrhoea status. AS - *Ascaris lumbricoides*, CRYP - *Cryptosporidium* sp., CYC - *Cyclospora cayetanensis*, EC - *Entamoeba coli*, EH - *Entamoeba histolytica*, GL - *Giardia lamblia*, HK - Hookworm, ST - *Strongyloides stercoralis*, TT - *Trichuris trichiura*. P < 0.05

Diarrhoeal status:	Organisms								
	AS	CRYP	CYC	EC	EH	GL	HK	ST	TT
Positive (n=100)	7	29	10	22	2	19	0	1	9
%	7.0	29.0	10.0	22.0	2.0	19.0	0.0	1.0	9.0
Negative (n=77)	22	5	1	12	2	9	1	3	23
%	28.6	6.5	1.3	15.6	2.6	11.7	1.3	3.9	29.9

**Table 2.** Frequency distribution of parasitic agents detected in stool samples in Lagos

Organisms	No of stools (%)	Prevalence (%) (n=1109)
<i>Cryptosporidium parvum</i>	34 (19.2)	3.1
<i>Entamoeba coli</i>	34 (19.2)	3.1
<i>Trichuris trichiura</i>	32 (18.1)	2.9
<i>Ascaris lumbricoides</i>	29 (16.4)	2.6
<i>Giardia lamblia</i>	28 (15.8)	2.5
<i>Cyclospora cayetanensis</i>	11 (6.2)	0.9
<i>Entamoeba histolytica</i>	4 (2.3)	0.3
<i>Strongyloides stercoralis</i>	4 (2.3)	0.3
Hookworm	1 (0.6)	0.1
Total	177 (100.0)	

( $p < 0.05$ ), but not with diarrhoeal status ( $p > 0.05$ ) (Tables 3, 4).

Based on diarrhoeal status; *Cryptosporidium* sp. was also the most prevalent pathogenic organism in diarrhoea patients in Lagos, followed by *Giardia* sp. In comparison, the prevalence of *Cryptosporidium*, *Giardia* and *Cyclospora* in diarrhoeal cases were 29.0%, 19.0%, and 10.0% respectively, while in the non-diarrhoeal cases, it was 6.5%, 11.7% and 1.3% ( $p < 0.05$ ) (Table 1).

### Diarrhoea stools and *C. cayetanensis*

Results from this cross-sectional study revealed that of the 216 watery-diarrhoeal-stools in the study, only 10 (4.62%) were positive. Majority (73.2%) of the diarrhoeic stool samples came from individuals aged between 21 to 50 years and 20.4% from those age 0-20years. One hundred and thirteen (52.3%) of the stools were from men and the remaining 44.4% (96) were from females. All stools were watery in nature. Eighty-three (38.4%) were from patients whose stools were submitted for investigation following GIT complaints, 39.4% (85) from those that came for HIV test and the remaining 45 (20.8%) from routinely collected stool samples (Table 5).

### Patients with stool positive for *Cyclospora*

All stool samples positive (eleven in total) for *Cyclospora* were from adults (age 22 to 45½years), however, 10 of these numbers were from diarrhoeic stools. Of these 10 three were males and the rest were females. All had histories of greater than 4 weeks of watery diarrhoea. Two of the 10 cases positive for *Cyclospora* were co-infected with *Cryptosporidium* sp. and both were also HIV positive patients with prolonged diarrhoea.

Generally, 7 of the 10 stools positive for *Cyclospora* oocysts, came from HIV seropositive diarrhoea patients

**Table 3.** Distribution of cysts/oocysts or ova in stool samples by patients' age, sex and diarrhoeal status. AS - *Ascaris lumbricoides*, CRYP - *Cryptosporidium parvum*, CYC - *Cyclospora cayetanensis*, EC - *Entamoeba coli*, EH - *Entamoeba histolytica*, GL - *Giardia lamblia*, HK - Hookworm, ST - *Strongyloides stercoralis*, TT - *Trichuris trichiura*

Organisms	AS	CRYP	CYC	EC	EH	GL	HK	ST	TT
Age group:									
0-5years	0	2	0	0	1	0	0	0	0
6-10 years	0	0	0	26	0	25	0	0	0
11-20years	18	0	0	21	1	0	0	0	11
21-50years	7	32	11	4	0	3	1	4	21
Not stated	4	0	0	2	2	0	0	0	0
Sex:									
Female	16	12	8	19	2	13	1	1	3
Male	13	20	3	15	1	9	0	3	12
Not stated	0	2	0	0	1	6	0	0	0
Diarrhoeal status:									
Positive	7	29	10	22	2	19	0	1	9
Negative	22	5	1	12	2	9	1	3	23

**Table 4.** Nature of infection by age, sex and diarrhoeal status

	Single	Multiple	P-value
<b>Age group:</b>			
0-5years	3	0	p < 0.05
6-10 years	1	25	
11-20years	18	10	
21-50years	67	5	
Not stated	6	1	
<b>Sex:</b>			
Female	44	24	p < 0.05
Male	48	11	
Not stated	3	6	
<b>Diarrhoeal status:</b>			
Positive	48	25	p > 0.05
Negative	47	16	

**Table 5.** Frequency distribution of diarrhoeal patients' stools

	Frequency (No)	%	<i>Cyclospora</i> oocysts positive No
<b>Age group:</b>			
Infants (0-5 years)	18	8.3	0
Children (6-10 years)	6	2.8	0
Teenager (11-20 years)	20	9.3	0
Adult (21-50 years)	158	73.2	10
Elderly (>51 years)	5	2.3	0
Not stated	9	4.2	0
<b>Sex:</b>			
Female	96	44.4	7
Male	113	52.3	3
Not stated	7	3.2	0
<b><i>Cyclospora</i>:</b>			
Detected	10	4.6	
Not detected	206	95.4	
<b>Reasons:</b>			
Routine	45	20.8	0
GIT	83	38.4	3
HIV testing	85	39.4	7
Check-up	0	0	0
Control	0	0	0
Others	3	1.4	0
Total	216	100	10

(Table 6). Though there was a statistically significant association between cyclosporiasis and diarrhoea (p < 0.05), there was no significant association between

**Table 6.** Nature of infection by age, sex and diarrhoeal status. N. S. - not stated, HIV - human immunodeficiency virus positive, GIT - gastrointestinal tract disorder, Cryp - *Cryptosporidium* sp.

Patients bio-data					
Age (years)	Sex	Diarrhoeal status	Nature of stool	Reason	Other organism
45.½	M	positive	watery	HIV	N. S.
25	F	positive	watery	HIV	<i>E. coli</i>
43	F	positive	watery	HIV	N. S.
23	F	positive	watery	HIV	<i>Giardia</i>
N. S.	F	negative	soft	GIT	<i>Ascaris</i>
32	F	positive	watery	GIT	N. S.
41	M	positive	watery	HIV	N. S.
32	F	positive	watery	GIT	<i>E. coli</i>
26	F	positive	watery	GIT	N. S.
45	M	positive	watery	HIV	Cryp
22	F	positive	watery	HIV	Cryp

the detection of *Cyclospora* oocysts in stools and age nor sex (p > 0.05). The study also revealed a statistical association between diarrhoea and consistency of stool, and reason for visiting the hospital (p < 0.05). The prevalence of *Cyclospora* and *Cryptosporidium* were found to be higher in stool from diarrhoeal patients, than in non-diarrhoeal patients (Table 2).

## DISCUSSION

The World Health Organization (WHO) reported in 1998 that 33% of global deaths are as a result of infectious and parasitic diseases, while the effect of mortality and morbidity are as a result of some parasitic infections (WHO 1998). The place of intestinal parasites in the over all health of man is also very important.

In terms of pathogenic importance, parasites such as *Cryptosporidium*, *Cyclospora*, *E. histolytica* and *Giardia*, have been shown to be responsible for severe diarrhoeal episodes especially in immunosuppressed individuals globally. Depending on the degree of infection especially in children, *Ascaris* and *Trichuris* have been known to cause complications. While *Ascaris* may result in pneumonitis characterized by cough, dyspnoea, fever, eosinophilia, intestinal obstruction, malnutrition and malabsorption following heavy infection. *Trichuris trichiura*, causes inflammatory eosinophilic reactions in the caecum or rectum, which causes chronic diarrhoea and sometimes rectal prolapse (Ihezuh 1998, Shanson 1999).

The results from this study have shown that *Cryptosporidium* is the most prevalent intestinal pathogenic parasite in Lagos, especially among diarrhoeal cases. While this organism was found mostly in adults, *Giardia* was found mostly among those aged 6-10 years. This result is similar to those reported by Marshall *et al.* (1997), Gilbert *et al.* (1998), and Okono (1999). The steady increase in the prevalence of *Cryptosporidium* in Nigeria may be attributed to increase in awareness about this pathogen among clinicians and laboratory scientists. Another factor could be the pandemic HIV/AIDS disease conditions, which is believed to have otherwise converted asymptomatic parasite-induced infection into life threatening diseases (Oyerinde 1999).

One of the "newer" emerging diarrhoeal pathogen detected in this study was *Cyclospora cayetanensis*, with a general prevalence of 0.9% (Table 2). Aderounmu (1999) reported *E. histolytica* as the most prevalent protozoan pathogen in Lagos, as against *Cryptosporidium*, as observed from this study. This difference may be attributed to the increase in awareness of *Cryptosporidium*, since this study was more extensive and distributed among more laboratories than that of Aderounmu.

In a study conducted in West Java - Indonesia, *C. cayetanensis* was found to be the dominant pathogenic intestinal parasite in that area, with 11.5% (29) of the 253 cases of GIT illness and diarrhoea being positive for the pathogen (Fryauff *et al.* 1999). From another study conducted, this time in Egypt where the stool samples of 130 immunocompetent diarrhoeal patients (80 children and 50 adults) were screened for *cayetanensis*, 9% (7) of the 80 children and 10% (5) of the adults were found to be positive, thus giving an incidence of 9.2% (11/130) (Nassef *et al.* 1998).

Results of this study show for the first time, the detection of *C. cayetanensis* oocysts in diarrhoeal stool in Lagos State. It has also shown the association between this pathogen and diarrhoea. Several studies have documented the fact that *C. cayetanensis* is a diarrhoea causing agent (Ortega *et al.* 1993, 1994; Clarke and McIntyre 1996a,b; Nassef *et al.* 1998; Fryauff *et al.* 1999). So far only very few countries in Africa have been documented to report case(s) of cyclosporiasis: South Africa (Markus and Frean 1993), Morocco (Ortega *et al.* 1998) and Egypt (Nassef *et al.* 1998). The low rate or report from Africa can well be attributed to lack of awareness, inadequate technology and limited studies. It is my belief, that the results of this study will stimulate

interest among medical professionals in this state, country and sub-region in cyclosporiasis. A much more detailed and specific hospital base study will however, be necessary to provide more information about this agent of diarrhoea in this country.

In summary, these results thus provide an important finding with the documentation for the first time, the detection and presence of *C. cayetanensis* in stool samples in the state and country. Also, it showed that *Cryptosporidium parvum* is the most prevalent intestinal parasite in the state. We do believe, this findings will generate some scientific interest among clinicians and scientist, so that the much needed more studies that will focus more on the epidemiology of this emerging pathogen, especially in relation to seasonal affect and its distribution will be investigated. We also therefore recommended that, there should be more intensive education to health practitioners about this emerging diarrhoeal pathogens, since *C. cayetanensis* can be easily mistaken for *Cryptosporidium* sp. by an inexperienced microbiologist as they both share some similar staining characteristics.

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## An Ultrastructural Study of *Nosema locustae* Canning (Microsporidia) from Three Species of Acrididae (Orthoptera)

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**Summary.** *Nosema locustae* is a pathogen of orthopterans with an unusually wide host range. It is the only microsporidian that has been developed as a microbial control agent. In spite of its practical importance the ultrastructure of *N. locustae* life stages other than spores, has not been studied. The insects used in this study, all in the family Acrididae, were the Migratory locust, *Locusta migratoria migratorioides* (Oedipodinae), the South American locust, *Schistocerca cancellata* (Cyrtacanthacridinae), and the grasshopper *Dichroplus schulzi* (Melanoplinae). All insects were reared routinely in the laboratory. The spores of *N. locustae* used for the experimental peroral inoculations were all of North American origin. Fat body cells were the predominant site of parasite development, though infection of tracheal epithelium cells and haemocytes also occurred. Ultrastructure of meronts, sporonts, sporoblasts and spores is described. The fine morphology of *N. locustae* stages is typical for microsporidians of the genus *Nosema*. Nuclei were always in diplokaryotic arrangement. Transition from meront to sporont was characterized by striking changes in parasite ultrastructure: appearance of electron dense granules (50-100 nm in diameter, presumable RNP complexes) in the nucleoplasm, and stacks of ER cisternae and prominent vacuoles in cytoplasm. The beginning of sporogony was marked by an increase in size of parasite cells due to extensive vacuolization. Tubule-like structures appeared in the host cell cytoplasm during parasite sporogony. Elongated conglomerates of electron dense material were scattered in the host cell and eventually ornamented the outersurface of the parasite membrane, forming an electron dense layer around sporonts. Spores were diplokaryotic, measured  $4.95 \pm 0.07 \times 2.65 \pm 0.04 \mu\text{m}$  (mean  $\pm$  SE, n=24) on fresh smears and  $3.49 \pm 0.18 \times 1.73 \pm 0.04 \mu\text{m}$  (n = 10) on ultrathin sections, and had electron-dense cytoplasm in which all internal structures typical of microsporidian spores were recognizable. The polaroplast was lamellar, the endospore was 200-300 nm thick, and the exospore was 40-50 nm. The polar filament was isofilar, arranged in 17-18 coils. Our study did not reveal any difference in the morphology of *N. locustae* while developing in the three different hosts.

**Key words:** *Dichroplus schulzi*, intracellular parasitism, *Locusta migratoria*, locusts, *Nosema*, *Schistocerca cancellata*, ultrastructure.

### INTRODUCTION

*Nosema locustae* is a pathogen of orthopterans that was developed as a microbial control agent of grasshoppers (Henry and Oma 1981, Johnson 1997,

Lockwood *et al.* 1999). Its development was in part possible because unlike most other microsporidia, *N. locustae* has an unusually broad host range. Natural or induced susceptibility to *N. locustae* has been recorded for as many as 102 species of Orthoptera (Brooks 1988, Habtewold *et al.* 1995, Lange and de Wysiecki 1996). The first findings of the species, albeit without naming it, belong to Goodwin (1949), who observed the invasion of the fat body of *Locusta migratoria* R. & F. during his studies on pigment metabolism, and to Steinhaus (1951) who found a new *Nosema* in fat bodies of three species of grasshoppers of the genus

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*Melanoplus* Stol. The formal description and naming of the species was done by Canning (1953, 1962) who studied the host cycle as it was seen in the light microscope. She observed mostly diplokaryotic proliferative and sporogonic stages but also reported early uninucleate bodies. She placed the new species within genus *Nosema* because "the sporont gives rise to a single spore". Comparative studies of nucleotide sequences of 16S rDNA (Baker *et al.* 1994) and RNA polymerase gene (Cheney *et al.* 2001) placed *N. locustae* in a separate branch on phylogenetic trees, apart from the type species, *N. bombycis*, and other species from lepidopteran hosts considered as "true Nosemas". Surprisingly for an organism that reached commercial development, very little is known about the ultrastructure of *N. locustae*. In what was the first detailed transmission electron microscopy study of a microsporidian spore, Huger (1960) provided the only available ultrastructural information on *N. locustae*, but developmental stages were not studied. Subsequently, some authors (Streett and Henry 1993, Cheney *et al.* 2001) mentioned that ultrastructural examinations of *Nosema* species not closely related to "true Nosemas from Lepidoptera" would be of value for a better understanding of phylogenetic and taxonomic relationships. In addition, an improved knowledge of the morphological characters of *N. locustae* at the ultrastructural level would facilitate its distinction from other known and yet undiscovered *Nosema* species of grasshoppers, particularly after introductions in field populations for control purposes. In this paper we present the results of an ultrastructural examination of *N. locustae* as revealed from infections induced in three species of grasshoppers.

## MATERIALS AND METHODS

The insects used in this study, all in the family Acrididae, were the Migratory locust, *Locusta migratoria migratorioides* R. & F. (Oedipodinae), the South American locust, *Schistocerca cancellata* (Serville) (Cyrtacanthacridinae), and the grasshopper *Dichroplus schulzi* Bruner (Melanoplinae). In addition to the availability of experimental insects from the established, healthy laboratory colonies, we chose these particular species for our study because *L. m. migratorioides* is the host type species for *N. locustae*, *D. schulzi* belongs to a subfamily, the melanoplinae, which is known for its members exhibiting high susceptibility to *N. locustae* (Henry 1969, Henry *et al.* 1973, Bomar *et al.* 1993, Lange and de Wysiecki 1996) and *S. cancellata* is not only a major agricultural pest in Argentina (Hunter and Cosenzo 1990), but is also closely related to the desert locust, *Schistocerca gregaria* (Forskål), a major pest in northern Africa and the Middle East (Steedman 1990). The colony of *L. m.*

*migratorioides* was maintained under controlled conditions (30°C, 16L : 8D photoperiod, 40-45% RH; diet of wheat seedlings and reed grass) in the insectarium at the Laboratory of Microbiological Control of the All-Russia Institute for Plant Protection, St. Petersburg. The colonies of *S. cancellata* and *D. schulzi* were maintained according to general procedures described by Henry (1985), in rearing rooms (30°C, 14L : 10D, 40% RH; diet of wheat seedlings and bran, lettuce, cabbage) at the Center for Parasitological Studies (CEPAVE) of La Plata National University.

The spores of *N. locustae* used for the experimental inoculations were all of North American origin. Those used for the inoculation of *L. m. migratorioides* were kindly provided by J. E. Henry and D. A. Streett from the United States Department of Agriculture - Agricultural Research Service, Rangeland Insect Laboratory, Bozeman, Montana. The spores employed for the inoculations of *S. cancellata* and *D. schulzi* were isolated and purified by the homogenization procedure (Henry and Oma 1974) from infected grasshoppers [the melanoplinae *Dichroplus pratensis* Bruner, *D. elongatus* Giglio-Tos, and *Baeacris punctulatus* (Thunberg)] collected in fields of central Argentina, after *N. locustae* became established in grasshopper communities following its introduction from North America between 1978-1982 (Lange and de Wysiecki 1996, Lange 1999, Lange and Cigliano 1999). The spore concentrates used in the introductions were also produced by J. E. Henry's group at Montana's Rangeland Insect Laboratory.

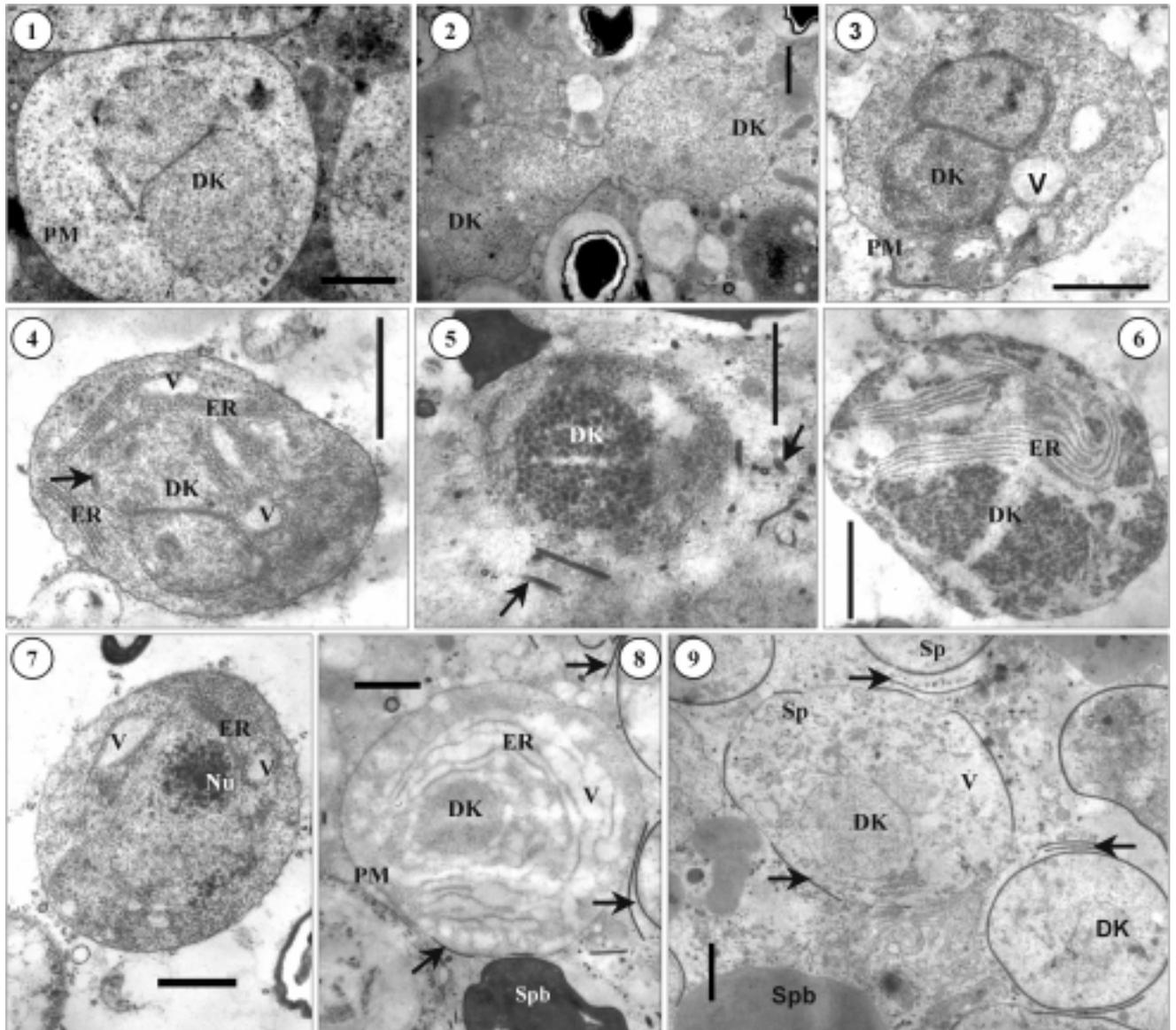
The *per os* standard inoculation protocol utilized to challenge grasshoppers with spore suspensions of microsporidia (Habtewold *et al.* 1995, Lange *et al.* 2000) was used to successfully induce infections in individuals of the three host species employed. Third-instar nymphs ingested  $10^5$  spores each.

For transmission electron microscopy, following ventral dissection of infected insects small samples of tissues were fixed for 1h at 4°C in 2.5% (v/v) glutaraldehyde buffered with 0.1 M cacodylate buffer (pH 7.4), postfixed in 1% aqueous  $\text{OsO}_4$  (w/v), and *en bloc* stained with 1% uranyl acetate. Dehydration was through an ascending acetone series after which samples were embedded in Epon-Araldite or Spurr's resins. Thick sections (1.0  $\mu\text{m}$ ) stained with methylene blue were observed under the light microscope, to determine the infected sites. Ultrathin sections were poststained with methanolic uranyl acetate followed by lead citrate, and were observed and photographed at an accelerating voltage of 70 kV with a Hitachi H-300 electron microscope.

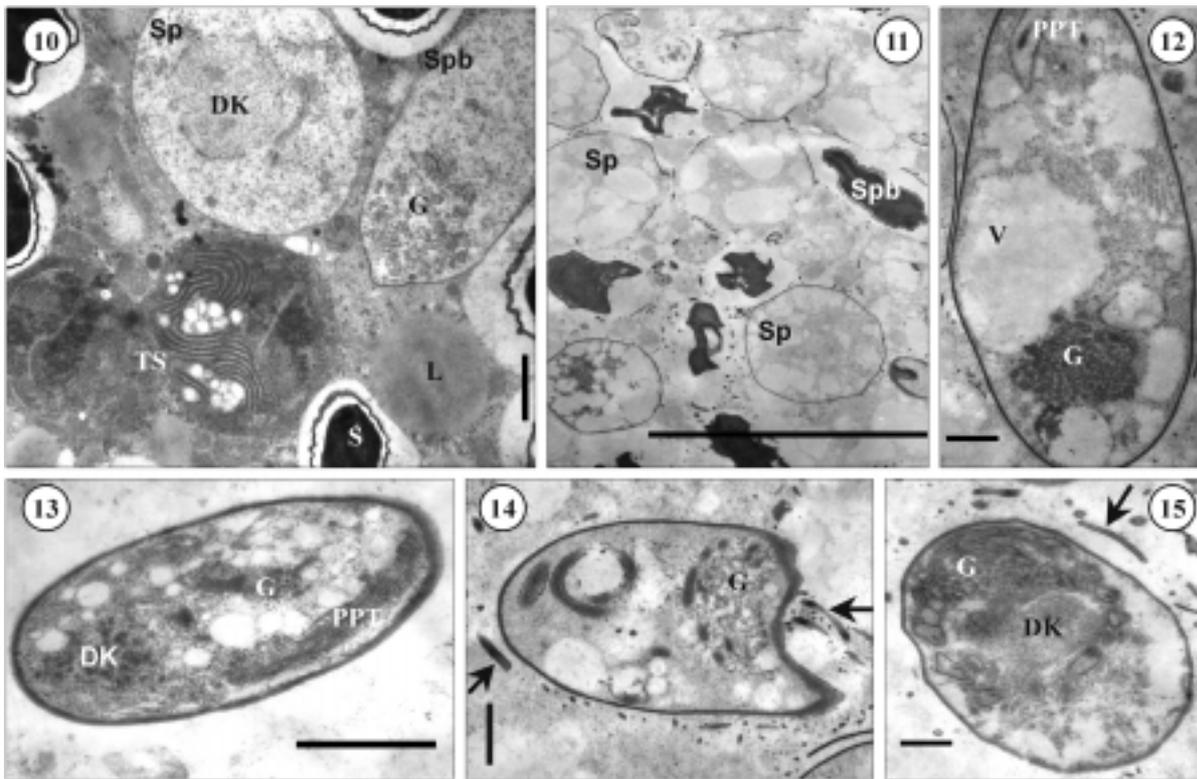
The contribution follows the terminology and conceptual basis proposed for the microsporidia by Sprague *et al.* (1992).

## RESULTS

We observed the same kind of stages and the same pattern of development of the pathogen in all three host species studied. The cytoplasm of the insect's adipose tissue cells was the overwhelmingly predominant site of development of *N. locustae*, although infection was also, albeit infrequently, present in cells of tracheal epithelium. The accumulation of parasites in the cytoplasm caused enlargement and fragility of host cells. As a result,



**Figs 1-9.** Fine morphology of proliferate stages of *Nosema locustae* development. **1** - meronts in a host adipocyte; **2** - binary fission of a tetranucleate meront; **3** - early meront/sporont transitional stage. Note appearance of large vacuoles outside nuclear envelope, electron dense globules inside the nucleus and thickening of the plasma membrane; **4** - transitional stage. Rough endoplasmic reticulum is arranged in regular multilayer patterns. Note vacuolization of the cytoplasm in the vicinity of ER stacks; **5** - transitional stage. Numerous electron dense particles are scattered throughout the nucleoplasm. Arrows indicate electron dense tubular structures inside host cytoplasm; **6** - typical characters of the transitional stage: diplokaryon (DK) with intranuclear particles, whorls of endoplasmic reticulum inside the cytoplasm. The electron dense envelope has not started to form yet; **7** - the same stage as on Fig. 6. Vacuoles appear as the expanded lumens of endoplasmic reticulum; electron dense granules are accumulated in nucleolus-like structure; **8** - early sporont. Electron dense layer is deposited on the external surface of its plasma membrane and on adjacent sporonts; similar elongated conglomerates of electron dense material are scattered inside the host cell (arrows). Expanded cisternae of rough endoplasmic reticulum (vacuoles) occupy the most space of the cell; **9** - roundish sporonts: nucleus/cytoplasm ratio is obviously lesser than in previous stages. Arrows indicate ribbons of electron dense material attached to the outer surface of the parasite cell or scattered free inside the host cytoplasm. DK - diplokaryon, ER - endoplasmic reticulum, Nu - nucleolus-like structure, PM - plasma membrane, V - vacuole, Sp - sporont, Spb - sporoblast. Scale bars - 1µm



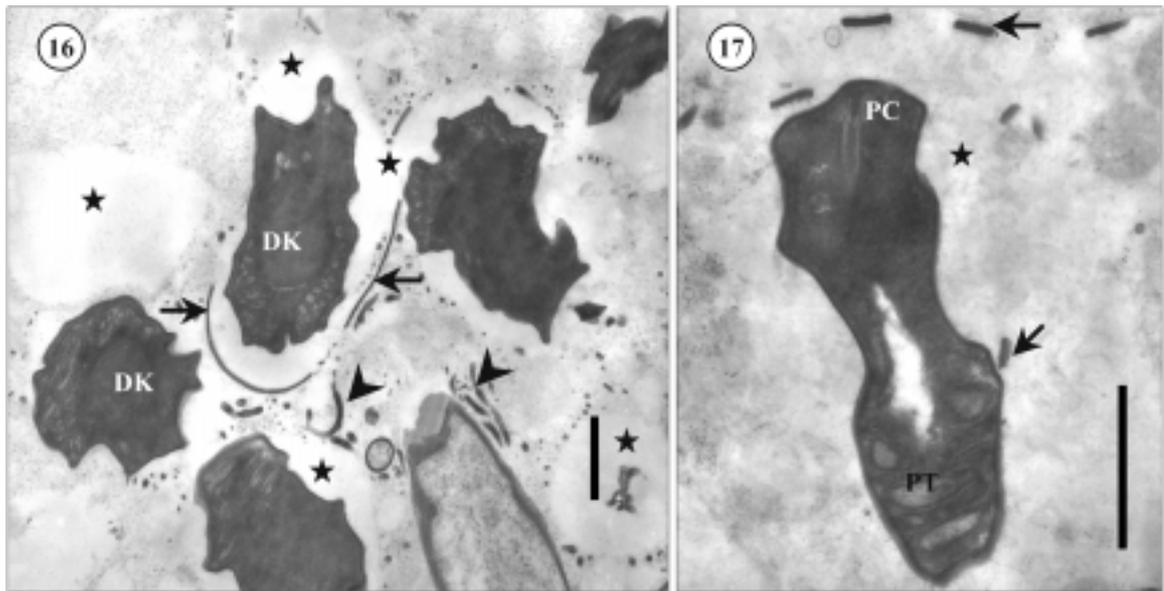
**Figs 10-15.** Fine morphology of *Nosema locustae* sporogony stages. **10, 11** - sections through the adipocyte, containing various pre-spore stages of *N. locustae*: pair of “transitional” stages; spores; sporonts; sporoblasts; **12** - parasite cell combining sporont (big cell size and prominent vacuolization of the cytoplasm) and sporoblast (cell polarization, thick wall, Golgi cluster and a primordial polar tube) characters; **13** - oblique section through an early sporoblast with primordial polar tube; **14, 15** - sporoblasts. Elements of the polar tube mature inside a peripheral region of the vesicular-tubular Golgi cluster. Tubular-like structures (arrows) are abundant in host cell cytoplasm during sporogony. DK - diplokaryon, G - Golgi apparatus (vesicular tubular cluster), L - lipid droplets, PPT - primordial polar tube, S - spore, Sp - sporont, Spb - sporoblast, TS - meront/sporont transitional stage, V - vacuole. Scale bars: Figs 10, 12-15 - 1  $\mu$ m; Fig. 11 - 10  $\mu$ m

heavily infected fat bodies were hypertrophied and easily disrupted. In some instances, mature spores were seen in membrane bounded compartments (presumably phagosomes) in haemocytes. Our attempts at observing early stages of development in intestinal epithelium at 48-96 h postinoculation failed. All stages of the parasite were in direct contact with the host cell cytoplasm (i.e., no interfacial envelopes were present).

Meronts (Fig. 1), as defined by Sprague *et al.* (1992), were the earliest stage in the life cycle of *N. locustae* that we recognized. They were round or slightly oval in shape, ranged from 3.5 to 5.0  $\mu$ m in diameter as measured in ultrathin sections. Meronts normally possessed two or four nuclei in diplokaryotic arrangement. Merogony was by binary fission of tetranucleate stages (Fig. 2). Meront ultrastructure was typical of microsporidia (Sprague *et al.* 1992, Becnel and Andreadis 1999). Meronts were surrounded by a simple plasmalemma of

7.5 to 10.0 nm thick. The cytoplasm had numerous free ribosomes and a few membrane profiles (Figs 1, 2). Condensed chromatin was not observed in nuclei of meronts even when they were undergoing binary fission (Fig. 2).

Transitional stages from merogony to sporogony (Figs 3-7, 10) were abundant in adipocytes of all three examined hosts. These intermediate stages were identifiable by the set of morphological features, mentioned beneath, which may be present in various combinations. (i) Appearance of numerous electron dense intranuclear particles scattered throughout the nucleoplasm (Figs 5, 6) or assembled in the nucleolus-like structure (Fig. 7). Sometimes the accumulation of electron dense particles 50-100 nm in diameter made the boundary between two nuclei of the diplokaryon nearly invisible (Fig. 5). The same structures were visible in parasite cytoplasm outside the nucleus (Fig. 6). (ii) Appearance of abundant



**Figs 16, 17.** *Nosema locustae* “late” sporoblasts. The shrinkage of the parasite cell during “early” sporoblast/ “late” sporoblast transition leaves behind the electron-lucid space (stars) limited by the electron dense layer (arrows), which eventually degrades in the host cell cytoplasm. Tubular-like structures (arrowheads), which are usually larger in diameter compared with the above-mentioned electron dense layer, sometimes can hardly be differentiated from the latter. DK - diplokaryon, PC - primordial polar cup, PT - primordial polar tube. Scale bar - 1  $\mu$ m

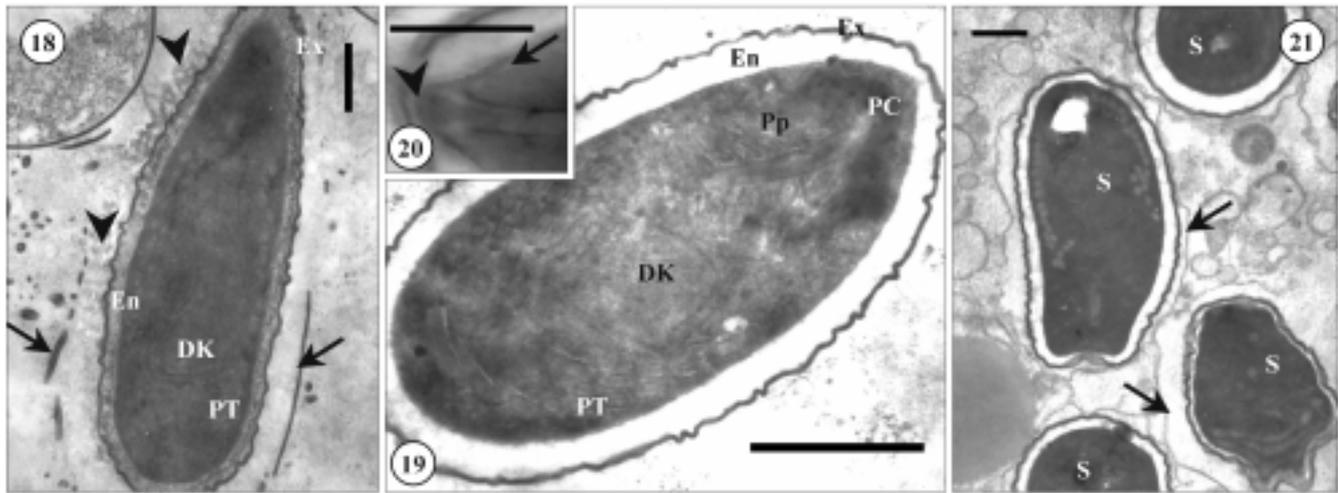
rough endoplasmic reticulum often arranged in regular multilayer patterns (Figs 4, 6, 11); (iii) conspicuous vacuolization of the cytoplasm in the vicinity of ER stacks or around the nuclei. (Figs 3, 4, 7). The plasmalemmas of transitional stages were *ca* 10-12 nm thick and showed an undulating profile with numerous evaginations. Small vesicles were normally present in close vicinity. The parasite cells at this stage were round or slightly elongated; their diameter, measured on ultrathin sections, varied from 2.9 to 3.6  $\mu$ m. Further vacuolization of the cytoplasm lead to an increase of the parasite’s cell volume.

Sporonts (Figs 8-11) were revealed in all studied hosts as round, large cells of 4.8-5.5  $\mu$ m in diameter, containing one diplokaryon with evidently lesser nuclear/cytoplasm ratio comparatively with the previous stages. Multiplication and development of sporonts were accompanied by emergence of tubule-like structures in the host cell cytoplasm. Elongated conglomerates of electron dense material were scattered inside the host cell and eventually ornamented the external surface of the parasite membrane, forming a continuous dense layer of about 25 nm thick (Figs 8, 9).

Sporoblasts were readily distinguishable from sporonts by their gradual elongation and polarization, and by

further thickening of the wall (Figs 12, 13). Polarization was manifested by the appearance of a distinct vesicular-tubular cluster [Golgi-like complex (Cali and Takvorian 1999)] in the posterior part of the cell and the primordial apical part of the polar filament anteriorly (Figs 12-15). The elements of the polar tube appeared to mature inside the peripheral (trans-Golgi) region of the vesicular-tubular cluster (Fig.15). From a certain point during sporogenesis, reduction in size of sporoblasts was evident (star-like stage) (Figs 16, 17). The shrinkage seem to occur abruptly, leaving behind the space formerly occupied by the sporont, recognizable by an electron dense layer, which eventually degraded in the host cell cytoplasm. The shrinkage, as a phenomenon of “extensive vacuolization” observed on the “transitional” stage, represents possibly a fixation artifact, which yet might reflect the alteration in membrane properties and its permeability taking place at these stages. As in earlier developmental stages (Fig. 5), tubular structures continued to be abundant in the host cytoplasm around sporoblasts (Fig. 16). Sporoblast sizes varied considerably: 2.2-3.8 x 1.0-2.8  $\mu$ m.

Spores were diplokaryotic, measured  $4.95 \pm 0.07 \times 2.65 \pm 0.04$  (mean  $\pm$  SE, n=24) in fresh smears and  $3.49 \pm 0.18 \times 1.73 \pm 0.04$  (n=10) in ultrathin sections and



**Figs 18-21.** *Nosema locustae* spores. **18** - young spore. The internal structure of the endospore is visible, as well as tubules (arrowheads) filling the zone of host cell cytoplasm between the parasite exospore and former borders of the “mother” sporont marked by the ribbons of electron dense material (arrows); **19** - mature spore with a typical set of organelles; **20** - section through the apical part of a polar filament, revealing an anchoring disk (arrowhead), a polar sac (arrow) and the anterior part of a polaroplast, composed of tightly packed membranes; **21** - in locust haemocytes spores were found residing inside membrane bound compartments (arrows), presumably parasitophorous vacuoles. DK - diplokaryon, En - endospore, Ex - exospore, PC - polar cap, Pp - polaroplast, PT - polar tube, S - spore. Scale bars: Figs 18, 19, 21 - 1  $\mu\text{m}$ ; Fig. 20 - 0.5  $\mu\text{m}$

had an electron-dense cytoplasm. Internal structures of the spore - anchoring disc, polar filament, lamellar polaroplast, diplokaryon, layers of endoplasmic reticulum and posterior vacuole (Figs 18-21) showed the characteristic organization of “typical microsporidia” (Vavra and Larsson 1999). The spore envelope was composed of the 200-300 nm thick electron-lucid endospore, which exhibited sometimes a fibrous structure (Fig. 18), and the undulating, electron-dense exospore 50-60 nm thick with no differentiated structure. The polar filament was isofilar, arranged in 17-18 coils. In *Locusta migratoria* haemocytes spores were sometimes enclosed in membrane-bounded compartments, presumably phagosomes (Fig. 20)

## DISCUSSION

This is the first study showing the ultrastructure of developmental stages of *N. locustae*. The only information at the electron microscopy level previously available was on spores (Huger 1960). We found justification in including in the present contribution our own observations on spores because although Huger (1960) did not specify the precise origin of the isolate of *N. locustae* he used for his study, he did not work with material of North

American origin but with infections in laboratory-reared *L. m. migratoroides*, as Canning (1953) did for the original description of the pathogen. Therefore, since we utilized material of North American origin we could conceivably have found some differences. However, our observations of spores are in good general agreement with those of Huger (1960). The slight discrepancies observed (measurements of endospore and exospore, and presence-absence of undulating profiles of exospore) can conceivably be attributed to the utilization of different fixation protocols. Huger (1960) used much longer fixation times (44 h) than we did (1½ h) and used  $\text{OsO}_4/\text{K}_2\text{Cr}_2\text{O}_7$  only while we used double fixation (glutaraldehyde/ $\text{OsO}_4$ ). Similarly, the stages observed by us by electron microscopy appeared consistent with those originally reported by Canning (1953, 1962) using light microscopy, not only in size and shape but also in nucleus/cytoplasm ratio. The only essential difference was that we did not observe uninucleate stages, but if we have had observed them it would not have been reliable information because sectioned material is misleading for establishing number of nuclei. Also, a stage like the one depicted in Fig. 5, having the nuclei of the diplokaryon in extremely intimate contact might have been easily recognized as uninucleated in light microscopy. Uninucleate stages have been shown in light microscopy studies of

**Table 1.** *Nosema* species from Orthoptera. A - adipose tissue, B - blood cells, C - gastric caeca, G - gonad, H - haemolymph, M - muscle, Mg - midgut, Mt - Malpighian tubules, N - neural tissue, P - pericardium, S - salivary glands, T - traqueal matrix

Species	Type host (family: subfamily)	Tissue/organ affected	Spore size (fresh) in µm	Nr. polar filament coils	Salient features	References
<i>N. acridophagus</i>	<i>Schistocerca americana</i> (Acrididae: Cyrtacanthacridinae)	Mg, G, P, N, A	4.1 x 2.6	10-12	Bi- or tetra nucleate meronts covered by layer of tubular elements	Henry 1967, Streett and Henry 1993
<i>N. asiaticus</i>	<i>Oedaleus asiaticus</i> (Acrididae: Oedipodinae)	Mg, Gc, G, Mt, A	4.2 x 1.8	11	Multinucleate meronts (up to 12 nuclei)	Wen 1996
<i>N. chorthippi</i>	<i>Chorthippus albomarginatus</i> (Acrididae Gomphocerinae)	A	3.5 x 1.9	?	Meront and sporont plasma membrane is ornamented with electron dense material arranged in "bead-like" structures	Issi and Krylova 1987
<i>N. cuneatum</i>	<i>Melanoplus confusus</i> (Acrididae: Melanoplinae)	P, A, G, T, Mg, Mt, N	4.8 x 3.4	10-12	Cuneate spores; globulo-vesicular layer around transitional stages	Henry 1971, Streett and Henry 1987
<i>N. grylli</i>	<i>Gryllus bimaculatus</i> (Gryllidae)	A, H	4-5.2 x 1.8-2.5	18-20	Transitional meront/sporont stage with numerous electron dense intranucleus particles	Sokolova <i>et al.</i> 1994
<i>N. locustae</i>	<i>Locusta migratoria</i> (Acrididae: Oedipodinae)	A	4-6.5 x 2.5-3.5	15-18	Transitional meront/sporont stage with numerous electron dense intranucleus particles	Canning 1953, Sokolova and Lange <i>ibidem</i>
<i>N. maroccanus</i>	<i>Docioctaurus maroccanus</i> (Acrididae: Gomphocerinae)	M, Mg	4.4-5 x 2.5-3.8	14-15	No information	Issi and Krylova 1987
<i>N. montanae</i>	<i>Melanoplus packardii</i> (Acrididae: Melanoplinae)	A	3.1 x 1.5	5-7	Multinucleate meronts (up to 8 nuclei). Chains of transitional stages	Wang <i>et al.</i> 1991
<i>N. pyrgomorphae</i>	<i>Pyrgomorpha conica</i> , <i>P. cognata</i> , <i>P. bispinosa</i> (Pyrgomorphidae)	Mg, A, M, S, G	3.9 x 2 3.4 x 1.2	7-9	Multinucleate transitional stages (up to 8 nuclei), frequently moniliform	Toguebaye <i>et al.</i> 1988, Lange <i>et al.</i> 1992
<i>N. trilophidiae</i>	<i>Trilophidia annulata</i> (Acrididae: Oedipodinae)	S, Mg, G, B, N, T, A	3.7 x 1.6	8	No information	Wen 1996

other *Nosema* species of Acrididae (Henry 1967, 1971; Wang *et al.* 1991; Lange *et al.* 1992).

Our study did not reveal any difference in the morphology of *N. locustae* while developing in the three different hosts employed. Since the hosts used are not only different species but also belong to distinct subfamilies (Melanoplinae, Oedipodinae, and Cyrtacanthacridinae), the results of the study strongly suggest that the morphology *N. locustae* remains constant regardless of the host involved. Host induced morphological plasticity has been reported in other microsporidia (Walters 1958, Armstrong *et al.* 1986, Mercer and Wigley 1987, Hayasaka and Kawarabata 1990), but appears not to be a trait of *N. locustae*. That the morphological characters of *N. locustae* remains the same regardless the host involved, at least for the isolates of North American origin, is a desirable characteristic from an applied point of view because the diagnosis after introductions will be much reliable.

Ten species of the genus *Nosema* Naegeli have been described from Orthoptera hosts (Table 1). The published information on the structure of *N. chorthippi* Issi and Krylova, *N. maroccanus* Issi and Krylova, and *N. trilophidia* Wen structure is not sufficient for a comparative analysis. *Nosema montanae* Wang *et al.* and *N. asiaticus* Wen form multinucleate merogonial plasmodia and thus can be easily differentiated from *N. locustae*. The ultrastructure of *N. locustae* was similar to the following species: *N. pyrgomorphae* Toguebaye *et al.* from grasshoppers of the genus *Pyrgomorpha* Serville, *N. cuneatum* Henry and *N. acridophagus* Henry, both studied ultrastructurally from grasshoppers of the genus *Melanoplus* Stål, and *N. grylli* Sokolova *et al.* from the cricket *Gryllus bimaculatus* Deg. (Issi and Krylova 1987; Street and Henry 1987, 1993; Toguebaye *et al.* 1988; Lange *et al.* 1992; Sokolova *et al.* 1994, 1998; Wen 1996). The meront fine structure is quite similar in all five species, though *N. acridophagus* meronts are elongated and possess fine tubular elements on the plasmalemma. The deposition of the homogenous electron dense material, deriving from the host cell cytoplasm, on the external part of the sporont plasmalemma is characteristic for *N. locustae*, *N. acridophagus* and *N. grylli*. The ultrastructure of sporonts and of the transitional (meront/ sporont) cells with a specific state of the nucleus, containing electron dense particles, is very similar in *N. locustae* and *N. grylli* (Sokolova *et al.* 1998). At the same time, the spore of *N. locustae* can be easily

distinguished from other spores of orthopteran "Nosemas" by the largest size in the group, and 17-18 polar filament coils. *Nosema grylli* is the only species that possess a similar number of coils (18-20) but it is parasitic for *G. bimaculatus*, which is not susceptible to *N. locustae* (Y. Sokolova, unpublished observation). Compared with *N. pyrgomorpha*, *N. locustae* differs in host and tissue specificity (Table 1).

The peculiar presumably transitional (meront/sporont) stage of microsporidian life cycle, characterized by the nucleus filled with numerous electron dense intranuclear bodies have been mentioned so far only for *N. grylli* (Sokolova *et al.* 1998). The same condition of nucleoplasm can be distinguished also in *N. pyrgomorphae* meronts (Fig. 14 in Lange *et al.* 1992). We believe this phase of nuclear cycle indicates the switch of parasite metabolism from proliferative (merogony) to morphogenic (sporogony) phase with intensification of biosynthetic processes, demanding rapid RNA processing and transcription. We suppose that electron dense intranuclear particles represent ribonucleoproteins (RNP). Their identification as well as investigation of their role in microsporidian host cycle is currently in progress. Among protists "RNP granules" are described from macronuclei of Infusoria and from nuclei of Foraminifera (Raikov 1982).

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## Morphology, Biometry and Ecology of *Nebela bigibbosa* Penard, 1890 (Protozoa: Rhizopoda)

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**Summary.** Testate amoeba *Nebela bigibbosa* Penard, 1890, isolated from the litter of beech forests in Bulgaria and its morphology, biometry and ecology have been investigated. Size frequency distribution analysis indicates that *N. bigibbosa* is a size-monomorphic species, characterized by a main-size class and a small size range (95% of all measured individuals have a shell length 145-170  $\mu\text{m}$  and 92% have a shell width 94-110  $\mu\text{m}$ ). The investigations of the ecology of *N. bigibbosa* show that it is not a typical inhabitant of *Sphagnum* mosses, but is closely related and frequently found in the litter of deciduous forests (mainly beech forests) and can be used as an indicator for these biotopes.

**Key words:** biometry, ecology, morphology, *Nebela bigibbosa*, Rhizopoda, Testacea.

### INTRODUCTION

The genus *Nebela* is one of the well-studied genera of the testaceans. It includes comparatively large species, which in most cases are clearly differentiated and well distinguished each other. More than 120 taxa of the genus *Nebela* have been described until now. Most of them live in mosses and litter, and rarely occur in other biotopes (soils, littoral, pelagial and benthal of the freshwater pools, etc.).

*Nebela bigibbosa* is one of the largest and well-distinguished species of the genus *Nebela*. It is characterized by the presence of two large pores on the broad

lateral face of the shell. The species was described by Penard (1890) in mosses from the environs of Wiesbaden (Germany). Although the studies of the freshwater and *Sphagnum*-dwelling testaceans were exceptionally intensive in the beginning of 20th century, this species has been reported only from Germany, Ireland, Great Britain, Spitsbergen, Canada and Java till 1960 (Penard 1890, 1903, 1905; Wailes and Penard 1911; Cash *et al.* 1919; Deflandre 1936; Hoogenraad and de Groot 1940; Jung 1942). The fact that the majority of the above authors have rarely found *N. bigibbosa* as single individuals might be the reason of the lack of more biometrical and ecological data about this species. The aim of the present study is to characterise morphologically, biometrically and ecologically *N. bigibbosa* using abundant material, isolated from the litter of beech forests in Bulgaria.

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## MATERIALS AND METHODS

The material for the present study was collected from the decomposing litter (Ao) of beech forests in Bulgaria. The samples were treated in laboratory immediately after their collecting. The flotation method of Bonnet and Thomas (1958) has been used to isolate the testate amoebae. Isolation was made under the stereomicroscope at 70x magnification. The brown mountain-forest soil is the main soil type of the studied beech forests (pH varies from 4.5 to 6.9). The altitude of the sampling sites ranges from 850 to 1800 m a.s.l. Detailed data about the sampling locations are given in Todorov (2001).

The morphometric characterization of the species and the construction of an ideal individual from the median of the shell measurements were made according to Schönborn *et al.* (1983). The following parameters were calculated:  $\bar{x}$  - arithmetic mean; M - median (this value is used to construct the ideal individual); SD - standard deviation; SE - standard error of the arithmetic mean; CV - coefficient of variation in %; Min, Max - minimum and maximum values; n - number of examined individuals. Shell's size was measured under the light microscope at x 400 magnification. Measurements in  $\mu\text{m}$ .

For scanning electron microscopy the shells were isolated, cleaned by several transfers through distilled water, mounted on coverslips and air-dried. The shells on coverslips were coated with platinum and examined with a JEOL Superprobe-733 operating at 15 kV.

## RESULTS AND DISCUSSION

### Description of species *Nebela bigibbosa* Penard, 1890 (Figs 1-8, Table 1, 2)

The shell is colourless or yellowish, laterally compressed (about 2 : 1), pyriform with a broad, slightly convex aperture (Figs 1-4). In broad lateral view, at approximately one third of the body length from the aperture, there are two large pores, located in lateral depressions (Figs 1-4). The shell is composed of oval, circular or elongate shell plates of different sizes (Figs 5-7). The aperture is oval, concave in narrow lateral view, surrounded by a distinct organic collar (Figs 1, 2, 5).

Geographical distribution: British Isles, Bulgaria, Canada, Chile, France, Germany, Greece, Java, Nepal, Philippines, Spitsbergen, Switzerland, Thailand.

References: Penard 1890, 1902, 1903, 1905; Wailes and Penard 1911; Cash *et al.* 1919; Deflandre 1936; Hoogenraad and de Groot 1940; Jung 1942; Bonnet 1965, 1966, 1967, 1974, 1977, 1980, 1981, 1987; Puytorac *et al.* 1972; Ogden and Hedley 1980; Golemansky and Todorov 1985, 1990; Todorov 1993, 1998, 2001; Todorov and Golemansky 1995).

## Analysis of characters

Table 1 shows the morphometric characterization of *N. bigibbosa*. The ideal individual of this species is constructed from median values of all characters in Fig. 8. The shell measurements with the exception of character 7 are fairly constant. The coefficients of variation of characters 1 - 6 are rather low and show a remarkable uniformity of *N. bigibbosa*. Only character 7 is more variable. Characters 1 - 4 correspond to the ranges given by Penard (1890), Wailes and Penard (1911), Cash *et al.* (1919), Deflandre (1936), Hoogenraad and de Groot (1940), and Ogden and Hedley (1980), (Table 2).

Size frequency distribution analysis indicates that *N. bigibbosa* is a size-monomorphic species, characterized by a main-size class and a small size range (Figs 9, 10). Figure 9 show that 95% of all measured individuals have a shell length 145-170  $\mu\text{m}$ . More than two thirds of them (71%) are within the limits of 150 - 165  $\mu\text{m}$ , since only 2% are less than 145  $\mu\text{m}$  long and only 3% are more than 170  $\mu\text{m}$  long.

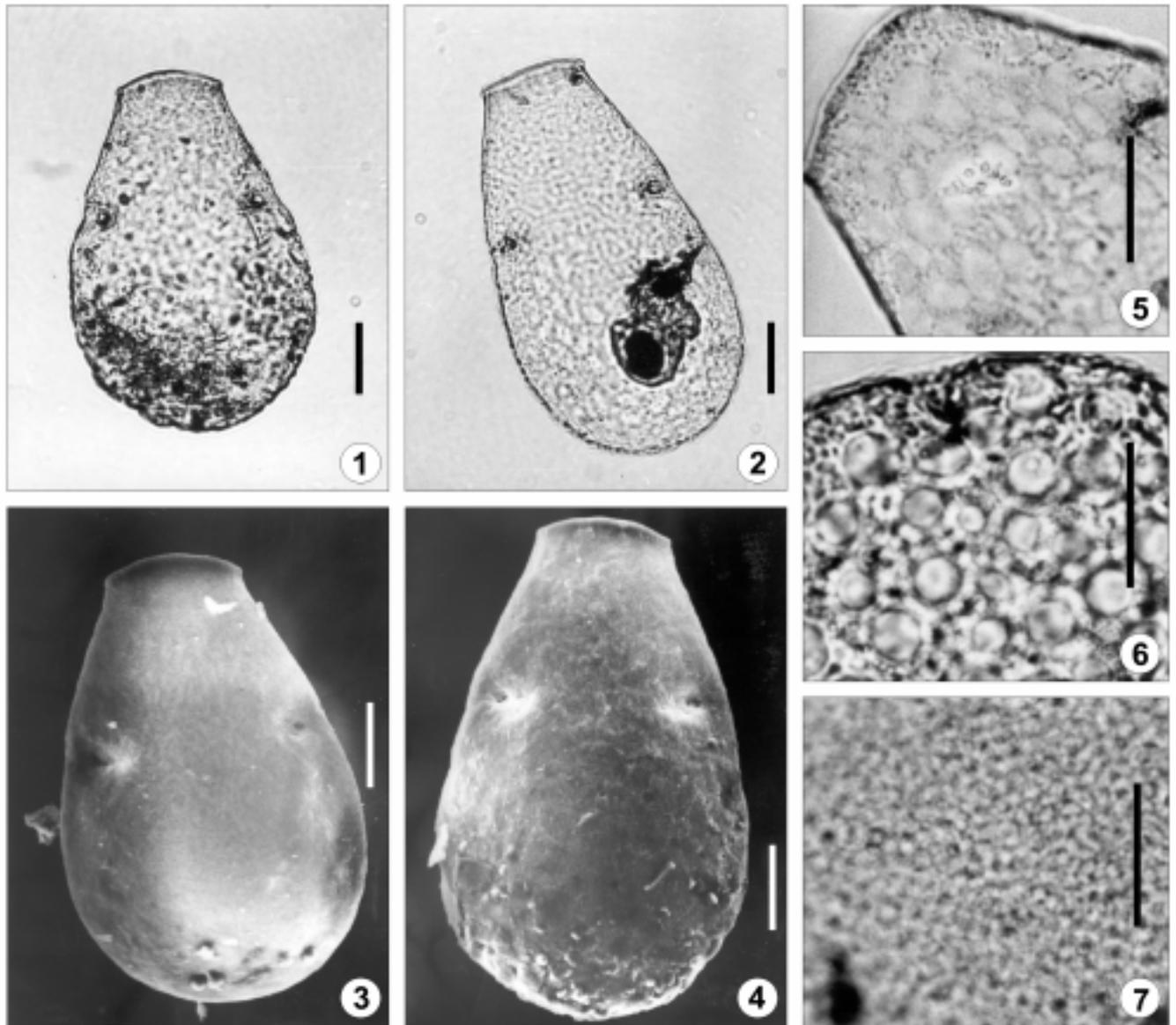
The frequency analysis of the shell width shows almost the same results (Fig. 10). Ninety-two percent of all measured individuals have a shell width between 94 and 110  $\mu\text{m}$  and about two thirds of them (64%) are within the limits 98-106  $\mu\text{m}$ . Only 4% have a shell width less than 94  $\mu\text{m}$  and 4% have a shell width above 110  $\mu\text{m}$ .

A scatter plot of shell length versus shell width of *N. bigibbosa* supports the above conclusion that this species is monomorphic and has a small size range (Fig. 11).

### Ecology of *N. bigibbosa*

Although this species was described at the end of the nineteenth century (Penard, 1890) its ecology is not yet fully clarified. Penard described the species from mosses in the environs of Wiesbaden (Germany). Later he, as well as some other authors, found *N. bigibbosa* in *Sphagnum* mosses (Penard 1903, Wailes and Penard 1911, Cash *et al.* 1919). In the monograph of the genus *Nebela* Deflandre (1936) also indicated that *N. bigibbosa* occurs in *Sphagnum* mosses. The majority of these authors found the species in single individuals and described it as rare.

Bonnet (1990) gave comparatively more detailed information about the ecology of *N. bigibbosa*. He



**Figs 1-7.** *Nebela bigibbosa*. 1, 2 - LM photographs of typical and elongated specimens; 3, 4 - SEM photographs of typical and elongated specimens; 5-7 - views, showing structure of a different shells composed of oval, circular or elongate shell plates. Scale bars: 1-4 - 30  $\mu\text{m}$ ; 5-7 - 20  $\mu\text{m}$

pointed out that this species has a reputation of a *Sphagnum*-dwelling, but it also occurs, although rarely, in some soil habitats as soil mosses, litter, neosols, etc. Bonnet also indicated that the habitats, where *N. bigibbosa* occurs, are characterized by high contents of organic matter, constant high moisture, absence of active Ca and acid reaction of the environment (pH between 3.6 and 6.8).

According to us the reputation of *N. bigibbosa* as a rare *Sphagnum*-dwelling species is due, first of all, to

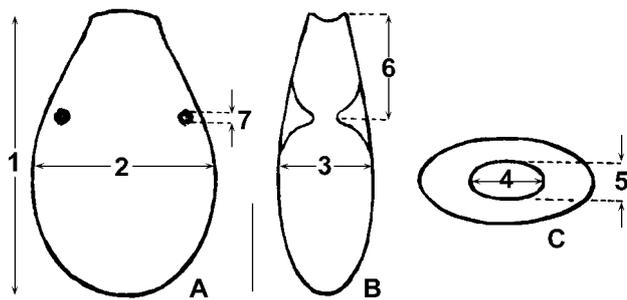
the fact that it is not a typical inhabitant of *Sphagnum* mosses, but occurs there only as an exception. A proof of that is the fact that *N. bigibbosa* was not found by many authors who studied the testacean fauna of the *Sphagnum* mosses (van Oye 1933; Hoogenraad 1934; Harnisch 1938; Hoogenraad and de Groot 1952; Grospietsch 1958; Golemansky 1966, 1967; Schönborn 1966; Moraczewski and Bonnet 1969; Meisterfeld 1973, 1977, 1979; Godeanu 1974; Chardez and Gaspar 1976; Beyens and Chardez 1984; Warner 1987). On the other

**Table 1.** Morphometric characterization of *N. bigibbosa* (measurements in  $\mu\text{m}$ ). Broad lateral view: 1 - length, 2 - breadth, 7 - pore diameter; B - narrow lateral view: 3 - breadth, 6 - pore collar aperture distance; C - apertural view: 5, 6 - aperture diameters. See Fig. 8 for character designation

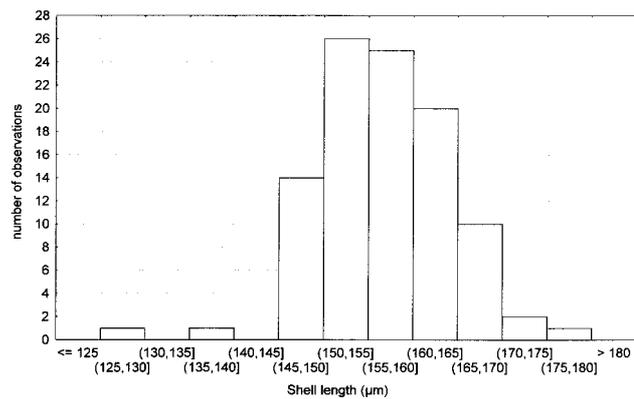
Character	$\bar{x}$	M	SD	SE	CV	Min	Max	n
(1)	157.5	157.0	7.36	0.74	4.7	128	177	100
(2)	101.9	102.0	4.8	0.49	4.7	90	115	100
(3)	51.6	52.0	1.8	0.18	4.2	48	56	100
(4)	40.4	40.0	2.8	0.28	7.0	35	49	100
(5)	20.9	21.0	1.2	0.12	5.8	19	23	100
(6)	59.6	60.0	3.4	0.34	5.7	52	70	100
(7)	3.8	3.5	0.5	0.05	12.4	3	5	100
proportion (2)/(1)	0.65	0.64	0.02	0.002	3.1	0.6	0.7	100

**Table 2.** Measurements (in  $\mu\text{m}$ ) of *N. bigibbosa* according to different authors

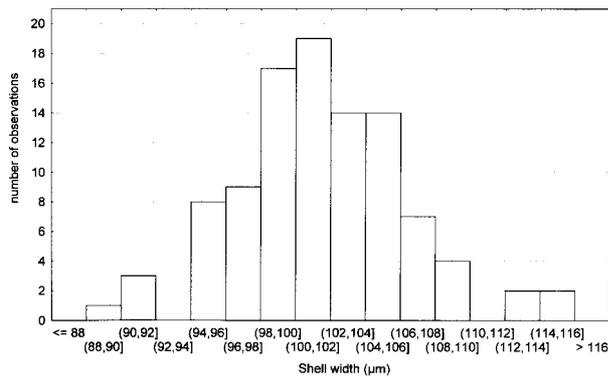
Authors	Length of shell	Breadth of shell	Depth of shell	Diameter of aperture
Penard, 1890	140-160	100-110	-	-
Wailes and Penard, 1911	135-170	87-110	50-55	34-45
Cash <i>et al.</i> 1919	135-170	87-110	50-55	34-45
Deflandre, 1936	135-170	87-110	-	34-45
Hoogenraad and de Groot, 1940	130-170	83-123	-	-
Ogden and Hedley, 1980	153-171	95-115	55-56	38-41
Present work	128-177	90-115	48-56	35-49



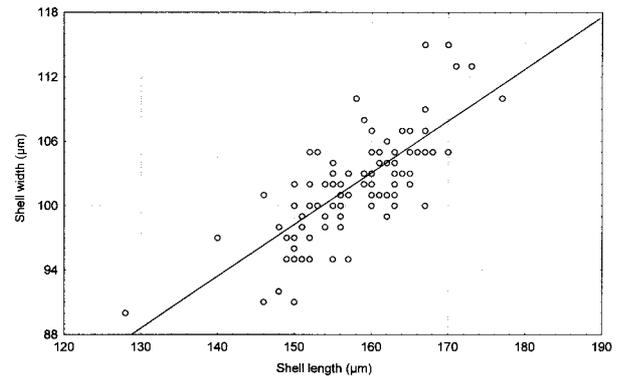
**Fig. 8.** A-C - Ideal individual of *Nebela bigibbosa* constructed from median values of all measured specimens; A - broad lateral view: 1 - length, 2 - breadth, 7 - pore diameter; B - narrow lateral view: 3 - depth, 6 - pore aperture distance; C - apertural view: 4, 5 - large and small axis of aperture; Scale bar - 50  $\mu\text{m}$



**Fig. 9.** Histogram showing size frequency of shell length of *N. bigibbosa*



**Fig. 10.** Histogram showing size frequency of shell width of *N. bigibbosa*



**Fig. 11.** Scatter plot of shell length versus shell width in *N. bigibbosa*

hand, *N. bigibbosa* has been found many times in the humus and litter of the forest ecosystems of different countries since 1965, when more intensive studies of the soil testate amoebae were begun (Bonnet 1966, 1967, 1977, 1980, 1981, 1987; Todorov 1993, 1998; Todorov and Golemansky 1995).

The results of our investigations show that *N. bigibbosa* is frequent and characteristic species for the litter of beech forests in Bulgaria (Todorov 2001). It was found in 48 of all 73 investigated samples and was constant species for this biotope (frequency of occurrence  $pF = 65.8\%$ ). The other common and constant species found together with *N. bigibbosa* were: *Arcella arenaria*, *Centropyxis aerophila*, *C. sylvatica*, *Cyclopyxis kahli*, *C. eurystoma*, *Corythion delamarei*, *Diffflugia lucida*, *Euglypha laevis*, *E. rotunda*, *Heleopera sylvatica*, *Nebela collaris*, *N. dentistoma*, *Plagiopyxis callida*, *Trinema complanatum*, *T. enchelys*, *T. lineare* and *Tracheleuglypha acolla*. Furthermore, *N. bigibbosa* had comparatively high dominance frequency ( $DF = 31.5\%$ ) and was a dominant species in many of the investigated samples.

All these facts suggest that *N. bigibbosa* is not a typical inhabitant of *Sphagnum* mosses but is closely related to the litter of deciduous forests (mainly beech forests) and can be used as an indicator species for these biotopes.

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## In Situ Confocal Laser Scanning Microscopy of Protozoans in Cultures and Complex Biofilm Communities

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**Summary.** A series of novel fluorescent compounds were used to stain protozoans in pure culture and in complex environmental biofilm communities. Confocal laser scanning microscopy was used to image fluor stained, NiSO<sub>4</sub> immobilized, living protozoans in situ. Most of the stains, specific for eucaryotic cell structures, such as Paclitaxel-BodipyFL, CellTracker, DiOC<sub>6</sub>(3), LysoTracker Red, MitoFluor Green and Rhodamine 6G showed potential for in situ staining of protozoa. However, some of the stains also stained bacterial or polymeric biofilm constituents. Nevertheless after staining, the usually transparent protozoan cells became clearly visible within the complex architecture of environmental biofilms. With respect to staining protozoan cells only and differentiation from other biofilm constituents, CellTracker showed the highest specificity. By using this approach, protozoa can be identified and distinguished within a complex habitat by virtue of (1) specific stains targeted for eucaryotic cell features, especially CellTracker, and (2) staining in combination with information on their size and morphology. With respect to fluorochrome concentration the approach has to be fine-tuned according to the specific characteristics of the sample as well as the identity and physiological status of the protozoa.

**Key words:** biofilms, confocal laser scanning microscopy (CLSM), fluorochromes, protozoans.

**Abbreviations:** CLSM - confocal laser scanning microscopy.

### INTRODUCTION

Environmental biofilms are very diverse and heterogeneous communities (Lock 1993). Their major cellular components may be bacteria, algae and the heterotrophic eukaryotes e.g. fungi, protozoa and micrometazoa. Furthermore, extracellular polymeric substances (EPS)

represent a major component of biofilm systems (Neu and Lawrence 1999). Although standard light and epifluorescence microscopy are effective tools in biofilm and protistan studies, they are limited to the examination of materials that are relatively thin in nature. In contrast, CLSM allows three-dimensional imaging of fully hydrated, living, thick, procaryotic and eukaryotic communities in their natural habitat. The large potential of this technique has been demonstrated for biofilms in a variety of studies (Lawrence *et al.* 1998a, b; Lawrence *et al.* 1991, 1994, 1998a, b; Bott *et al.* 1997; Møller *et al.* 1997, 1998; Neu and Lawrence 1997;

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Manz *et al.* 1999; Neu 2000). To date most studies investigating biofilms by means of confocal laser scanning microscopy (CLSM) have focused on the prokaryotic part of biofilms. Nevertheless, heterotrophic protozoans represent an important yet understudied component of biofilms. Their movement and grazing behaviour results in changes in the biofilm biocoenosis (Jackson and Jones 1991, McCormick 1991, Møller *et al.* 1997, Lawrence and Snyder 1998). However, in situ investigations of motile eukaryotic organisms in biofilms are hampered by methodological limitations.

A range of approaches have been used to study protozoa, direct microscopic examination with bright field or phase contrast optics or general staining and epifluorescence microscopy (Pedersen 1982). The application of specific antibodies to stain the cytoskeleton of protists has also been carried out (Fleury 1991, Jeanmaire-Wolf *et al.* 1993). However, these studies were morphological or developmental in nature and did not focus on the natural environment. In situ hybridisation has also been used to examine and identify protists in situ and in pure cultures (Lim *et al.* 1993, 1996). Most of these methods (i.e., antibodies, hybridisation) have the drawback of requiring fixation resulting in distortion, loss of protists and loss of context or growth habit. As eukaryotes, members of the protista have a variety of structural and chemical characteristics that may be exploited to separate them from bacterial, exopolymeric, algal and other components of biofilm communities. These include an extensive cytoplasm, endoplasmic reticulum (ER), Golgi apparatus, vacuoles, tubulin containing cytoskeleton, numerous mitochondria, ciliary bands etc.

The fluorescent stains often used for investigating microbial communities with epifluorescence microscopy are general nucleic acid or protein stains such as Acridine Orange, DAPI, SYTO or Proflavin (Strugger 1948, Porter and Feig 1980, Sherr *et al.* 1993, Neu and Lawrence 1997, Lawrence *et al.* 1998a). The application of these stains leads to a non-specific binding to all cells present in the biofilm. In the case of algae, the autofluorescence signal of chlorophyll, can be used to distinguish them from bacteria and other biofilm components (Lawrence *et al.* 1998a). Nevertheless, in complex biofilms under most staining conditions it is difficult to distinguish the bacterial signal from the protozoan signal. However, the protozoa, as members of the eukaryotes, offer the possibility to use stains specific for cell organelles and other cellular structures. A variety of cellular stains have been developed in order to visualise and identify subcellular compartments, storage products

and other metabolites of eukaryotes. In addition to their specificity for protozoans, these stains also allow the analysis of fully hydrated living biofilms without prior fixation. A further challenge for observation of protozoa in situ using a point scanning CLSM is the motility of these organisms. During the scanning process it is necessary that the cells are stationary, in order to perform optical sectioning and averaging for final three-dimensional reconstruction.

The aim of this study was to find a suitable fluorescent staining protocol for protozoa in pure culture as well as in complex biofilm communities. For this purpose we evaluated a range of potential fluorescent stains specific for eukaryotic cell features. In addition, the staining protocol was tested in combination with an immobilisation technique in order to apply CLSM to motile protozoan cells.

## MATERIALS AND METHODS

**Cultures.** The ciliates (*Paramecium caudatum*) and heterotrophic flagellates were co-cultivated in Petri dishes with Eau de Volvic (French table water) enriched with some rice grains. For examination an aliquot of culture medium (500 or 1000 µl) containing cells was used to fill four well coverslip chambers (Nunc, Roskilde, Denmark). Immobilisation tests and staining were carried out in these chambers.

**Biofilms.** The complex biofilms were grown in a rotating annular reactor (RAR) on polycarbonate slides (Sinis, Dettingen, Germany). The reactors were fed with raw water from the South Saskatchewan River, Saskatchewan, Canada. For further details of the operating conditions see Neu and Lawrence (1997).

**Microscopy.** A MRC-1000 CLSM (BioRad, Hemel Hempstead, UK) equipped with an argon-krypton laser and mounted on a Microphot SA microscope (Nikon, Tokyo, Japan) was used to obtain images of biofilms and stained protozoa. Observations of RAR slides were made with water immersible lenses including a 63x, 0.9 numerical aperture (NA) Zeiss lens and 10x 0.3 NA, 20x 0.4 NA, and 40x 0.55 NA Nikon lenses. The system was controlled by a Pentium host computer with the operating software Comos 6.01. Pieces of the polycarbonate slides (*ca* 1.0 cm<sup>2</sup>) with biofilm (grown for 38 to 48 days) were glued in Petri dishes and covered with filtered river water for subsequent staining. Images were obtained using green, excitation (ex) 488/32, emission (em) 522/32, red (ex = 540, em = 580/32 nm) and far red (ex = 647, em 680/32). In addition, phase contrast transmission laser images were obtained. Images were also obtained with a TCS-4D CLSM system (Leica, Heidelberg, Germany) equipped with an argon-krypton laser and mounted on a DM-IRBE inverted microscope (Leica). For observation, the following lenses were available: 20x 0.6 NA, 40x 0.75 NA, 63x 1.2 W NA and 100x 1.4 NA. The CLSM system was controlled by Scanware Ver. 5.1A (Leica).

**Preparation of stains.** A variety of potential stains were obtained from Molecular Probes Inc. (Eugene, Oregon, USA) and are listed in Table 1. The stains were stored according to the instructions

of the supplier (at 4 °C or -20 °C), warmed to room temperature, and if provided as a solution, briefly centrifuged. Stock solutions were prepared by adding distilled water, phosphate buffered saline (PBS), DMSO or ethanol to the stain (see Table 1). The stock solution was further diluted with filter-sterilised distilled water, river water, Eau de Volvic or PBS-Buffer to create a working solution. The working solutions were directly applied to the culture medium or the biofilm. Incubation time was 5-10 min., although for MitoFluor Green we used an incubation time of 30 min.

**Immobilisation.** A solution of nickel sulfate (stock solution 1% w/v for NiSO<sub>4</sub>) was used for the immobilisation of protozoa (Lee *et al.* 1985). The solution was applied at a final concentration of 0.002% (w/v) to culture medium containing ciliates or flagellates. To the biofilm samples 50-100 µl of a 0.01 % working solution was added.

## RESULTS AND DISCUSSION

For staining eucaryotic cells a large number of fluorescent stains are now available for use in combination with epifluorescence microscopy or confocal laser scanning microscopy (Haugland 1999). These stains target many of the organelles and other structures present in eukaryotic cells but have not been extensively tested using protists either in pure culture or in environmental microbial communities. In the present study we assessed whether some of these stains are suitable for staining and detecting protists in natural biofilm communities and in pure culture. Staining protocols were optimized for the specific biofilm system and cultures used. The dilution factors and concentration of fluorochromes listed in Table 1 are intended as guidelines. We recommend testing various concentrations of stains and incubation times in order to achieve the best results. In the examples given, the protozoan cell was visualized by concurrent transmission/fluorescence imaging.

**Paclitaxel-BodipyFL.** Paclitaxel (approved generic name for the anti-cancer pharmaceutical Taxol) is known to interfere with tubulin polymerisation. It also blocks cells in the G<sub>2</sub> and M phases of the cell cycle. It has been reported that live cells incubated with Paclitaxel-Bodipy FL showed a staining pattern that may correspond to labelled tubulin filaments (Haugland 1999).

We observed that the cytoplasm was labelled to a certain degree with Paclitaxel-BodipyFL (Figs 1A, 2B). Cilia were also brightly stained, as were the fibrillary structures located near or connected to the peristomal regions such as the oral region of hypostome ciliates (Fig. 1A). To our knowledge, PaclitaxelBodipyFL has not previously been used as a vital stain for protozoan cells. Although we did not observe the characteristic

ciliation pattern observed after silver staining, the bright staining of cilia and fibrillary peristomal structures indicates that microtubules/tubulin may be a major target of this stain in protists. Consequently this compound may be a useful stain for various applications.

**Rhodamine 123.** Rhodamine123 has been applied to a variety of cell types, e.g. live bacteria, plants and human cells. This cationic fluorescent dye accumulates in active mitochondria (e.g. Johnson *et al.* 1980) without inducing cytotoxic effects (Haugland 1999). Other studies have used Rhodamine 123 to label the mitochondria of the protozoan malarial parasite *Plasmodium* (Divo *et al.* 1985) as well as the mitochondria in phytoplankton cells (Klut *et al.* 1989).

Rhodamine123 applied to ciliate cultures and biofilms did not work as a specific stain. We found that application of Rhodamine123 at high concentration to cultured ciliates resulted in a negative staining similar to that described by Caldwell *et al.* (1992) when staining biofilms and cells with fluorescein. In the case of pure cultures there was a rather bright background staining with protozoan cells appearing dark. The application to complex biofilm communities resulted in staining of the whole biofilm, particularly bacterial cells, with no evident staining of ciliates or other protozoa (data not shown).

**Rhodamine 6G chloride.** This stain is a rhodamine derivative which has been shown to be selectively accumulated in mitochondria (Johnson *et al.* 1981). Haugland (1999) suggested that at low concentrations lipophilic rhodamine dyes selectively stain mitochondria in live cells, whereas at high concentrations they stain the ER of animal cells. Thus this dye may have some potential for applications in studies of protozoa.

In our studies the stain was effective when applied to pure cultures of both ciliates (Fig. 1B) and flagellates. In contrast, when staining protozoa in biofilms the signal was not very intense and non-specific binding to microbial polymers and bacterial cells was observed. This indicated that the stain has some potential for pure culture studies but was not really suitable for environmental applications.

**DiOC<sub>6</sub>(3), Dicarboyanine.** This stain is a carboyanine with short (C1-C6) alkyl-chains. Terasaki and Reese (1992) confirmed that DiOC<sub>6</sub>(3) labelled the ER and other membrane compartments in epithelial cells. They suggested that DiOC<sub>6</sub>(3) stains all intracellular membranes. DiOC<sub>6</sub>(3) has also been reported to stain mitochondria in live yeast and other eukaryotic cells, as well as sarcoplasmic reticulum in beating heart cells (Haugland 1999).

In our applications this fluorochrome stained ciliated protozoans both in pure culture and in complex microbial biofilms. Observations in environmental biofilm samples indicated that staining also occurred in bacterial and algal cells. This makes it difficult to separate the protozoan signal from other signals if digital image analysis is applied to quantify certain features (Fig. 2A).

**LysoTracker Red.** LysoTracker probes consist of a fluorophore or biotin moiety linked to a weak base that is only partially protonated at neutral pH. According to Haugland (1999) this compound is thought to be freely permeable through cell membranes and typically concentrates in spherical organelles.

CLSM observations indicated that LysoTracker Red stained specific regions of the cytoplasm which may correspond to the lysosomal vesicles. This can be clearly seen in the image of a *Paramecium* (Fig. 1D). LysoTracker Red was particularly suited for staining of heliozoans in biofilms where the cytoplasm was brightly stained and even the axiopods were visible (Figs 2 C, D).

**MitoFluor Green.** MitoFluor Green has a chemical structure similar to MitoTrackerGreen. The MitoTracker probes are cell permeant mitochondrion-selective dyes. They are non-fluorescent in aqueous solutions and become fluorescent once they accumulate in the lipid environment of mitochondria. Mitochondria are visualised in live and fixed cells and exhibit bright green, fluorescein-like fluorescence (Haugland 1999). MitoTracker probes were previously used to stain mitochondria in protist cells (Vassella *et al.* 1997) as well as arthropod and mammalian cells (Hoth *et al.* 1997, Pereira *et al.* 1997).

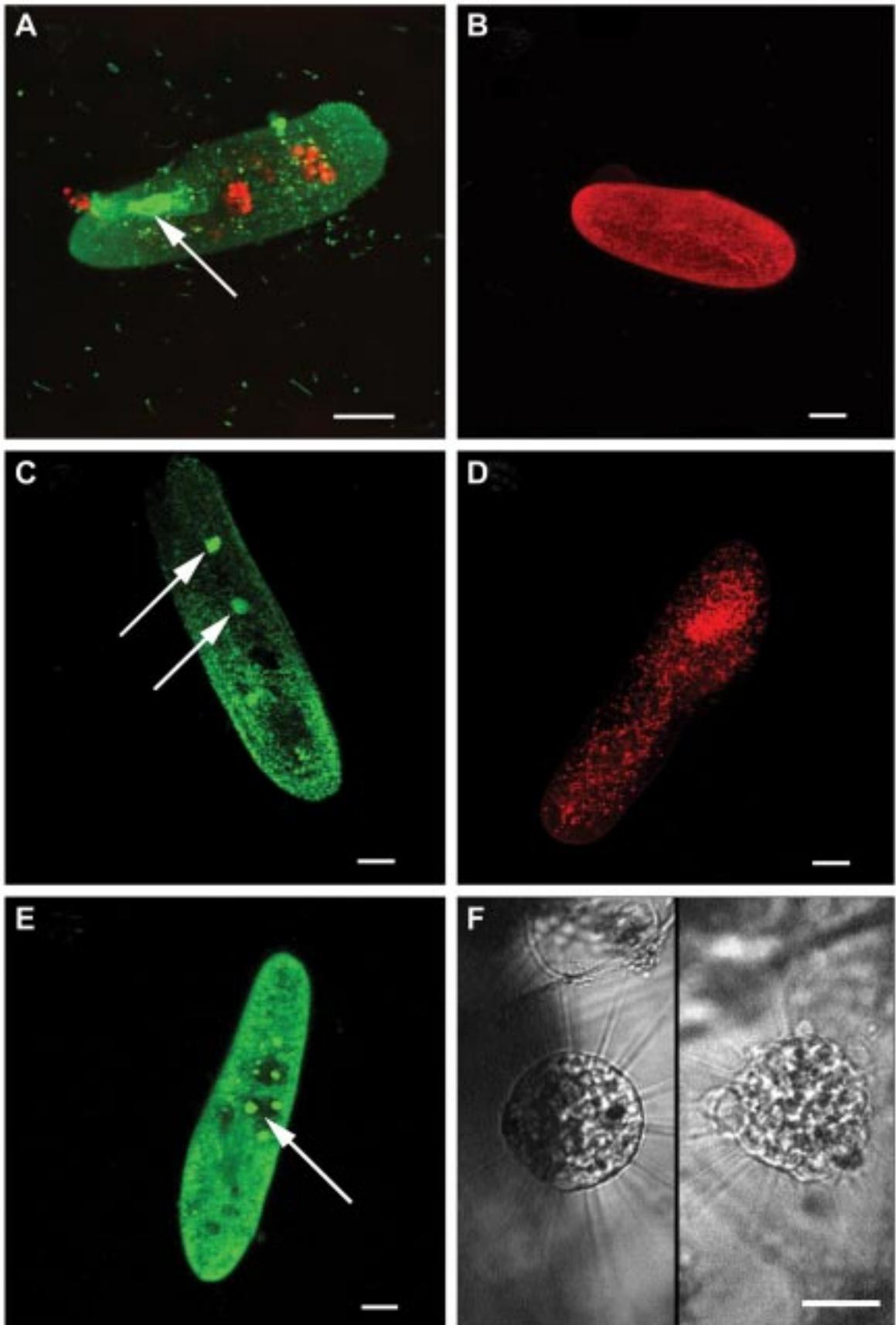
In our experiments MitoFluor Green labelled *Paramecium* cells in cultures (Fig. 1C) as well as peritrich ciliates and the cell body of heliozoans in complex biofilms. The staining was bright, but faded quickly. Some peritrich ciliate cells stained brightly but labelling was concentrated at the outer rim of the cells. MitoFluor Green was not suited for all ciliate groups, due to the fact that specimens of the Scuticociliatida or Hypostomatida were not stained at all.

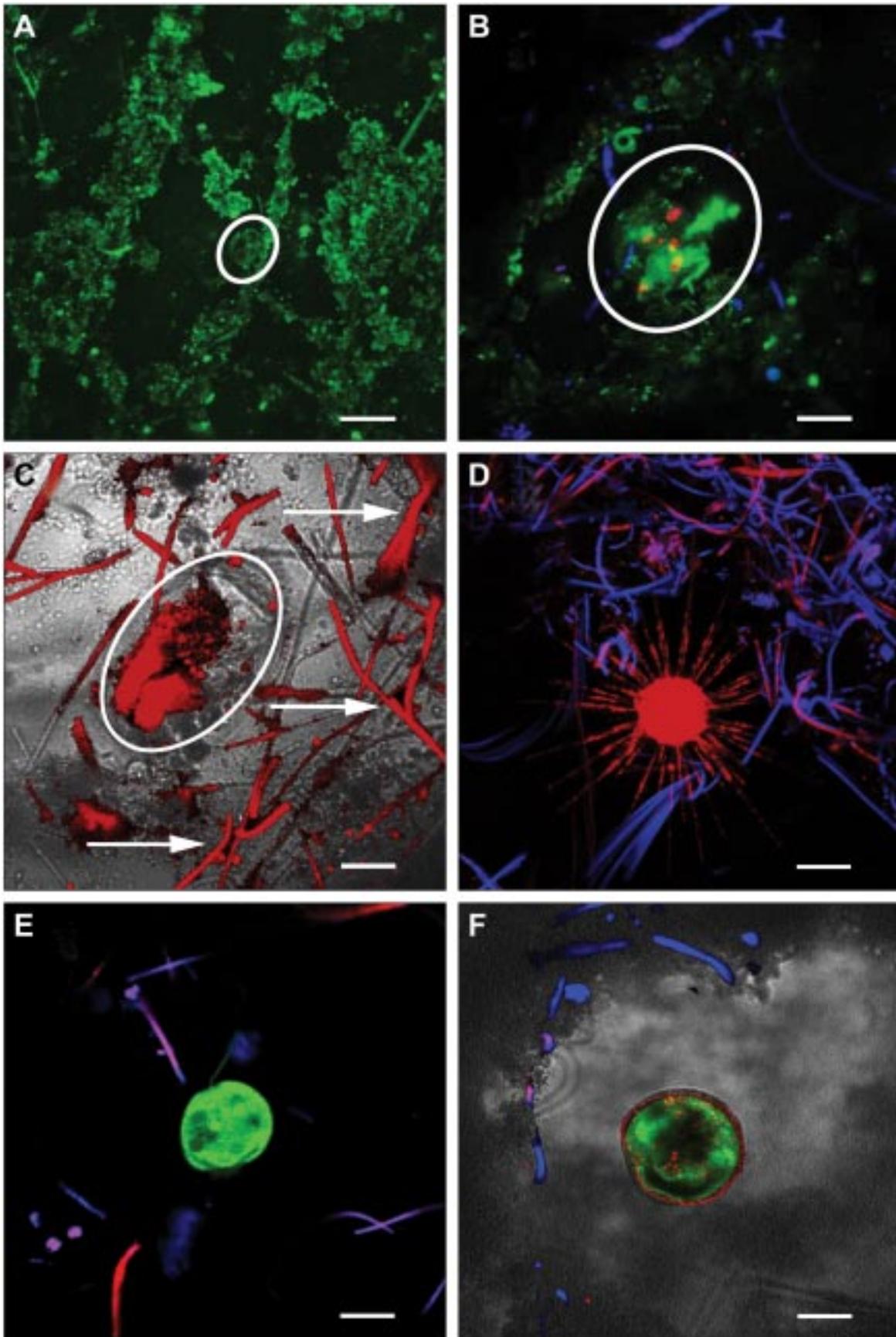
**CellTracker, CMFDA.** Chloromethylfluorescein diacetate (CMFDA) is a fluorescent chloromethyl derivative that freely diffuses through the membranes of live cells. Once inside the cell, these mildly thiol-reactive probes undergo what is believed to be a glutathione S-transferase-mediated reaction to produce membrane-impermeant glutathione fluorescent dye adducts. Haugland (1999) indicated a variety of applications for these dyes including cell tracing in mixed cultures (Yoshida *et al.* 1996), long-term viability assays (Poole *et al.* 1996) or measuring cellular glutathione content using flow cytometry (Hedley and Chow 1994). Furthermore, CMFDA was used to label live protozoan cells during grazing experiments (Li *et al.* 1996).

Both in cultures and in the biofilm samples, CellTracker stained the whole cell body of various protozoan groups (Figs 1E, 2E). In some cases structures like nuclei, oral organelles or basal bodies (flagellates) were more intensively stained. Staining of protozoans in the biofilm was very specific, and was limited to protozoans only. No other signals were visible using epifluorescence microscopy with different filter settings or the various excitation/emission options of the CLSM. Cell bodies of heliozoans were well stained and the vacuolized structure of the cytoplasm became visible. The axiopods were usually weakly stained but in some cases not at all. CellTracker may be also combined with LysoTracker which may reveal additional structural details (Fig. 2F).

**Imaging of Protists.** The application of CLSM to observe protozoans in situ presents special challenges due to their motile and responsive nature. Bacterial cells have proven relatively insensitive to scanning during CLSM (Caldwell *et al.* 1992). In contrast, we frequently observed that during the scanning process protozoan cells tried to escape from the beam or scanned region. We observed that sessile ciliates, frequently contracted their stalks, *Paramecium* exhibited fast swimming movements, and even semi-sessile heliozoa moved away. Immobilization could be achieved by adding a nickel sulfate solution to the samples. The reaction of cells differed from treatment to treatment, possibly depending

**Fig. 1 A-F.** Staining of cultured *Paramecium* sp. with different fluorescent stains. **A** - Dual channel image with Paclitaxel-Bodipy signal (= green) showing brightly stained fibrillary structures in the peristomal region (arrow) and autofluorescence inclusions (= red). The small green rods surrounding the protozoan cell are stained bacteria. **B** - Single channel image after staining with Rhodamine 6G chloride, staining may indicate mitochondria. **C** - Single channel image after staining with MitoFluor Green, small bodies may be mitochondria, large bright fluorescent bodies may indicate food vacuoles (arrows). **D** - Single channel image after staining with LysoTracker Red, staining may indicate lysosomal vesicles. **E** - Single channel image after staining with CellTracker (CMFDA), large brightly fluorescent bodies may indicate food vacuoles (arrow), other fluorescent regions are presumably thiol-rich areas. **F** - Effect of constant radiation with the laser beam on the integrity of heliozoan cells. Image shows transmission micrograph before (left) and after (right) exposure to laser light. Scale bars - 20  $\mu$ m





**Table 1.** List of stains used with indication of proposed target within the cells, wavelength of absorption and emission maximum, concentration of stock solution and dilution

Fluorochrome	Target (proposed)	Absorption [nm]	Emission [nm]	Stock-solution/ Dissolving reagent	Working solution	Amount of working solution	
						Cultures [µl/ml medium]	Biofilm [µl/sample]
Paclitaxel-BodipyFL	Tubulin	504	511	10 µM Ethanol	-	40	10-50
Rhodamine 123	Cytoplasm, Mitochondria	507	529	2,6 mM Water	1:100	100	100
Rhodamine 6G chloride	Mitochondria, Endoplasmatic Reticulum	528	551	1,7 mM Water	1:10	100	10
DiOC <sub>6</sub> (3)	Intracellular Membranes, Endoplasmatic Reticulum, Mitochondria	484	501	2,6 mM Ethanol	1:100	100	10
LysoTracker Red	Acid Organells and Vesicels, Lysosoms	577	590	1 M DMSO/ Water	1:10, 1:20 <sup>a</sup>	10	5 (stock) 100 (work.)
MitoFluor Green	Mitochondria	489	517	1,65 mM DMSO	1:200	40	100-400
CellTracker, CMFDA	Proteins and Peptides, (Thiols, e.g. Glutathione)	490	520	2,15 mM DMSO	1:10 <sup>a</sup> to 1:40	10	100-200

<sup>a</sup>Dilutions used for biofilm samples only

on the physiological condition of the cells. In some instances cells reacted with lysis after a rather short incubation time, whereas others were not affected over a longer period of time. For example, with some cell types such as heliozoans, lysis occurred within a short interval (< 1 min) after the beginning of scanning indicat-

ing the limitation of this imaging technique with this specific group (Fig. 1 F). Nevertheless, by stepwise lowering the concentration of nickel sulfate it was possible to immobilise the protozoa without lysis. In most cases staining and immobilisation alone had no negative impact on the protozoan cells and if observed by epi-

**Fig. 2 A-F.** Staining of protozoa in complex lotic biofilms grown with river water. Differentiation of biofilm and protozoa was done by concurrent imaging in the fluorescence and transmission mode. **A** - Single channel image after staining with DiOC<sub>6</sub>(3) showing equal staining of biofilm material and protozoa (circled). **B** - Triple channel image after staining with Paclitaxel-Bodipy demonstrating equal staining of biofilm bacteria and protozoa (circled), autofluorescence signals (red and blue) can be separated. **C** - Transmission image and single channel fluorescent image after staining with LysoTracker Red. The autofluorescence of algae and cyanobacteria (arrows) and the protozoan signal (circled) appear in the same fluorescence channel. **D** - Dual channel image after staining with LysoTracker Red, the 2-channel mode separated the autofluorescence of algae (blue) and cyanobacteria (pink) from the signal of the protozoan cell (red). **E** - Triple channel image after staining with CellTracker. The 3-channel mode clearly separated the autofluorescence of phototrophic biofilm cells from the signal of the protozoan cell (green). **F** - Transmission image and triple channel fluorescent image after staining with LysoTracker (red) and CellTracker (green). In the 3-channel mode the autofluorescence of phototrophic biofilm cells can be clearly separated from the signal of the double stained protozoan cell. Scale bars - 20 µm

fluorescence, phase contrast, or bright field microscopy no lysis was observed. Furthermore, it was usually possible to perform CLSM on most samples.

CLSM allowed detailed observation of protozoa in pure culture and in most cases in complex biofilm communities. Not all of the stains selectively stained protists in complex microbial communities. Although stains such as Paclitaxel-BodipyFL, DiOC<sub>6</sub>(3), LysoTracker, MitoFluor Green, and Rhodamine 6G demonstrated some specificity for protists they lacked the broad range necessary for general staining of all protozoa. In particular staining of small ciliates remained difficult. The most suitable fluorochrome for generally staining protozoa in complex environmental biofilms was CellTracker. This stain allowed specific staining of eukaryotic cells without non-specific binding to other biofilm constituents. The conditions for staining listed in Table 1 may be used as guidelines and require assessment for each specific application. For example, the various protozoa react differently to the stains, most ciliates and heliozoans are well stained, whereas some of the flagellates and amoebae are often only weakly or not stained at all. When staining protozoa with stains specific for mitochondria it becomes difficult to distinguish between ingested bacteria and mitochondria. In this case the bacteria fed to the protozoa may be pre-stained with nucleic acid specific fluorochromes having an emission signal in a different channel. Finally, the autofluorescence signal of chlorophyll allowed observation of grazed algae, cyanobacteria or endosymbionts within the protozoans.

In conclusion, this study reports the application of an in situ approach using CLSM for imaging protists in complex interfacial communities. The staining procedure offers the possibility of imaging protozoa alive without fixation and/or embedding. In some cases the fluorochromes also stained bacterial or polymeric biofilm constituents. Despite this fact, the usually transparent protozoan cells became visible within the complex architecture of environmental biofilms. By using this approach, protozoa can be identified and distinguished within the complex interfacial habitat by specific stains targeted for eukaryotic cell features (CellTracker) and by staining in combination with the information on protozoan size and morphology. The approach as suggested may have to be adjusted according to the fluorescence characteristics of the sample and the growth status of the protozoa. Finally, there is the potential to stain bacterial and polymeric biofilm constituents with other fluorochromes and fluor conjugated probes (Lawrence

*et al.* 1998a, Neu *et al.* 2001) which then may be clearly separated from the protozoan signal by co-localisation.

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## Complete Elimination of Endosymbiotic Algae from *Paramecium bursaria* and its Confirmation by Diagnostic PCR

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**Summary.** The green paramecium, *Paramecium bursaria*, has several hundred green algae in the cytoplasm. Symbiotic algae can be removed from their host cells by treatment with a herbicide, paraquat. The presence of symbiotic algae in *P. bursaria* can be microscopically examined by detecting the red fluorescence of the algal chlorophyll. However, etiolated algae could not be detected by fluorescent microscopic analyses. Therefore, the absence of symbiotic algae should be confirmed by examining the presence or absence of algal DNA in *P. bursaria*. In this study, we tried to detect the DNA of symbiotic algae by polymerase chain reaction (PCR) using plant genome-specific primers designed to amplify the DNA sequence in the ribulose-1,5-bisphosphate carboxylase small subunit (*rbcS*) encoding gene. Using this technique, it was confirmed that the DNA of endosymbiotic algae was absent in paraquat-treated paramecia.

**Key words:** algae-free paramecia, constant darkness, endosymbiosis, paraquat, ribulose-1,5-bisphosphate carboxylase small subunit, small subunit rDNA.

### INTRODUCTION

*Paramecium bursaria* is an interesting model for the study of a coexisting system in intracellular symbiosis. One cell of *P. bursaria* contains several hundred green algae in the cytoplasm as endosymbionts (Loefer 1936). The host and symbionts can be separated and cultured independently, and the algae-free paramecia

can be re-infected with the ex-symbiotic algae (Siegel 1960, Bomford 1965, Weis and Ayala 1979). Several studies have reported that *P. bursaria* can be freed from symbiotic algae by culturing in constant darkness (Siegel 1960, Karakashian 1963, Weis 1978), by irradiation with X-ray (Wichterman 1943, 1948), or by exposure to photosynthesis inhibitors such as 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) (Reisser 1976). However, in these experiments, reproducibilities are lacking, because defined experimental conditions have not been described.

To study the mechanism of endosymbiosis, it is important to establish a simple model using algae-free paramecia and homogeneous (cloned from a single cell) but not heterogeneous algae. It has not been elucidated whether the symbiotic algae in *P. bursaria* are composed of a

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single species or not. We have previously cloned endosymbiotic algae from *P. bursaria* and characterization of each clone was carried out (Nishihara *et al.* 1998). On the other hand, we have reported a new technique to produce algae-free paramecia by treating green paramecia with a herbicide, paraquat (Hosoya *et al.* 1995). We have shown that an appropriate exposure of *P. bursaria* to paraquat produces symbiotic algae-free strains without cellular damage or physiological distortion of cell division and conjugation. The newly established algae-free paramecia showed the same growth rate as that of normal green paramecia. These cloned algae and algae-free paramecia will be useful tools to elucidate a mechanism for the establishment of symbiosis between algae and host paramecia.

In our previous report, the absence or presence of symbiotic algae was simply assessed by observing paramecium cells under a fluorescence microscope, since chlorophylls in symbiotic algae emit red fluorescence (Hosoya *et al.* 1995). However, the presence of some chlorophyll-free algae that survived after the herbicide treatment could not be detected by microscopic observation. Therefore, an alternative method is required to clarify that the symbiotic algae are completely removed from their host cells.

In the present study, we attempted to detect the presence of algal genomic DNA by diagnostic polymerase chain reaction (PCR) using a set of plant-specific primers designed to amplify the DNA sequence in the ribulose-1,5-bisphosphate carboxylase small subunit (*rbcS*) encoding gene. Using this technique, it was confirmed that paraquat is useful for completely removing the algae from green paramecia.

## MATERIALS AND METHODS

### Strains and culture

One strain of *Paramecium bursaria* syngen 1 (NF-1, mating type I) was used in this work. The NF-1 strain was collected from the Koori-ike pond in Toyota-Gun, Hiroshima, Japan in 1999. The cells were cultured in a lettuce infusion supplemented with *Klebsiella pneumoniae* at 23°C under a light-dark cycle (LD=12:12h) at *ca* 1,000 lx of natural-white fluorescent light or in constant darkness (DD).

### Treatment with paraquat

The NF-1 cells in the stationary phase were treated with paraquat to remove the symbiotic algae. The cells were harvested using a nylon mesh (10 µm mesh size) and washed three times with a fresh lettuce

infusion. The cells were suspended in 50 ml of a fresh lettuce infusion containing 10 µg/ml of paraquat (Wako Pure Chem. Ind. Ltd., Osaka, Japan) at an initial cell density of 1,000 cells/ml and were incubated at 23°C under the LD condition. After treatment for 5 days, the cells were harvested and washed three times with a fresh lettuce infusion. Then the cells were transferred to a bacterized lettuce infusion and grown at 23°C under the LD condition. For the observation of paramecia, a Nikon Nomarski differential interference contrast (DIC) microscope (Nikon, Tokyo, Japan) and a fluorescence microscope (Optiphot BFD2, Nikon) were used.

### Preparation of DNA from *Paramecium*

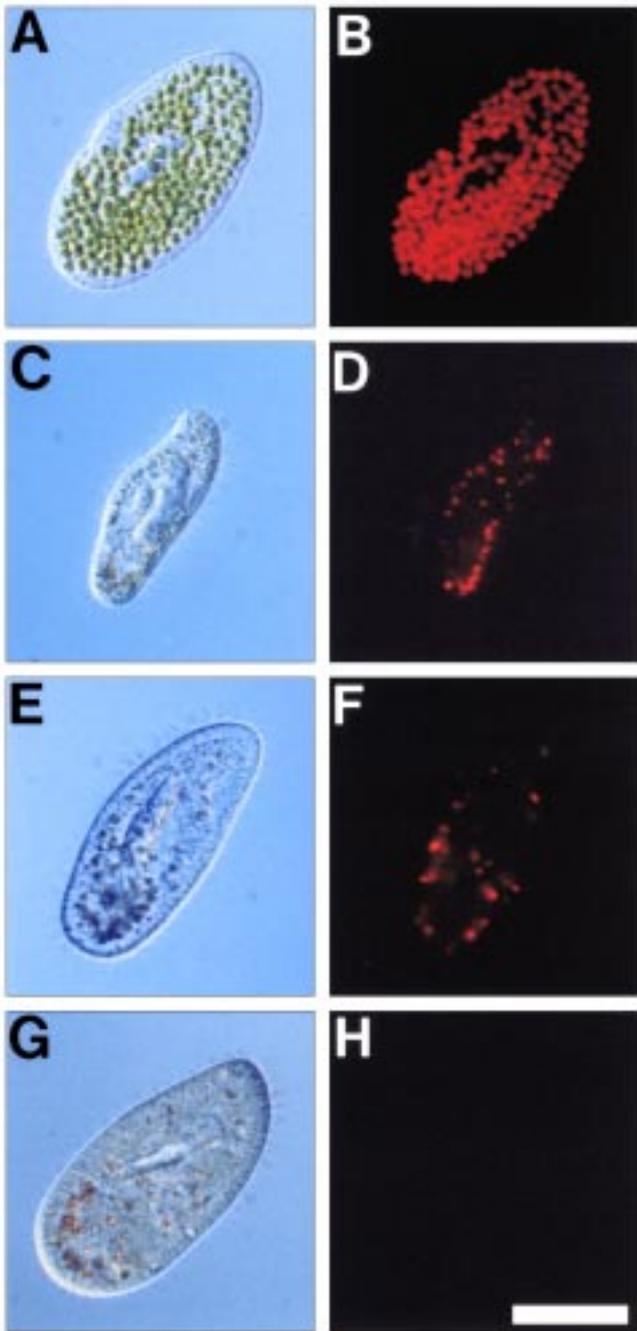
For the preparation of DNA, paramecia cells were harvested using a nylon mesh and washed three times with a CA medium (Nishihara *et al.* 1998). The cells (*ca* 2 x 10<sup>5</sup> cells) were frozen with liquid nitrogen and were crushed with glass beads (GMB-60, Nippon Rikagaku Kikai Co. Ltd., Tokyo, Japan) in DNA extraction buffer (100 mM Tris-HCl, pH 8.0, 10 mM EDTA and 1% sodium N-lauroyl sarcosinate) with phenol. Total DNA was subjected to a standard phenol/chloroform extraction and precipitated with ethanol.

### PCR amplification and sequence analysis

Two sets of oligonucleotide primers were used in this experiment. One set of primers designed to amplify the small subunit (SSU) rRNA-encoding gene (rDNA) was used for universal detection. The forward (SSU-5') and reverse (SSU-3') primers were 5'-TTGGAGGGCAAGTCTGGTGC-3' and 5'-TCCTTGGCAAATGCTTTCGC-3', respectively. It was confirmed that these sequences were found in SSU rDNA of green algae *Chlorella* sp. and *P. bursaria* 18S rDNA (DDBJ/EMBL/GenBank databases accession nos. AY004348 and AF100314). For the detection of an algae-specific DNA sequence, a second set of primers was designed using the sequence of *rbcS* encoding gene (cDNA) of *Chlorella vulgaris* (DDBJ/EMBL/GenBank databases accession no. AB058647). The forward (*rbcS*-1-5') and reverse (*rbcS*-1-3') primers were 5'-TTCTCCTACCTGCCCTCTG-3' and 5'-GCGTCAGTGCAGCCGAACAT-3', respectively. Partial sequences of SSU rDNA and *rbcS* DNA were amplified by PCR using the above primers and *Taq* DNA polymerase (TOYOBO Co. Ltd., Osaka, Japan). After denaturation for 3 min at 98°C, amplification was performed for 30 cycles of 45 s at 95°C, 45 s at 57°C and 1 min at 72°C and an additional extension period for 5 min at 72°C. The amplified PCR fragments sub-cloned into the pGEM-T vector (Promega, Madison, USA) were used for sequence analysis. Sequencing was carried out using a DNA sequencer ALF Express II (Amersham Pharmacia Biotech Inc., Uppsala, Sweden). The sequences were analyzed with the software DNASIS (Hitachi Software Engineering, Kanagawa, Japan).

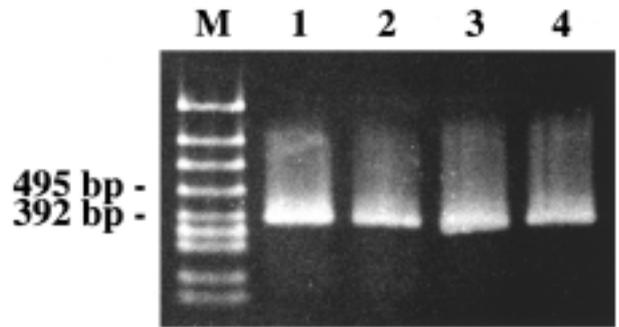
## Results and Discussion

Siegel (1960) reported that culturing green paramecia in DD for 24 days produced algae-free paramecia. However, in our previous study, because no algae-free paramecia were obtained by culturing green paramecia



**Fig. 1.** Nomarski differential interference contrast images (A, C, E and G) and their fluorescence images (B, D, F and H) of *Paramecium bursaria*. The images of algae-containing green paramecia (A and B), cells grown in constant darkness for 20 days (C and D) or 50 days (E and F) and symbiotic algae-free paramecia treated with paraquat (G and H) are shown. Symbiotic algae-free paramecia were produced by treatment with 10  $\mu\text{g/ml}$  of paraquat for 5 days. The algal chlorophyll fluoresces red (B, D and F) but no fluorescence was observed in the cells treated with paraquat (H). Scale bar - 40  $\mu\text{m}$

in DD for 22 days, we developed a new technique to produce algae-free paramecia by treatment with paraquat



**Fig. 2.** Diagnostic PCR analyses using primers SSUb-5' and SSUb-3'. The genomes of *Paramecium bursaria* cells treated with paraquat (lane 1), grown under constant darkness for 50 days (lane 2) and 20 days (lane 3) and algae-containing green paramecia (lane 4). PCR products were separated on 2% agarose gels and photographed after ethidium bromide staining. The molecular size markers (lane M) were  $\Phi\text{x}174/\text{Hinc II}$  digest. About 390-bp bands were observed in lanes 1 to 4

(Hosoya *et al.* 1995). In the present study, we compared the algae-eliminating efficiency of the conventional method (culturing in DD) and the newly proposed chemical method (using of paraquat) in green paramecia (NF-1). According to classical studies, we examined the effect of culturing green paramecia in DD for a period of 20 days, and in addition, the impact of 50 days of culturing in DD (the long-term DD condition) was also examined. We prepared the paraquat-treated cells (pqNF-1) and dark-grown cells cultured for 20 days (cd20NF-1) or 50 days (cd50NF-1) in the DD condition. The Nomarski DIC images and their fluorescence images of NF-1, cd20NF-1, cd50NF-1 and pqNF-1 cells are shown in Fig. 1. When NF-1 was observed under a fluorescence microscope, endosymbiotic algae could be visualized as red objects, due to the algal chlorophyll's red fluorescence (Fig. 1B). Notably, no fluorescence was detected in pqNF-1 (Fig. 1H). In contrast, several fluorescent particles were detected in both cd20NF-1 (Fig. 1D) and cd50NF-1 (Fig. 1F). To determine the average number of algae that remained in the dark-grown cells (cd20NF-1 and cd50NF-1), 25 cells were sampled from each culture and the remaining algae were counted under a fluorescence microscope (Table 1). The average number of symbiotic algae in each cd20NF-1 and cd50NF-1 cells was  $41.3 \pm 17.6$  cells and  $37.5 \pm 14.5$  cells, respectively. There were no algae-free paramecia in the dark-grown culture. The number of symbiotic algae was markedly reduced during the long-term culture in DD, but this method failed to complete the

**A**

NF-1  
*P. bursaria*  
TTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCAGCTCCAATAGCGTATACTTA  
TTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCAGCTCCAATAGCGTATACTTA

NF-1  
*P. bursaria*  
AGTTGTTGCAGTTAAAAAGCTCGTAGTTGAAATTCGGTCAGCCAGCTCTCAGGACATC  
AGTTGTTGCAGTTAAAAAGCTCGTAGTTGAAATTCGGTCAGCCAGCTCTCAGGACATC

NF-1  
*P. bursaria*  
-ATGGCGGATCTCCGCTTACAATCTACTCGAGCTTTAAACCTTGAGTTGTAGAGTAGGCA  
-ATGGCGGATCTCCGCTTACAATCTACTCGAGCTTTAAACCTTGAGTTGTAGAGTAGGCA

NF-1  
*P. bursaria*  
GTTTACCTTGAAAAAATTAGAGTGTTCAGGCAGGTTTTCGCCGGAATACATTAGCATG  
GTTTACCTTGAAAAAATTAGAGTGTTCAGGCAGGTTTTCGCCGGAATACATTAGCATG

NF-1  
*P. bursaria*  
GAATAATGGAATAGGACTTTAGCCTGATGTTGGTTTTGGCTATTGTAATGATTAATAGG  
GAATAATGGAATAGGACTTTAGCCTGATGTTGGTTTTGGCTATTGTAATGATTAATAGG

NF-1  
*P. bursaria*  
GACAGATGGGGCATTAGTATTTAATTGTCAGAGGTGAAATTCCTGGATTTATTAAGAC  
GACAGATGGGGCATTAGTATTTAATTGTCAGAGGTGAAATTCCTGGATTTATTAAGAC

NF-1  
*P. bursaria*  
TAACTTATGCCAAAGCATTGCGCAAGGA  
TAACTTATGCCAAAGCATTGCGCAAGGA

**B**

NF-1  
*Chlorella*  
TTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCAGCTCCAATAGCGTATACTTA  
TTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCAGCTCCAATAGCGTATACTTA

NF-1  
*Chlorella*  
AGTTGTTGCAGTTAAAAAGCTCGTAGTTGAAATTCGGT-----C-T-G-CCAGTC-T  
AGTTGTTGCAGTTAAAAAGCTCGTAGTTGAAATTCGGTAGGGTTCCTGCGGTCC-SCGGT

NF-1  
*Chlorella*  
T-CGGA--CA-TCAATGGCG--ATCTC-C-GCCT--ACAATCTACTCGAGCCTTAAAC  
TTCGGTGTGCACTGACGGCGCTATCTTGCTGCCGGGGACGGGCTCTGGGC--TTAA-

NF-1  
*Chlorella*  
CT-T-GAGT-T-GTAGAGTAGGC-AGTTTACCTTGAAAAAATTAGAGTGTTTTCAG-GC  
CTGTCCGGGACTCGG--AGYCGGCAGGTTACTTTGAGTAATTAATAGTG-TTCAAAGC

NF-1  
*Chlorella*  
AGGTTTTTCGC-CGGAATACATTAGCATGGAATAATG-CATAGGACTTTAGCCTGAT--  
AGGCTTACGCTCTGAATACATTAGCATGGAATAACAGCATAGGACTCTGGCCT-ATCT

NF-1  
*Chlorella*  
-GTTGGTTTTT--GG-CT--ATTGTAATGATTAATAGGGACAGATGGGGCATTAGTATT  
TGTGGTCTGTAGGACC-GGA-GTAATGATTAATAGGGACAGTGGGGCATTAGTATT

NF-1  
*Chlorella*  
TAATTGTCAGAGGTGAAATTCCTGGATTTATTAAGACTTAACTTATGCGAAAGCATTGCG  
TCATTGTCAGAGGTGAAATTCCTGGATTTATTAAGACTTAACTTATGCGAAAGCATTGCG

NF-1  
*Chlorella*  
CAAGGA  
CAAGGA

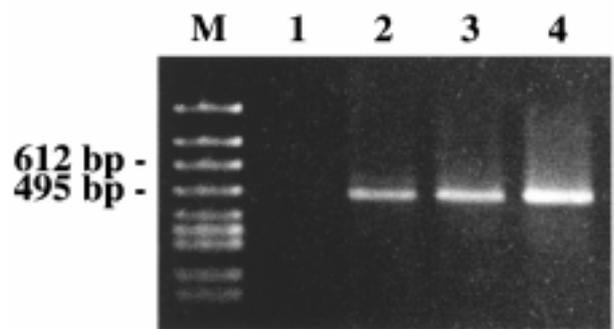
**Fig. 3.** Partial sequences of SSU rDNA amplified from NF-1 genome, 18S rDNA of *Paramecium bursaria* and *Chlorella* sp. SSU rDNA. A - comparison of nucleotide sequences between NF-1 SSU rDNA and 18S rDNA of *P. bursaria*. B - comparison of nucleotide sequences between NF-1 SSU rDNA and *Chlorella* sp. SSU rDNA. The SSU rDNA sequence of *Chlorella* sp. and the reported sequence of 18S rDNA of an unnamed *P. bursaria* strain collected from S. Nation River (Ontario, Canada) (Strüder-Kypke *et al.* 2000) were available from DDBJ/EMBL/GenBank databases (accession nos. AY004348 and AF100314, respectively). The differences in sequence length were compensated for by introducing alignment gaps (-) in the sequences and matched sites are framed. Arrows indicate the primer positions designed from 18S rDNA of *P. bursaria* and *Chlorella* sp. SSU rDNA. The sequences of NF-1 SSU rDNA shared 97.9% homology with *P. bursaria* 18S rDNA and relatively much weaker homology (74.6%) with *Chlorella* sp. SSU rDNA

**Table 1.** The number of symbiotic algae that remained in a single *Paramecium bursaria* sampled from three groups, cd20NF-1, cd50NF-1 and pqNF-1. The number of algae was counted under a fluorescence microscope. n - number of individuals examined, SD - standard deviation

Character	No. of symbiotic algae/paramecium (cells)				Rate of algae-free paramecium (%)	n
	Minimum	Maximum	Mean	SD		
cd20NF-1	13	81	41.3	17.6	0	25
cd50NF-1	9	64	37.5	14.5	0	25
pqNF-1	0	0	0	-	100	25

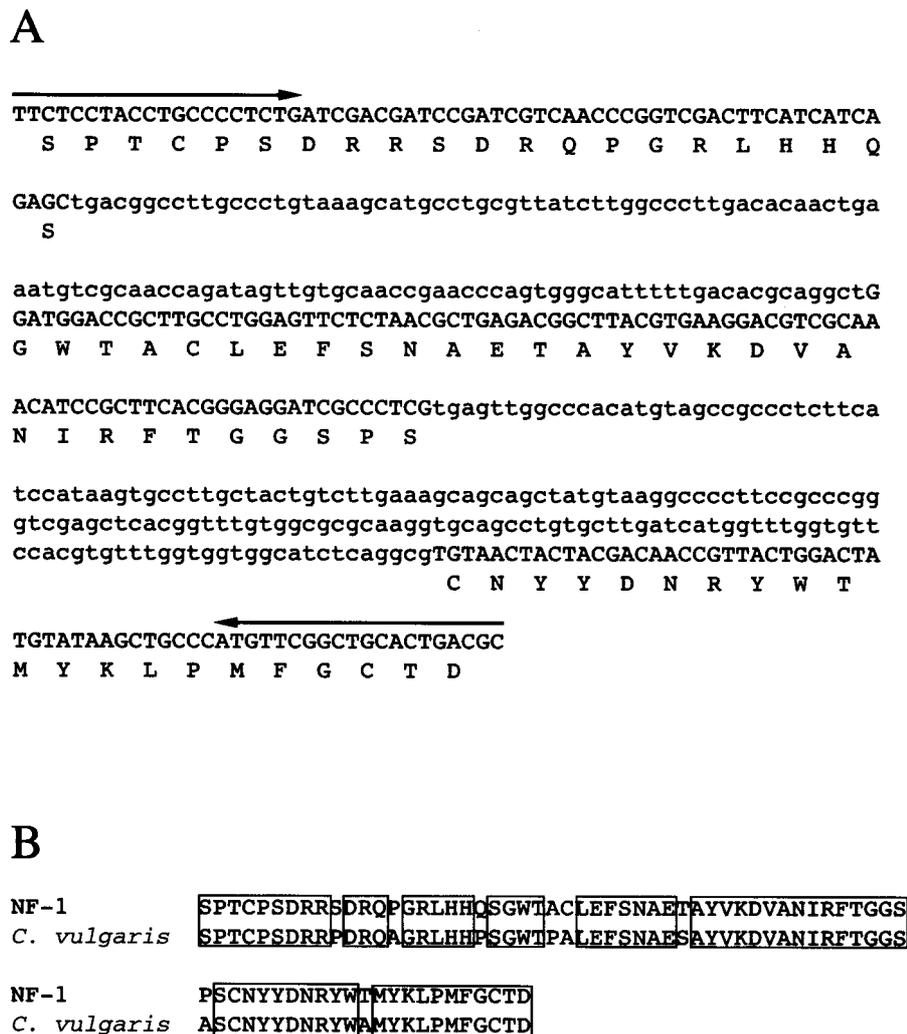
elimination of algae from the host cells. In contrast, no symbiotic algae were detected in the pqNF-1 cells (Table 1).

It is possible that some etiolated algae which lost chlorophyll survived in the pqNF-1 cells. Thus, the absence or presence of such algae should be proven by examining the presence of algal genomic DNA in the host paramecia. Electron microscopic observation may be applicable for this purpose, but the evidence is weaker compared to that obtained by a molecular method. Therefore, we attempted to detect the DNA of endosymbiotic algae that survived in host paramecia by diagnostic PCR using plant gene-specific primers. Total genomes were collected from pqNF-1, cd20NF-1, cd50NF-1 and NF-1 cells. The SSU primers were used as a positive control so that the yield of PCR products from four different samples could be normalized. As seen in Fig. 2, about 390-bp fragments were amplified from all of the pqNF-1, cd20NF-1, cd50NF-1 and NF-1 genomes. The size of the amplified DNA was consistent with the expected size of the partial sequence of *P. bursaria* 18S rDNA to be amplified by SSU primers. The PCR products were subjected to DNA sequencing. In Fig. 3, the determined nucleotide sequence of SSU rDNA amplified from the NF-1 genome was compared with the *P. bursaria* 18S rDNA sequence and *Chlorella* sp. SSU rDNA sequence. The sequence of NF-1 SSU rDNA shared 97.9% similarity with *P. bursaria* 18S rDNA, while only 74.6% similarity in the DNA sequence was found between NF-1 SSU rDNA and *Chlorella* sp. SSU rDNA. These results suggested that the sequence of SSU rDNA amplified from the NF-1 genome corresponded to 18S rDNA of the host paramecia. Although it was expected that the PCR products amplified from cd20NF-1, cd50NF-1 and NF-1 would include both the *P. bursaria* 18S rDNA and

**Fig. 4.** Diagnostic PCR analyses using primers *rbcS*-1-5' and *rbcS*-1-3'. The genomes of *Paramecium bursaria* cells treated with paraquat (lane 1), grown under constant darkness for 50 days (lane 2) and 20 days (lane 3) and algae-containing green paramecia (lane 4). The molecular size markers (lane M) were the same as Fig. 2. About 500-bp PCR products were observed in lanes 2 to 4 but not in lane 1

alga SSU rDNA fragments, the sequence analysis showed that only 18S rDNA was amplified.

Secondly, we carried out algal DNA-specific detection using the *rbcS* primers. About 500-bp PCR products were obtained from the cd20NF-1, cd50NF-1 and NF-1 genomes, but no signal was detected in pqNF-1 (Fig. 4). The 500-bp fragments obtained from cd20NF-1, cd50NF-1 and NF-1 were subjected to sequence analysis. The determined nucleotide sequence in the 500-bp fragment amplified from the NF-1 genome is shown in Fig. 5. Since the 500-bp fragment was amplified by genomic PCR, the obtained nucleotide sequence included the introns. The deduced amino acid sequence of the encoded protein was compared with that of *Chlorella vulgaris* C-169 *rbcS*. The deduced amino acid sequence of the NF-1 PCR product shared 88.8% similarity with that of *C. vulgaris* *rbcS*. The deduced



**Fig. 5.** Nucleotide and deduced amino acid sequences of *rbcS* DNA amplified from the NF-1 genome. A - the determined nucleotide sequence of *rbcS* DNA amplified from the NF-1 genome is shown, together with the deduced amino acid sequence of the encoded protein. The sequences estimated to be introns were uncapitalized. Arrows indicate the primer positions designed from *Chlorella vulgaris* C-169 *rbcS* cDNA (DDBJ/EMBL/GenBank databases accession no. AB058647). B - deduced amino acid sequence of NF-1 *rbcS* was compared with that of *C. vulgaris* C-169 *rbcS*

amino acid sequence of 500-bp fragments obtained from cd20NF-1 and cd50NF-1 were identical to that of the NF-1 PCR product. These results suggested that the *rbcS*-targeting primers are adequate for symbiotic algae-specific detection.

In conclusion, it became clear that treatment of *P. bursaria* with paraquat only for 5 days completely eliminated the genomic DNA of the symbiotic algae, indicating that algae-free *P. bursaria* were produced. In contrast, the present study showed that no algae-free paramecia were produced by a conventional method in which *P. bursaria* was cultured in DD. In addition, the rapid growth of the host cells in DD will accelerate the

clonal aging. These results suggest that culturing in DD is not suitable for producing algae-free paramecia. The new technique using paraquat can produce algae-free paramecia without physiological damage or distortion in cell division and conjugation (Hosoya *et al.* 1995, Nishihara *et al.* 1996). As presented here, treatment with paraquat is a powerful technique to produce completely algae-free paramecia.

### Perspectives

Our results also showed that symbiotic algae could survive without photosynthesizing under the long-term DD condition. It is possible that the host cells supply the

least amount of nutrition required for survival of symbiotic algae. To elucidate the endosymbiotic mechanism of *P. bursaria* and symbiotic algae, it is important to investigate the flow of nutrition and metabolites between hosts and symbionts.

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## Comparative Studies on Two Closely Related Species *Uronemella filificum* (Kahl, 1931) and *Uronema elegans* Maupas, 1883 with Redescription of *Paranophrys marina* Thompson et Berger, 1965 (Ciliophora: Scuticociliatida) from China Seas

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**Summary.** The living morphology, infraciliature and silverline system of 3 known marine scuticociliates, *Uronemella filificum* (Kahl, 1931) Song et Wilbert, 2002 (formerly *Uronema filificum* Kahl, 1931), *Uronema elegans* Maupas, 1883 and *Paranophrys marina* Thompson et Berger, 1965 are reinvestigated and described. Based on the populations isolated from China seas, improved diagnoses for all three species are supplied. Diagnosis for *Paranophrys marina*: marine slender *Paranophrys in vivo* ca 35-45 x 10-15 µm with sharp apical and narrowed posterior end; membranelle 1 two-rowed and dominant long, almost conjoint to membranelle 2; scutica with several singled basal bodies arranged in line; 10 densely ciliated somatic kineties; contractile vacuole large and caudally positioned, opens at the posterior end of the somatic kinety 2; one oval macro- and one small micronucleus. The well-known *Uronemella filificum* can be recognized by the following characters: marine pear-shaped *Uronemella in vivo* ca 25-45 x 12-30 µm with conspicuously large apical plate and typically thigmotactic living behaviour; membranelle 1 one- or partly two-rowed, only slightly away from apical area; 16-23 somatic kineties with densely packed cilia; contractile vacuole pore at posterior end of somatic kinety 2; one macro- and one micronucleus; extrusomes present. *Uronema elegans* is characterized by cylindrical or kidney-like body shape *in vivo* ca 30-50 x 20-30 µm with small apical plate and conspicuously reticulate pellicle ridges; membranelle 1 one- or partly two-rowed, which is considerably away from the apical area; 23-26 somatic kineties; contractile vacuole positioned sub-caudally near ventral margin with its pore at posterior end of somatic kinety 2; one macro- and one micronucleus; extrusomes bar-like, densely distributed.

**Key words:** marine ciliates, morphology, *Paranophrys marina*, Scuticociliatida, taxonomy, *Uronema elegans*, *Uronemella filificum*.

### INTRODUCTION

Morphological researches on the order Scuticociliatida using modern methods have demonstrated that diversity in this specialized group exhibits a greater richness -not

only in species number, but also in ecological or physiological phenotypes- than considered before. Accordingly, more and more work has revealed that taxonomic studies based only on the silver impregnations have led to new problems in species identification even for the well-known scuticociliates (Berger and Thompson 1960; Borror 1963; Dragesco 1963; Small 1967; Thompson 1972; Grolière 1974, 1980; Agamaliev 1978; Small and Lynn 1985; Fernandez-Leborans and Novillo 1994). This is mainly because many criteria used for species sepa-

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ration, e.g. the number of somatic kineties, body shape and size *et al.*, are confluent or overlap among many morphologically-similar organisms. Thus, it is difficult to recognize a species merely depending on data which derive from merely few specimens or a certain population (Wilbert 1986, Foissner *et al.* 1994, Petz *et al.* 1995, Foissner 1996).

In some of our recent work, many little-known marine forms have been reinvestigated (Song 1993, 2000; Xu and Song 1999; Song and Wilbert 2000), and as a part of that series, three scuticociliates collected from the Yellow Sea and the South China Sea are described in the present paper.

## MATERIALS AND METHODS

*Paranophrys marina* was collected in summer of 1998 from an open off-shore maricultural pond for mollusc-farming (*Argopecten irradians*) near Qingdao (Tsingtao, 36° 08' N; 120° 43' E); *Uronema elegans* was isolated from the mantle cavity of a marine mollusc (*Sinonovacula constricta*) near Qingdao in June 2001, while *Uronemella filificum* was found in July 2001 from coastal water in the suburb area of Zhanjiang (21° 12' N; 110° 18' E), Guangdong Province (Fig. 1).

Observations on living cells were carried out with a microscope equipped with Normarski differential interference optics. Protargol (Wilbert 1975) and Chatton-Lwoff method (Corliss 1953) were used for revealing the infraciliature and silverline system.

Drawings of impregnated specimens were conducted with the help of camera lucida; measurement was performed under the 1250 x magnification. Terminology is mainly according to Corliss (1979).

## RESULTS AND DISCUSSION

### About the definition of the genus *Uronemella* Song et Wilbert, 2002 (in press)

The newly-established genus *Uronemella* Song et Wilbert, 2002 differs from the well-known *Uronema* Dujardin, 1841 basically in living features: (1) the former exhibits a dominant frontal plate, and hence has an inverted pear-shaped or stout-oval body shape (*vs.* oval, ellipsoid to cylindrical); (2) the cytostome is positioned post-equatorially (*vs.* located equatorially) and (3) thigmotactic locomotion (*vs.* non-thigmotactic in the latter). The movement of *Uronema* demonstrate a particular thigmotactic manner, the "rotation-movement": with help of a thread-like structure which derives from the caudal cilium and hence attaching temporarily to substrate and making continuously a rotation behaviour.

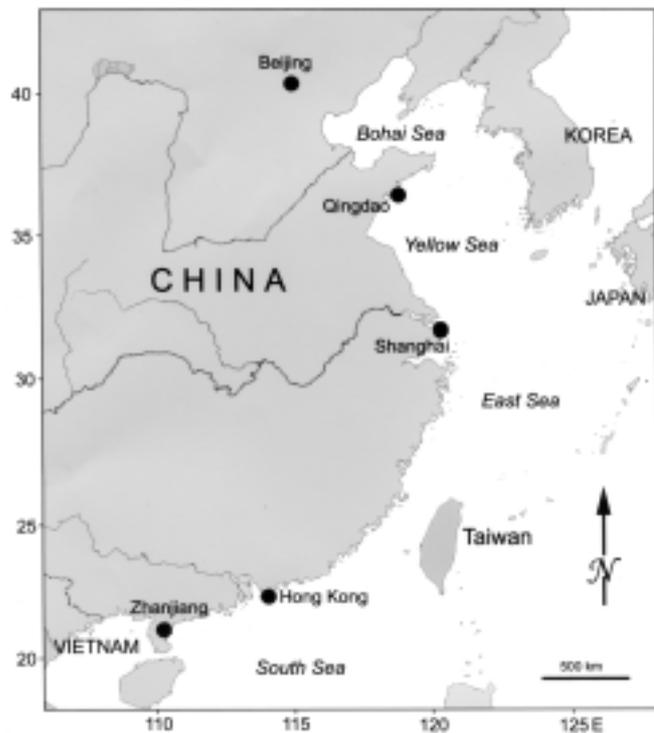


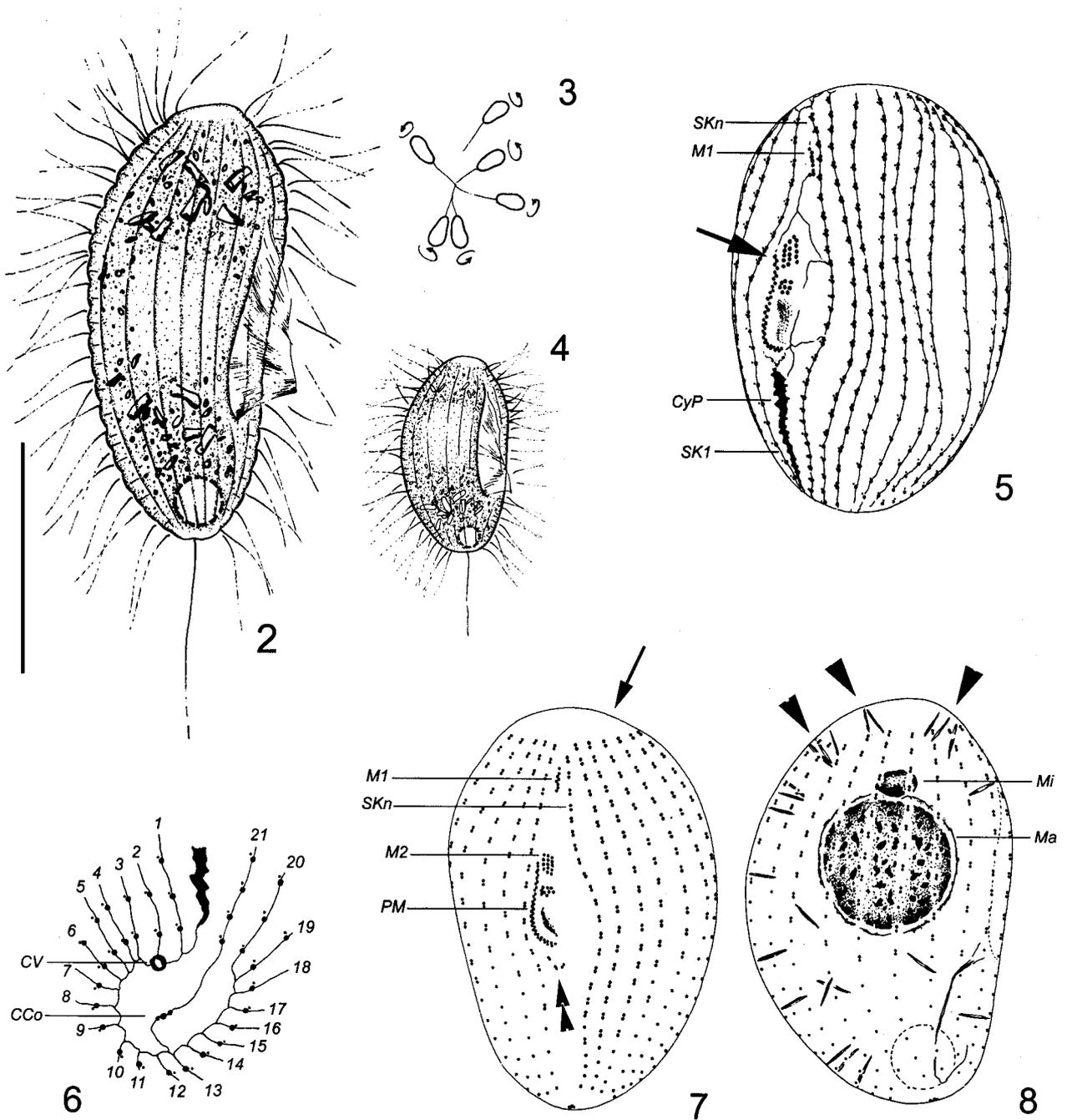
Fig. 1. Map of sampling sites (Qingdao and Zhanjiang)

Kahl (1931, p. 356) described the movement for *Uronema filificum* as follows: "...nach einiger Zeit der Ruhe sieht man auf dem Objektträger mehr und mehr Individuen, die sich an Detritus angeheftet haben und einen Faden langsam bis auf 1/2 mm ausziehen, an dem sie rotierend pendeln; sie lösen sich aber oft ab..." According to this definition, *Uronema filificum* Kahl, 1931 was transferred into the genus *Uronemella* by Song and Wilbert (2002).

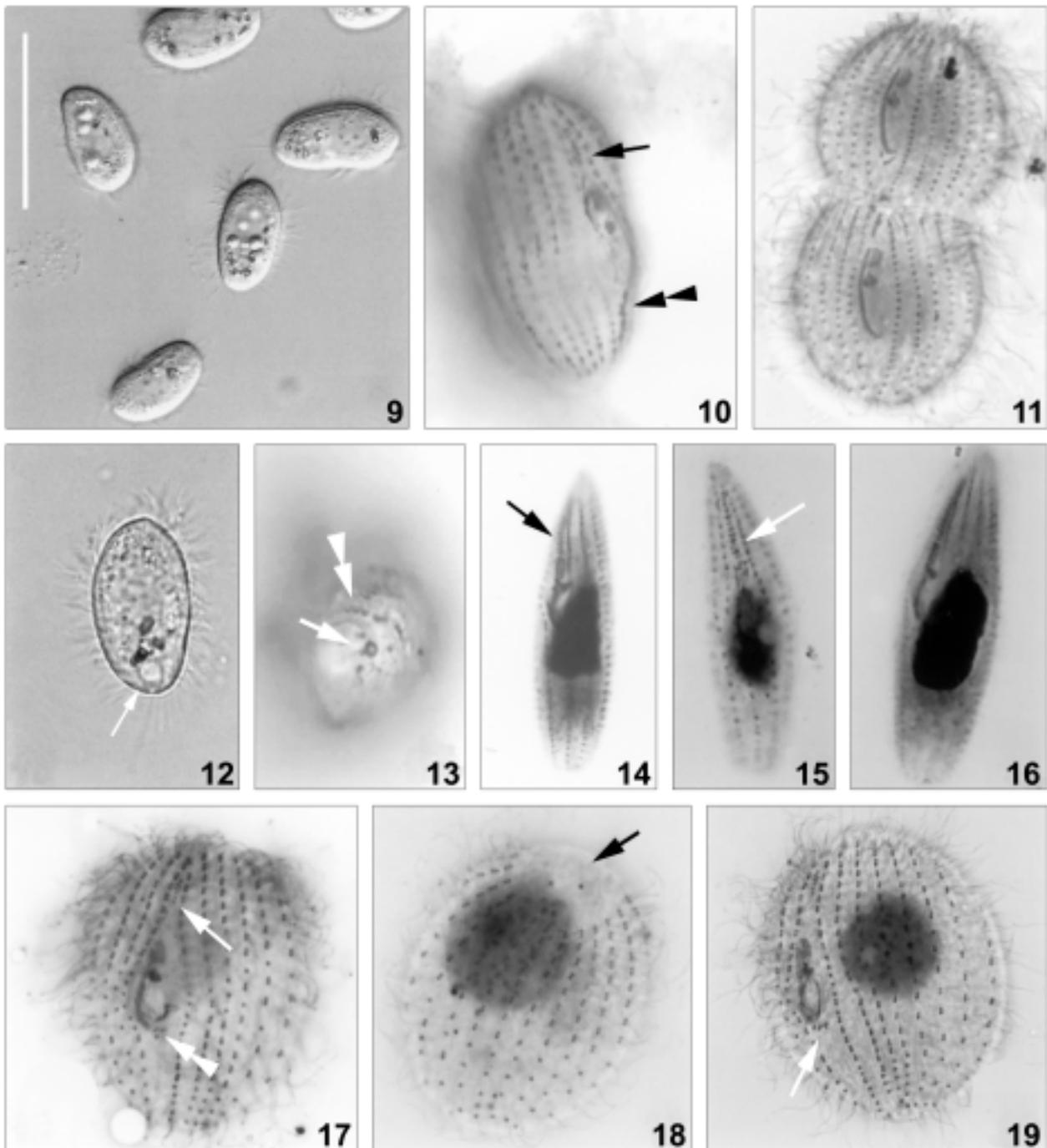
In 1980, Jankowski proposed a new genus *Uronemita* with *U. filificum* as its type species (Jankowski 1980). However, according to art 13 of the Code of Zoological Nomenclature (ICZN 1999), *Uronemita* is an invalid name because no definition or description has been supplied in the original report for this new taxon.

### *Uronemella filificum* (Kahl, 1931) Song et Wilbert, 2002 (Figs 2-8, 9-13, 17-19, 28, 33; Table 1, 2)

- Syn. 1931 *Uronema filificum* - Kahl, *Tierwelt Dtl.*, **21**: 356 (original)  
 1963 *Uronema filificum* - Borror, *Trans. Am. Microsc. Soc.* **82**: 125  
 1968 *Uronema elegans* - Czapik, *Acta Protozool.* **5**: 225  
 1968 *Uronema filificum* - Thompson et Kaneshiro, *J. Protozool.* **15**: 141  
 1981 *Uronema filificum* - Wilbert et Kahan, *Arch. Protistenkd.* **124**: 78  
 1985 *Urocyclon filificum* - Small et Lynn, In: *An Illustrated Guide to the Protozoa*  
 1986 *Uronema elegans* - Dragesco et Dragesco-Kernéis, *Faune Trop.* **26**: 347  
 1996 *Uronema filificum* - Pérez-Uz, Song et Warren, *Europ. J. Protistol.* **32**: 263



**Figs 2-8.** *Uronemella filificum* *in vivo* (2-4), after silver nitrate (5, 6) and protargol impregnations (7, 8). 2 - right lateral view of a representative individual; 3 - motion scheme, to show 4 cells conjoint together with the caudal thread attached on the bottom; 4 - a well-fed specimens after culture; 5 - ventro-lateral view of silverline system; arrow marks the anterior end of the paroral membrane; 6 - caudal view, to show the silverline pattern; 7 - ventral view of infraciliature, note the conspicuous cilia-free apical plate (arrow) and the small scuticia with only 3 pairs of basal bodies (arrowheads); 8 - right lateral view of infraciliature, to show the extrusomes (arrowheads) and nuclear apparatus. Abbreviations: CCo - caudal cilium complex, CV - pore of contractile vacuole, CyP - cytophyge, M1-2 - membranelle 1-2, Ma - macronucleus, Mi - micronucleus, PM - paroral membrane, SK1, n - the first and the last somatic kinety. Scale bar - 20  $\mu$ m



**Figs 9-19.** Photomicrographs of *Uronemella filificum* (9-13, 17-19) and *Paranophrys marina* (14-16) from life (9, 12), after silver nitrate (10, 13) and protargol impregnations (11, 14- 19). **9** - slightly pressed cells; **10** - lateral view, to show the buccal apparatus (arrow marks the membranelle 1) and the cytopyge (arrowheads); **11** - ventral view of a late divider; **12** - lateral view, arrow indicates the contractile vacuole; **13** - caudal view, arrow marks the contractile vacuole pore, while arrowheads indicate the last somatic kinety which extends through the caudal cilia complex; **14, 15** - lateral and ventral view, to show the prolonged membranelle 1 (arrows in 14, 15); **16** - lateral view, to show macronucleus; **17** - ventral view, to show the buccal apparatus; arrow indicates the membranelle 1, while the arrowheads mark the scuticum; **18** - dorsal view, to show the dominant apical plate (arrow); **19** - ventral view, arrow demonstrates the scuticum. Scale bar - 40  $\mu$ m

**Table 1.** Morphometrical characterization of *Uronemella filificum* population I (first line, original), *U. filificum* population II (second line, original), *Uronema elegans* (third line, original) and *Paranophrys marina* (fourth line, data after Xu and Song 1999). Data based on protargol impregnated specimens (according to the present authors). All measurements in  $\mu\text{m}$ . CV- coefficient of variation, Max - maximum, Mean - arithmetic mean, Min - minimum, n - sample size, SD - standard deviation, SE - standard error of the mean.

Character	Min	Max	Mean	SD	SE	CV	n
Body length	20	36	31.9	8.19	2.05	25.7	16
	28	43	34.4	3.31	0.74	9.6	20
	33	45	36.8	3.45	0.92	9.4	14
	35	53	46.6	4.05	1.08	8.7	14
Body width	10	25	21.1	8.18	2.04	38.8	16
	16	28	23.2	3.64	0.81	15.7	20
	19	25	20.9	1.77	0.47	8.5	14
	11	16	12.9	1.44	0.39	11.2	14
Length of buccal field	11	23	17.1	3.87	1.12	22.7	12
	18	24	21.0	1.75	0.44	8.3	16
	18	24	20.3	2.01	0.58	9.9	12
	23	29	26.6	2.30	0.73	8.7	10
Number of somatic kineties	21	22	21.3	0.48	0.12	2.2	16
	22	23	22.8	0.41	0.09	1.8	20
	23	24	23.1	0.36	0.10	1.6	14
	10	10	10	0	0	0	14
Number of basal bodies in somatic kinety 1*	17	23	19.6	2.15	0.14	11.0	7
	18	23	20.3	1.56	0.45	7.6	12
	ca 23	-	-	-	-	-	-
	-	-	-	-	-	-	-
Number of basal bodies in somatic Kinety n	21	28	23.3	1.82	0.49	7.8	14
	23	32	27.6	3.28	0.88	7.6	14
	ca 28	-	-	-	-	-	-
	-	-	-	-	-	-	-
Number of basal bodies in membranelle 1	7	9	-	-	-	-	3
	ca 6	-	-	-	-	-	-
	ca 7	-	-	-	-	-	-
	20	27	-	-	-	-	-
Number of macronucleus	1	1	1	0	0	0	>100
	1	1	1	0	0	0	>100
	1	1	1	0	0	0	>100
	1	1	1	0	0	0	>100
Length of macronucleus	7	20	13.0	4.80	0.30	36.9	15
	10	14	11.5	1.37	0.34	11.9	16
	11	18	13.8	2.68	0.74	19.5	13
	12	18	15.0	1.95	0.59	13.0	11

\* Dikinetids counted as single ones

Though this “well-known” species has been mentioned or redescribed for several times after Kahl (Borror 1963, Thompson and Kaneshiro 1968, Wilbert and Kahan 1981, Small and Lynn 1985), all these studies supplied little living information which is, in authors’ opinion, insufficient for species identification. On the basis of the previous descriptions as well as the data obtained from the Chinese population, an improved diagnosis is added hence.

**Improved diagnosis:** marine *Uronemella in vivo* ca 25-45 x 12-30  $\mu\text{m}$  with conspicuously large apical

plate and typically thigmotactic living behaviour; membranelle 1 one- or partly two-rowed, slightly away from apical area; 16-23 somatic kineties with densely packed cilia; contractile vacuole pore at posterior end of somatic kinety 2; one macro- and one micronucleus; extrusomes present.

**Description of Chinese populations:** cells *in vivo* about 25-35 x 15-25  $\mu\text{m}$  in the Zhanjiang population, while 30-45 x 20-30  $\mu\text{m}$  in the Qingdao population. Body shape rather constant, inverted pear-shaped or oval when well-fed with ventral side slightly concave, while

**Table 2.** Morphological and morphometrical characterization of *Uronemella filificum* and *Uronema elegans*. All measurements in  $\mu\text{m}$ . [CVP - contractile vacuole pore; EP - about equatorially positioned; Ex - extrusomes; BM - behind mid-body; SK - somatic kinety(ies); SS - silverline system; ? - data not available]

Character	<i>Uronemella filificum</i>	<i>U. filificum</i>	<i>U. filificum</i>	<i>U. filificum</i>	<i>U. filificum</i>	<i>Uronema elegans</i>	<i>U. elegans</i>	<i>U. elegans</i>	<i>U. filificum</i> *	<i>U. elegans</i>
Cell size <i>in vivo</i>	ca 30 (length)	23-43 x 12-27	27-32 x 12-20	25-35 x 15-25	30-45 x 20-30	35-50 x 20-25	?	?	?	40-90
Cell size after impregnation	?	23-31 x 14-21	?	20-36 x 10-25	28-43 x 16-28	33-45 x 19-25	40-50 (length)	35-58 x 20-34	33-37 x 24-31	?
Number of SK	ca 18	16-19	16-17	21-22	22-23	23-24	25-26	23-24	ca 21	?
Position of CVP	end of SK2	end of SK2	end of SK2	end of SK2	end of SK2	end of SK2	?	?	?	?
Reticulate -stripes on cell surface ( <i>in vivo</i> )	?	?	?	absent	absent	present	?	?	?	present
Body shape	oval, inverted pear-shaped	inverted pear-shaped	inverted pear-shaped	slender, inverted pear-shaped	plump D-shaped	cylindrical	?	elongate cylinder-shaped	oval	elongate, cylinder-shaped
Apical plate	very large	very large	very large	very large	very large	relatively small	?	relatively small	?	relatively small
Glutinous thread extended from caudal cilium	present	present	present	present	present	absent	?	absent	?	absent
Buccal field	?	?	?	shallow and long	shallow and long	very deep and short	?	?	?	?
Position of cytostome	BM	BM	BM	BM	BM	EP	EP	EP	BM	EP
Extrusomes	?	?	?	present	present	present	?	?	?	?
Structure / position of membranelle 1	one-rowed slightly sub-apical	one-rowed slightly sub-apical	one-rowed slightly sub-apical	partly two-rowed slightly sub-apical	one-rowed slightly sub-apical	partly two-rowed considerably sub-apical	one-rowed considerably sub-apical	one-rowed considerably sub-apical	one-rowed slightly sub-apical	?
Sample location	Red Sea, Israel	Virginia coast, USA	Florida coast, USA	South China Sea Zhanjiang, China	Yellow Sea, Qingdao, China	Yellow Sea, Qingdao, China	Caspian Sea, Georgia	Virginia coast, USA	Cotonou, Africa	North Sea, Germany
Data resource	Wilbert, Kahan 1981	Thompson, Kaneshiro 1968	Borrer 1963	original	original	Song, Wilbert 2002	Agamaliev 1974	Thompson, Kaneshiro 1968	Dragesco, Dragesco-Kernéis 1986	Kahl 1931

\* Described as *Uronema filificum*

**Table 3.** Morphological comparison of *Uronemella filificum* and *Uronema elegans*.

Character	<i>Uronemella filificum</i>	<i>Uronema elegans</i>
Body shape	inverted pear-shaped	cylindrical to kidney-shaped
Apical plate	conspicuously large, about 1/3 of body width	small, definitely <1/3 of body width
Anterior end of membranelle 1	slightly away from apical plate	conspicuously away from apical plate
Pellicle feature	slightly notched	strongly notched with reticulate ridges on cell surface
Extrusomes	positioned within kineties, not highly developed, generally not recognizable after silver nitrate impregnation	highly developed and densely arranged, positioned often slightly away from kineties; always clearly to discern after silver nitrate impregnation
Number of somatic kineties	16-23 (mostly <i>ca</i> 18)	23-27
Arrangement of basal bodies within somatic kinety n and n-1	closely packed till posterior end	loosely distributed (especially in the posterior portion)
Buccal field (buccal apparatus) after impregnation	area between somatic kinety 1 and n proportionally wide and dominant	(as an area) small and narrow, relatively inconspicuous
Buccal cavity <i>in vivo</i>		
(1) when viewed from side	inconspicuous (shallow and often difficult to recognize)	as a clear area, small but very deep; clearly to discern
(2) when viewed ventrally	slender water-drop-shaped, long and always as a hyaline area	almost not recognizable
Position of cytostome	posterior to equatorial level	on the equatorial level
Cytopyge	relatively long	proportionally short
Position of contractile vacuole	caudally positioned	sub-caudally near ventral side
Behaviour	typical thigmotactic, always making “ <i>filificum</i> ”- movement	non-thigmotactic, completely quiet on substrate
Sticky thread deriving from caudal cilium	present	absent

dorsally conspicuously convex; cross section more or less bilaterally flattened. Frontal end flat, truncated with conspicuous apical plate, which is about 1/3 of body width, while posteriorly narrowly rounded (Fig. 2). In culture, cells usually plump and more oval than pear-shaped (Fig. 4). Buccal field about 3/5 of body length; cytostome conspicuously posterior to mid-body level (Figs 2, 4), which is as a narrow field (or slender-water-drop-shaped) when viewed from ventral side. Pellicle thin and inconspicuously notched. Extrusomes about 1.5  $\mu\text{m}$  long, bar-shaped, closely beneath pellicle, *ca* 3  $\mu\text{m}$  long after protargol impregnation (Figs 2, 8).

Cytoplasm colourless to slightly greyish, contains many to numerous granules *ca* 0.5-1  $\mu\text{m}$  across and crystals of different size and shape (Fig. 2). Macronucleus large, round to oval, located centrally. Contractile vacuole caudally positioned (Figs 2, 4, 12).

Cilia about 8  $\mu\text{m}$  long, densely arranged; caudal cilium *ca* 15  $\mu\text{m}$  long. Two manners of locomotion: (1) as attaching to substrate, making typical rotation movement. In this case, cells often several together conjoined with their threads (about 10  $\mu\text{m}$  long) deriving from caudal cilium, or sticking to debris while slowly rotating around longitudinal axis of body in both cases (Fig. 3); (2) when disturbed, cells moving very quickly, swimming in zig-zag pattern.

Somatic ciliature of the Zhanjiang population as shown in Figs 7 and 8: mostly 21 ciliary rows extending over entire length of body, composed of close-set dikinetids in anterior half of body, while monokinetids loosely-arranged in posterior half. As exception, left-most kineties (or SKn, SKn-1; to left of buccal field) conspicuously more densely ciliated than other nearby ones with almost entirely dikinetids (Figs 7, 17, 19).

Buccal apparatus similar to its congeners. Membranelle 1 positioned near apical plate and clearly separated from other membranelles, consisting of *ca* 7-9 basal bodies, which form often 2-rowed structure in the middle portion (Figs 7, 19). Membranelle 2 and 3 short, near to each other, each consisting of 3 rows of basal bodies. Paroral membrane on right of shallow buccal cavity, composed zigzagging row of basal bodies, extending anteriorly to about middle of membranelle 2. Scutica consisting of 3 to 4 pairs of basal bodies (Figs 7, 17; arrowheads).

Silverline system as shown in Figs 5, 6, 10 and 13: pore of contractile vacuole at posterior end of somatic kinety 2 (Fig. 13, arrow); line from somatic kinety n (left-most one to buccal field) extending posteriorly through caudal complex (CCo) and connecting dorsally with

about kinety 12-13 (Fig. 6). Cytopyge (CyP) long, irregularly shaped (Fig. 5).

**Ecological features:** This species was isolated from a shrimp-incubating pond, salinity was about 20 ‰, pH and temperature were 8.2 and *ca* 30°C respectively.

**Comparison:** With establishment of *Uronemella*, Song and Wilbert (2002) included three morphotypes in the genus: *U. binucleata* (Song, 1993) Song et Wilbert, 2002 (= *Homalogastra binucleata* Song, 1993), *U. filificum* (Kahl, 1931) Song et Wilbert, 2002 and *U. cymruensis* (Pérez-Uz et Hope, 1997) Song et Wilbert, 2002 (= *Urocyclon cymruensis* Pérez-Uz et Hope, 1997).

As a widely distributed species, *Uronemella filificum* has been redescribed for many times during last decades from various geographical regions (Borror 1963, Thompson and Kaneshiro 1968, Wilbert and Kahan 1981, Pérez-Uz *et al.* 1996). But considering its identification, there is always some disagreement remained: e.g. the number of somatic kineties seems highly variable and population-dependent (Table 2), which is usually relatively constant in most other scuticociliates (Foissner and Wilbert 1981, Wilbert 1986, Foissner *et al.* 1994, Petz *et al.* 1995). This character is hence somehow confluent in some cases between *U. filificum* and *Uronema elegans* (Table 2), and this is also the main reason why these two forms were repeatedly confused in previous reports (Borror 1963, Czapik 1968, Agamaliev 1974, Dragesco and Dragesco-Kernéis 1986).

Based on the information obtained in the present work as well as in previous papers, *Uronemella filificum* can be separated from *Uronema elegans* in many “minor” dissimilarities: (1) the body size in the former is usually smaller (25-40 vs. 35-50  $\mu\text{m}$  in length); (2) the body shape *in vivo* is basically inverted pear-like with broadest region in anterior portion (vs. elongate or cylindrical with small apical plate in *U. elegans*); (3) cell surface is only inconspicuously notched (vs. conspicuously reticulate stripes and ridges on cell surface - even at low magnification); (4) the former possesses thigmotactic manner with sticky thread deriving from the caudal cilium (vs. lacking such thread and non-thigmotactic as observed by the authors recently); (5) buccal ciliary organelles are clearly to recognize from life (vs. within the large buccal cavity and somewhat difficult to discern from outside); (6) contractile vacuole is located at the posterior-most end (vs. near ventral side sub-caudally); (7) membranelle 1 only slightly away from the apical plate (vs. far away in *U. elegans*); (8) usually lower number of somatic kineties (16-23 vs. 23-27).

In addition, both forms demonstrate different appearances of the buccal apparatus (buccal region conspicuously wider in *U. filificum* than in *U. elegans*) and silverline system (e.g. extrusomes usually not recognizable after silver nitrate impregnation vs. highly developed and usually slightly away from kineties in the latter) (Table 3) (Borror 1963, Czapik 1968, Thompson and Kaneshiro 1968, Agamaliyev 1974, Wilbert and Kahan 1981, Small and Lynn 1985, Dragesco and Dragesco-Kernéis 1986).

Song and Wilbert (2002) described a morphotype found from Qingdao, which differs from the Zhanjiang population only in possessing slightly higher number of somatic kineties (22-23 vs. 21-22) and a plump body shape. Considering the variation of these features among different populations, it should be conspecific, as an extreme form, with *U. filificum* according to the new understanding.

With reference to the position of cytostome, the body shape, the general appearance of living morphology, the living behaviour, the number of somatic kineties and other infraciliatural features, the following two morphotypes were very possibly misidentified and hence should be also conspecific with *Uronemella filificum*: *Uronema elegans* sensu Dragesco et Dragesco-Kernéis, 1986 and *Uronema elegans* sensu Czapik, 1968.

***Uronema elegans* Maupas, 1883 (Figs 20-26, 27, 29-32, 37-46; Table 1, 2)**

Syn. 1883 *Cryptochilum elegans* - Maupas, *Arch. Zool. Exp. Gén.* 1: 663 (original)

1931 *Uronema elegans* - Kahl, *Tierwelt Dtl.*, 21: 357

1968 *Uronema elegans* - Thompson et Kaneshiro, *J. Protozool.* 15: 142

1974 *Uronema elegans* - Agamaliyev, *Acta Protozool.* 13: 69

This species was often confused with its morphologically similar form, *Uronemella filificum* considering the taxonomic definition and species separation. Based on the data obtained, we supply here a new definition for this “well-known” organism.

**Improved diagnosis:** Marine cylindrical or kidney-shaped *Uronema in vivo* mostly ca 30-50 x 20-30 µm with small apical plate and conspicuously reticulate pellicle ridges; membranelle 1 short and one- or partly two-rowed, which is considerably away from the apical area; 23-26 somatic kineties; contractile vacuole sub-caudally positioned near ventral margin with its opening pore at posterior end of somatic kinety 2; one macro- and one micronucleus; extrusomes bar-like, densely distributed.

**Description of Chinese population:** Cells *in vivo* usually about 35-45 x 20-25 µm in newly-sampled specimens, body shape rather constant, generally cylindrical or kidney-shaped when viewed from lateral with ventral side slightly concave in mid-body, while dorsally convex (Figs 20, 26, 38); cross section rounded. Frontal end truncated, that is, with small apical plate (Figs 20, 26, 40). In culture, cells might be slightly plumper and more oval (Figs 26, 38). Buccal field about 1/2 of body length, with cytostome at bottom of deep buccal cavity (Fig. 25). Pellicle thick and strongly notched on outline with conspicuous reticulate ridges (Figs 24, 37). Extrusomes bar-shaped, about 2 µm long and closely arranged beneath pellicle (Figs 21, 25, 44).

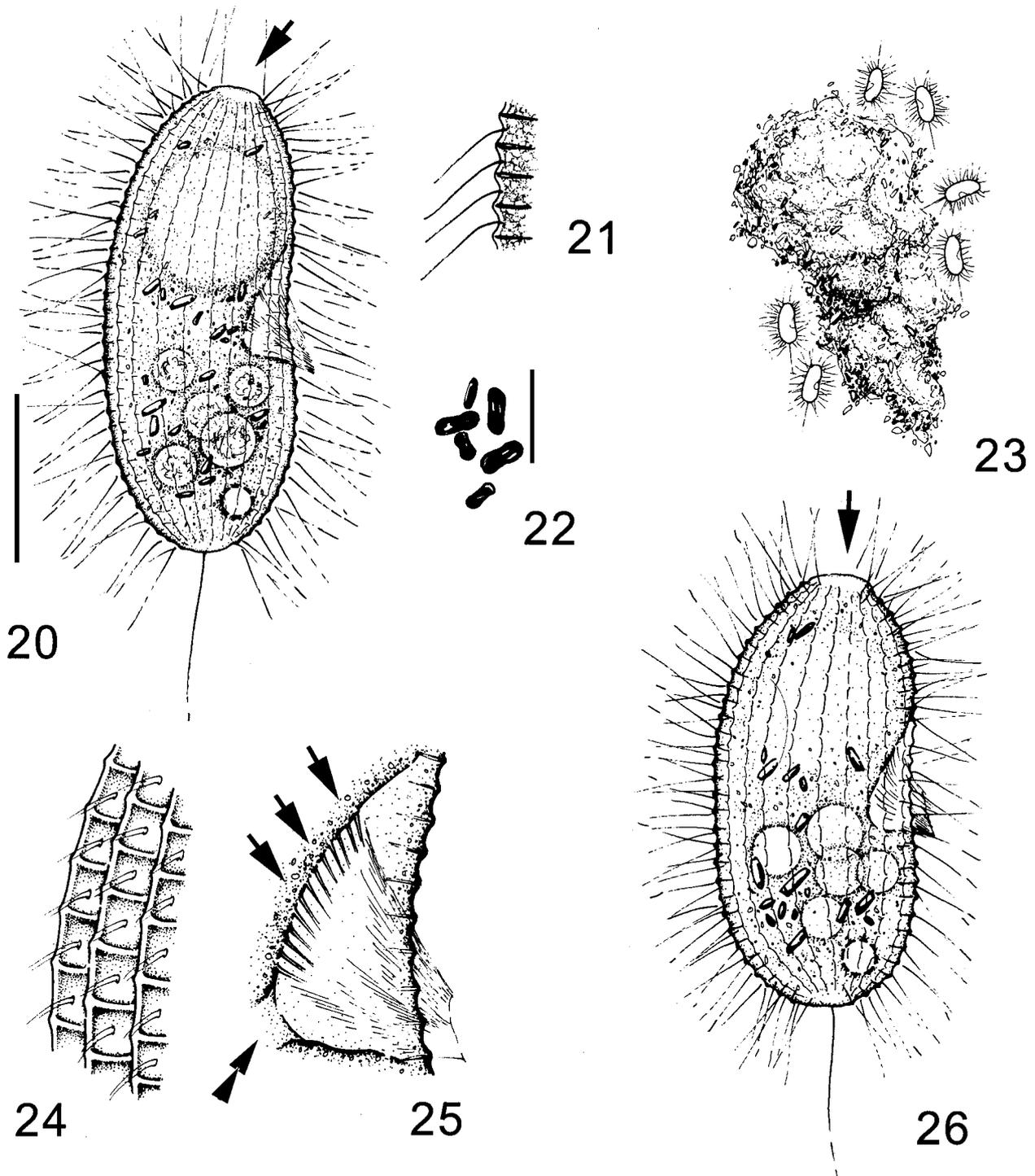
Cytoplasm colourless to greyish, containing several to many large (ca 5 µm across) food vacuoles and bar- or dumbbell-like crystals, which are usually 2-3 µm in size (Figs 25, 44). Macronucleus large and rounded, located mostly in anterior region (Figs 20, 26). Contractile vacuole small, sub-caudally near ventral side positioned (Figs 20, 26, 39).

Cilia about 8-10 µm long, densely arranged; caudal cilium ca 15-18 µm long. Cilia within buccal cavity about 6-8 µm long, usually difficult to recognize (Figs 20, 25). Swimming behaviour generally slow with no peculiarities. But mostly, when no disturbing, always completely quiet on the bottom. In this case, cells seem to attach to substrate using any part of cell region (i.e. no special thigmotactic area) (Figs 23).

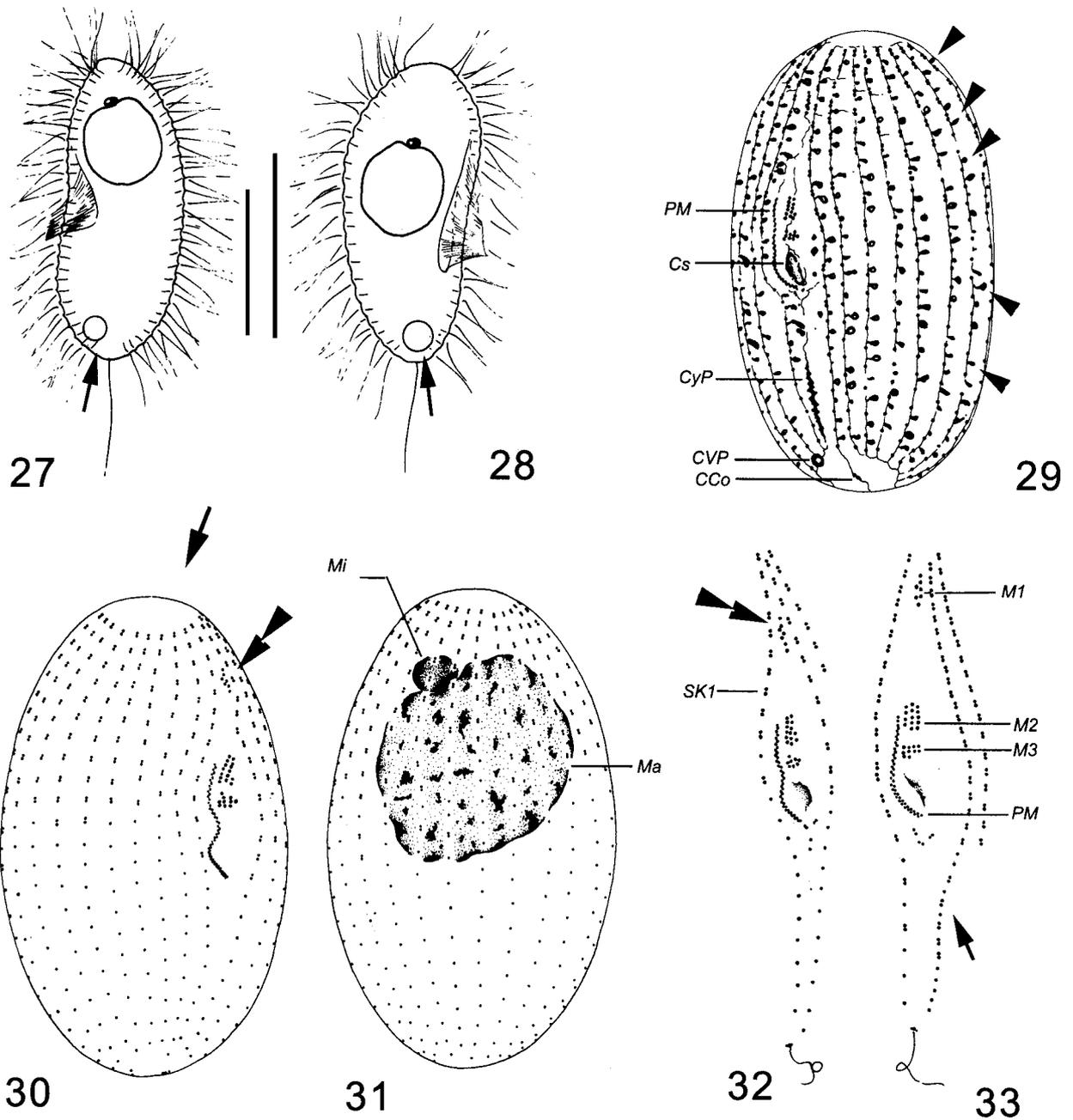
Somatic ciliature as shown in Figs 30 and 31: mostly 23 somatic kineties extending over entire length of body, composed of dikinetids in anterior 40 % of body length, while loosely-arranged monokinetids in posterior portion (Fig. 45).

Buccal apparatus slightly different from its congeners: membranelle 1 conspicuously sub-apically positioned and “far away” from other membranelles, consisting of ca 7 basal bodies, which are often arranged somehow in 2 rows in the middle portion (Figs 32, 43). Membranelle 2 and 3 relatively small, each consisting of 3 rows of basal bodies. Paroral membrane on right of narrow buccal field, with zig-zag rows of basal bodies, extending anteriorly to about middle of membranelle 2. Scutica with ca 3 pairs of basal bodies, closely behind posterior end of paroral membrane (Fig. 32).

Silverline system rather unique: extrusomes always densely distributed after silver nitrate impregnation (arrowheads in Fig. 29), which are often more or less away from direct silverline (thus between kineties)



**Figs 20-26.** *Uronema elegans* from life. **20** - a slender specimen, note that the contractile vacuole is located near ventral side caudally; arrow marks the small apical plate; **21** - portion of pellicle, to show extrusomes and the ridges; **22** - crystals; **23** - six specimens on the detritus, note that cells may attach on the debris using any part of the body; **24** - top view of a portion of the pellicle, to show the reticulate ridges; **25** - portion of the buccal field, arrows mark the cilia of the membranelles on the bottom of the buccal cavity; note that the cytostome is located at the deepest area of the buccal cavity (arrowheads); **26** - a plump specimen, arrow indicates the apical plate. Scale bars -15  $\mu\text{m}$  (in 20), 5  $\mu\text{m}$  (in 22)



**Figs 27-33.** *Uronema elegans* (27, 29-32) and *Uronemella filificum* (28, 33) from life (27, 28), after silver nitrate (29) and protargol impregnations (30-33). **27, 28** - lateral views, to show the typical body shapes of two species, note that the widest part of the body are in different regions; arrow marks the contractile vacuole; **29** - silverline system of ventral side, note that the extrusomes are arranged slightly away from the somatic kineties (arrowheads); **30, 31** - ventral and dorsal view of infraciliature; arrow marks the apical plate, arrowheads indicate the sub-apically positioned membranelle 1; **32, 33** - comparison of buccal apparatus. Note that the position of the membranelle 1 is considerably antieriad positioned in *filificum* than in *elegans* (arrowheads); arrow in 33 refers to the close-set basal bodies in the last somatic kinety. Abbreviations: CCo - caudal cilium complex, Cs - cystostome, CVP - pore of contractile vacuole, CyP - cytophyge, M1-3 - membranelle 1-3, Ma - macronucleus, Mi - micronucleus, PM - paroral membrane, SK1, - the first somatic kinety. Scale bars - 20  $\mu$ m

(Figs 29, 42, 46). Contractile vacuole pore positioned at posterior end of somatic kinety 2. Cytopyge (CyP) proportionally short, irregularly shaped (Fig. 29).

**Ecological features:** This species was isolated from the mantle cavity of marine mollusc (*Sinonovacula constricta*), salinity was about 32 ‰, pH was 8.0 and temperature was *ca* 24°C.

**Comparison and discussion:** We recognized the Qingdao population basically due to the following features: body shape including the small apical plate, appearance of the pellicle, high number of ciliary rows, ventrally positioned contractile vacuole, small but deep buccal cavity, and habitat. Thus, we believe that the identification is correct. As to the only exception, i.e. the body size, which was claimed to be up to 90 µm long in some cases (Maupas 1883, Kahl 1931), a reasonable explanation is that Maupas might mix this species with other forms or due to an optical misinterpretation as many other researchers did in that time.

As mentioned above, because of the variable number of somatic kineties (23-26 in *U. elegans* vs. 16-23 in *U. filificum*), this species has been repeatedly confused with *Uronemella filificum* (see comparison part for *Uronemella filificum*). A summarized comparison between these two organisms is hence supplied in Table 3.

The African population described by Dragesco and Dragesco-Kernéis (1986) under the name of *Uronema elegans* is likely a misidentification. According to the position of membranelle 1, it should be a population of *Uronema filificum*.

***Paranophrys marina* Thompson et Berger, 1965 (Figs 14-16, 34-36; Table 1)**

This species was described first by Thompson and Berger (1965) and has never been reinvestigated ever since. As no adequate living data were supplied in original work, a comprehensive redescription based on the Chinese population is hence given here.

**Improved diagnosis:** Marine slender *Paranophrys in vivo* *ca* 30-45 x 10-15 µm with pointed apical and narrowly rounded posterior end; membranelle 1 very long and two-rowed, almost conjoint to membranelle 2; 10 somatic kineties with densely spaced cilia; scutica composed of several singled basal bodies arranged in line; contractile vacuole large and caudally positioned, opens at posterior end of somatic kinety 2; one oval macro- and one small micronucleus.

**Redescription:** Body shape generally constant, slim and spindle-shaped with sharply pointed anterior end and narrowly rounded caudal end (Fig. 34). Cell size *in vivo*

about 30-45 x 10-15 µm, in some giant forms (not uncommon in fresh samples) length up to 55 µm. Buccal cavity inconspicuous and about 2/5 of body length. Pellicle smooth, no extrusomes observed. Cytoplasm colourless to greyish, often filled with many small (*ca* 2-3 µm across) light-reflecting granules (inactive food vacuoles) and several bar-shaped crystals (Fig. 34). One large oval macronucleus centrally located with many nucleoli on surface; one small micronucleus anteriorly attached to macronucleus (Fig. 35). Contractile vacuole large, terminally located at posterior end of cell (Fig. 34).

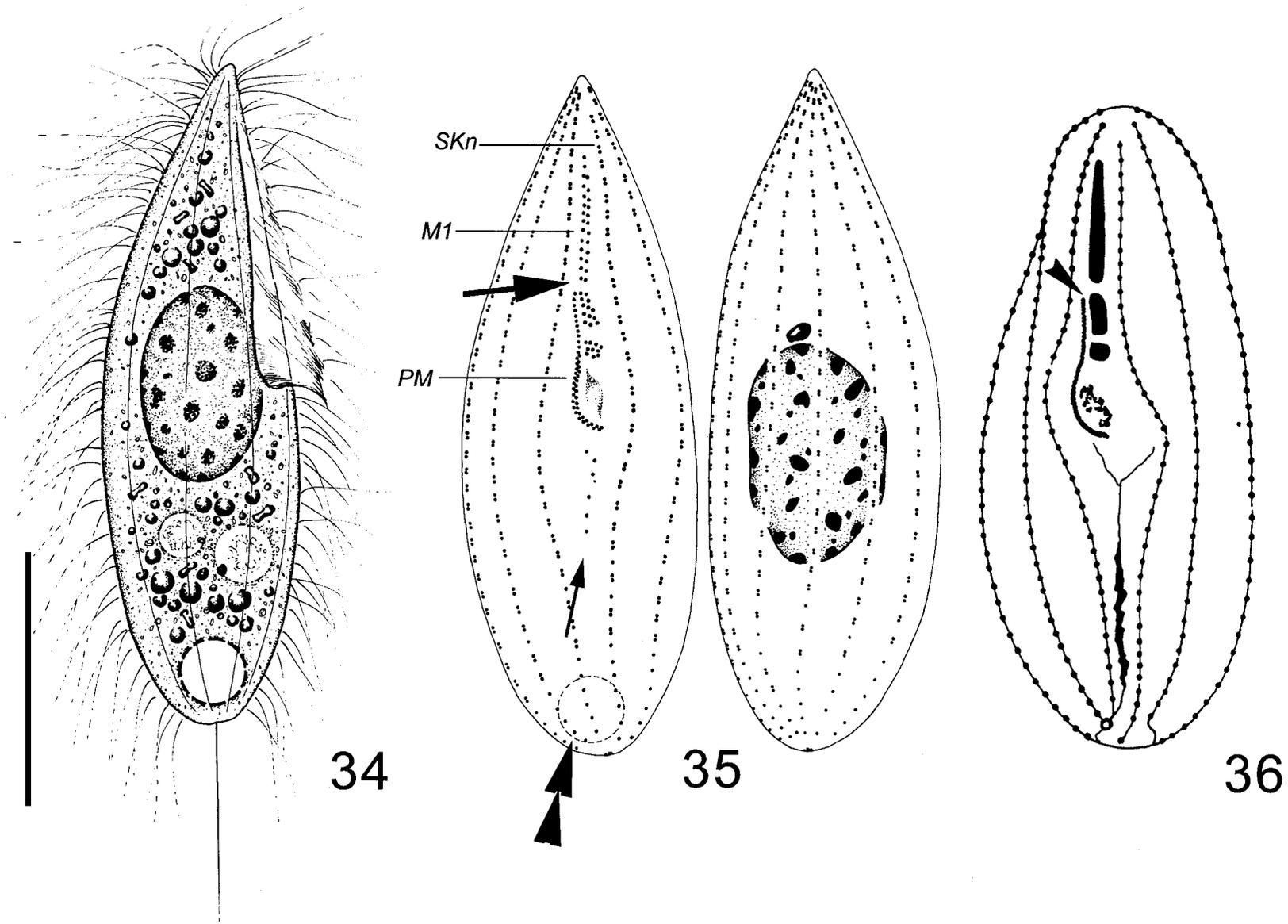
Cilia densely arranged, *ca* 8 µm long, one single caudal cilium about 15 µm in length. Movement active, or pretty quiet while crawling on bottom of petri dish.

Infraciliature as shown in Fig. 35. Buccal apparatus consisting of 3 closely distributed membranelles on left side of buccal cavity and one paroral membrane on right side (Figs 35, 15). Membranelle 1 slightly away from apex, consisting of 2 long rows of kinetids with about 10-12 basal bodies each, hence about 2.5 - 3 times as long as membranelle 2; membranelle 2 with 2 long and 1 short longitudinal rows (sometimes difficult to distinguish), each containing about 2-6 basal bodies; membranelle 3 located close to membranelle 2, with normally 3 short rows of basal bodies (Figs 35, 15). Paroral membrane characteristic of genus extending anteriorly to about anterior level of membranelle 2, composed of zig-zag rows of basal bodies (Figs 10, 15). Scutica generally in linear form, consisting of about 6 basal bodies (Fig. 35).

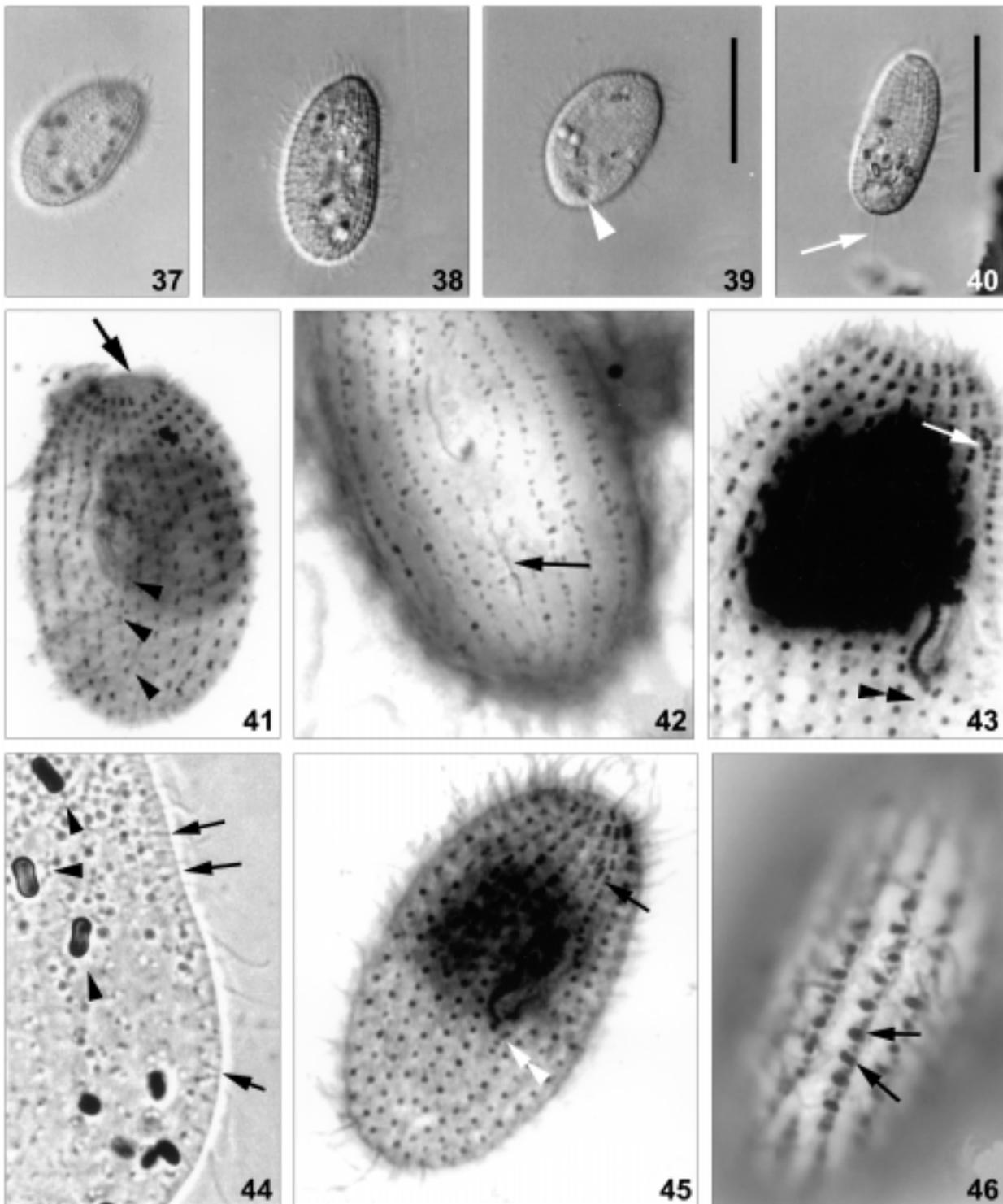
Invariably 10 bipolar somatic kineties composed of densely-arranged dikinetids over usually more than 4/5 of the length, only in caudal portion with monokinetids (Fig. 35).

**Ecological features:** This species was found as an ectocommensal within the mantle cavity of farmed scallop (*Argopecten irradians*) near Qingdao, where the water was clean (salinity about 31‰, pH 8.2, water temperature *ca* 25°C). This ciliate can be easily maintained as free-living one in seawater with rice grains.

**Discussion:** Thompson and Berger (1965) described *Paranophrys marina*, which was isolated from hydroids (*Plumularia* sp.) in the waters near Friday Harbor (Washington, USA), as follows: "... the body is round in cross-section and bluntly tapered in the anterior third of the body. Both the anterior and posterior ends are rounded. The anterior ventral surface, containing the buccal apparatus, is somewhat flattened...". Since no detailed living information was supplied, we identified our organism more or less inferentially. In the original



**Figs 34-36.** *Paranophrys marina* in vivo (34), after protargol (35) and silver nitrate impregnations (36, redrawn from Thompson et Berger, 1965). 34 - right lateral view of a representative specimen; 35 - ventral and dorsal view of the same specimen; small arrow marks the scutica where basal body pairs are aligned in a long row; arrowheads indicate the large contractile vacuole. Note that the membranelle 1 is almost conjoined with membranelle 2 (large arrow); 36 - ventral view of silverline system, arrowhead marks the anterior end of paroral membrane which extends to the anterior level of membranelle 2. Abbreviations: CV - contractile vacuole, M1 - membranelle 1, PM - paroral membrane, SKn - somatic kinety n. Scale bar - 20  $\mu$ m



**Figs 37-46.** Photomicrographs of *Uronemella elegans* from life (37-40, 44), after protargol (41, 43, 45) and silver nitrate impregnations (42, 46). **37** - a slightly pressed specimen, focusing on the cell surface to show the reticulate appearance of the pellicle; **38** - a plump form, note the strongly notched surface; **39** - to show the contractile vacuole (arrowhead); **40** - slender form, note the small apical plate; arrow marks the caudal cilium; **41** - ventral view of infraciliature, arrow marks the small apical plate, while the arrowheads indicate the somatic kinety n; **42** - ventral view, to show the narrow buccal field and the short cytophyge (arrow); **43** - anterior portion of right-lateral side, arrow indicates the membranelle 1, while the arrowheads mark the scuticula; **44** - detail of cortex to show the extrusomes (arrows) and crystals (arrowheads); **45** - general view of infraciliature, note that the basal bodies within kineties are relatively loosely arranged; arrow marks the membranelle 1, arrowheads refer to the scuticula; **46** - to demonstrate extrusomes (arrows), which are away from the kineties. Scale bars - 20  $\mu$ m

description, *Paranophrys marina*, as an ectocommensal form, possesses (1) constant 10 somatic kineties; (2) long and dominant membranelle 1, which is close to the other membranelles; (3) conspicuously small cilia-free apical area and (4) paroral membrane begins at anterior end of membranelle 2. All these features resemble our form perfectly (Figs 35, 36).

The only dissimilarity is possibly the body shape: the Qingdao population has a sharply narrowed anterior cell end, while Thompson and Berger depicted their form with a "rounded" one. We assume that their description was possibly deduced from the silver-impregnated specimens instead of deriving *in vivo* observations (as Thompson mentioned that this species was collected by the junior author several years before it was described). Thus the actual body form of this species was likely misinterpreted: its apical end is pointed but not rounded (this supposition might be confirmed by the photos in the original report, p.528, Figs 2, 3) (Thompson and Berger 1965).

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## Redescription and Histopathology of *Myxobolus cyprinicola* Reuss, 1906, an Intestinal Parasite of the Common Carp (*Cyprinus carpio* L.)

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**Summary.** Although *Myxobolus* spores can often be detected from the gut of fish, from the mucus covering the intestinal wall and from the intestinal content, the number of species actually developing in the gut wall is rather low. *Myxobolus cyprinicola* can be considered a parasite rarely occurring in Europe. This parasite was found to cause infection in the gut of common carp caught from Lake Balaton. Its pinhead-sized plasmodia were located in the lamina propria at the tip of the mucosal folds of the intestine, immediately below the basement membrane. The plasmodia were closely connected with the capillaries of the lamina propria. The large number of red blood cells found between the basement membrane and the plasmodium wall suggests that they started their development in the lumen of a capillary. By their morphological characteristics, the spores of *M. cyprinicola* are well distinguishable from those of *M. cyprini*, which more commonly occur in the gut within necrotic macrophages (yellow bodies) after having reached that site as a result of a secondary process. Because of the small size of the plasmodia, the subepithelial location, and the low prevalence and intensity, *M. cyprinicola* can be considered a less pathogenic species.

**Key words:** carp, histology, intestine, *Myxobolus*, Myxosporea, Pisces.

### INTRODUCTION

Of the approx. 500 *Myxobolus* species known at present, various authors described a large number of species from the common carp (*Cyprinus carpio*). In their monograph Donec and Shulman (1984) recorded 25 *Myxobolus* species in common carp, while Chen and Ma (1998) have reported the occurrence of 50 carp-parasitic *Myxobolus* species in China. Landsberg and Lom (1991) regard 19 of these species

as species originally described from the common carp. To date, four species (*Myxobolus basillamellaris*, *M. cyprini*, *M. dispar*, *M. intrachondrealis*) have been known to occur in Hungary (Molnár 1979, 2000; Lom and Molnár 1983; Molnár and Kovács-Gayer 1985). All species recorded in Hungary were characterised by distinct tissue and organ specificity: *M. cyprini* proved to be a typical muscle cell parasite (Molnár and Kovács-Gayer 1985), *M. dispar* formed plasmodia at the apical end and *M. basillamellaris* at the base of the gill filaments (Lom and Molnár 1983), while the plasmodia of *M. intrachondrealis* developed in the cartilaginous substance of the gill arch (Molnár 2000). In addition to developing and mature spores of plasmodia in the wall of the intestine, different organs (skin, gills, kidney, gut,

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etc.) often contained solitary spores that corresponded to *M. cyprini* spores dispersed throughout the body by the blood stream (Molnár and Kovács-Gayer 1985). According to the above authors, such spores were particularly often detectable in the mucus drawn off the gut wall, in which they occurred as solitary spores or as small clusters of spores embedded in yellow bodies.

This paper reports the occurrence and specific location of *M. cyprinicola* Reuss, 1906, a species forming plasmodia in the gut wall, in common carp from Lake Balaton, and it also presents a redescription of this rare species.

## MATERIALS AND METHODS

The studies were conducted from September 1999 to July 2001 in the framework of a survey of the parasite fauna of Lake Balaton fishes. The samples originated from four different biotopes of the lake, from the area of Siófok, Balatonszemes, Tihany and Keszthely. A total of 48 two- to four-year-old common carp measuring 18 to 48 cm (average: 33 cm) in length were examined. The carp were subjected to complete parasitological examination that included studying *Myxobolus* infection of the gut. To remove the intestinal content, the fish were kept in flow-through water in the laboratory for 1-2 days, then killed by exposure to MS-222 (3-aminobenzoic acid ethyl ester) solution, and dissected. The gut was cut open in its entire length, the remaining gut content was carefully removed, and gut segments were examined thoroughly under stereomicroscope at ten-fold magnification. To avoid shining, 0.65% saline solution was sprayed onto the intestinal mucosa in a thin layer, and top lighting was applied to search for nodules that contrasted with the gut mucosa by their whitish colour. From the plasmodia located within the nodules the spores were sucked out with a pipette. Unfixed spores were studied by an Olympus BH2 microscope using Nomarski differential interference contrast. The spores were recorded on video, digitised images were obtained according to the method of Székely (1997), and the measurements of the spores were taken. For histological examination, small samples were excised from the infected gut segments and fixed in Bouin's solution for 4 h. From the paraplast-embedded blocks 4 µm thick sections were made by cutting through the intestine, and the sections were stained with haematoxylin and eosin.

## RESULTS

Seven out of the 48 common carp examined from Lake Balaton (14.6%) proved to be infected. Infection occurred at all sampling sites and in all periods, and was found in the smallest (18 cm) and bigger (40 cm) fish specimens alike. The intensity of infection was low, 1-11 (4) plasmodia. Only mature plasmodia containing spores (Fig. 1) were found during the survey. The spherical

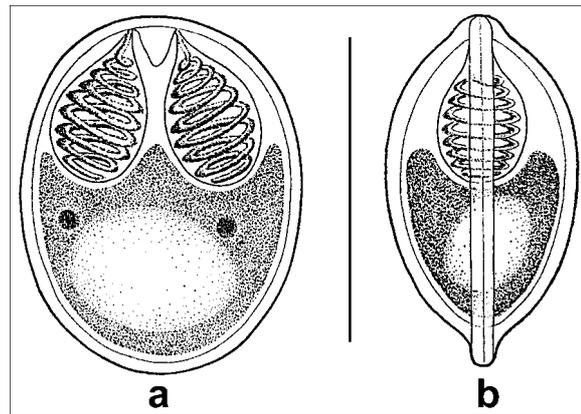
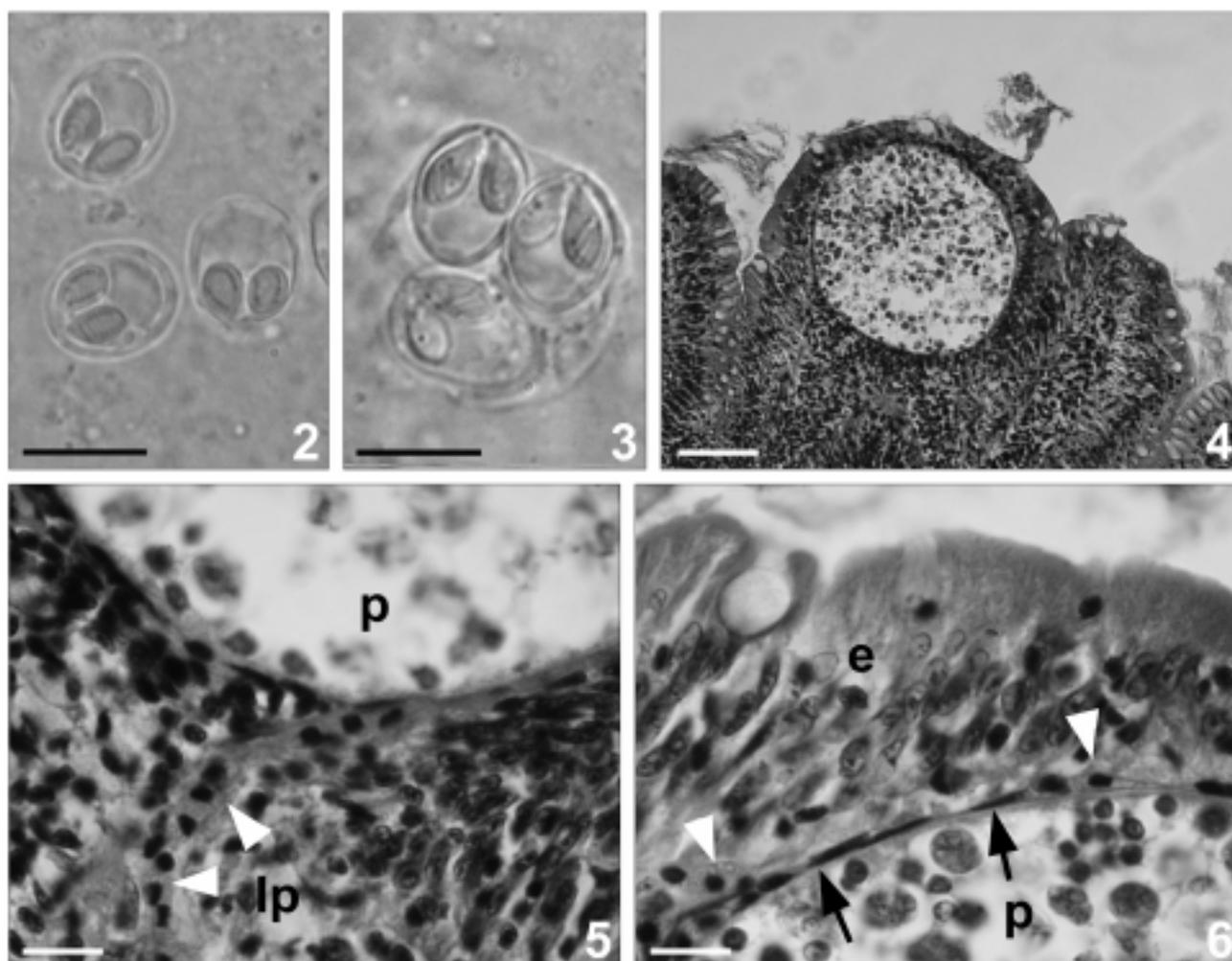


Fig. 1. Schematic representation of *Myxobolus cyprinicola* spores in frontal view (a) and in lateral view (b). Scale bar - 10 µm

plasmodia 0.3 to 1.5 mm in diameter were located in the gut wall. In 9 fish, solitary spores located outside plasmodia, in yellow bodies, were also found in the mucus covering the enterocytes; these, however, proved to be spores of the species *Myxobolus cyprini* by their shape and size (Fig. 2). In the framework of other projects and routine diagnostic examinations several hundred common carp of similar age and size were examined from fish farms, but *M. cyprinicola* infection could not be detected in any of them.

On the basis of 50 spores released from the plasmodia the redescription of the species *M. cyprinicola* Reuss, 1906 is given as follows: spores (Figs 1, 3) are ellipsoidal in frontal view and lemon shaped in lateral view. Spore valves are relatively thick, symmetrical and smooth. Sutural line distinct, sutural edge protruding, forming a longer process in the posterior and a shorter protrusion on the anterior end in lateral view. Spores measure 11.8 (11-12.2) µm in length, 9.0 (8.1-9.4) µm in width and 6.2 (6.0-6.4) µm in thickness. Two polar capsules are pyriform in shape, equal in size and measure 5.0 (4.8-5.2) µm in length and 3.2 (3.0-3.4) µm in width and thickness. Polar capsules are slightly converging toward the posterior end of the spores and open at the base of the intercapsular appendix. Polar filaments are closely coiled with 8 turns in the polar capsule, situated perpendicularly to the longitudinal axis of the capsule. The extruded polar filament measures 41 (38-47) µm in length. A large, triangular intercapsular appendix is located between the polar capsules at the anterior end of the spore. A large iodophilous vacuole and two nuclei of the sporoplasm were well discernible in spores.

**Histopathological findings.** In histological sections, the spores of *M. cyprinicola* were found in the lamina



**Fig. 2.** *Myxobolus cyprinicola* spores released from a mature plasmodium. x 1800

**Fig. 3.** *Myxobolus cyprini* spores embedded in a yellow body from the gut of common carp. x 1800

**Fig. 4.** *Myxobolus cyprinicola* plasmodium at the tip of the mucosal folds of the gut in common carp. Histological section. Haematoxylin and eosin (H & E.), x 200

**Fig. 5.** *Myxobolus cyprinicola* plasmodium (p) containing spores in the lamina propria of the intestinal wall (lp). A capillary (arrowheads) is seen to be connected with the wall of the plasmodium. H & E, x 1200

**Fig. 6.** The very thin ectoplasm of the *M. cyprinicola* plasmodium (p) located under the epithelial layer (e) of the gut is bordered by flattened cells (arrowheads) which contain red blood cells (arrows) in some places. H & E, x 1200

propria of the gut, in spherical, relatively small plasmodia 0.3-1.5 mm in diameter (Fig. 4). The plasmodia were usually located at the tip of the mucosal folds of the intestine, closely adhering to the basement membrane of the epithelium. The spore-filled endoplasm of the plasmodium was surrounded only by a very thin ectoplasm, and the latter was bordered by a very thin capsule of host origin, consisting of a cell row presumably formed from the propria, fine capillaries (Fig. 5) led to the capsule, and the red blood cells seen scattered around the capsule were also indicative of the remnants of a capillary covering the ectoplasm of the

plasmodium (Fig. 6). Although we could not observe young plasmodia, the close connection with the capillaries suggests that the formation of plasmodia may commence inside a capillary. Infection caused by the relatively small plasmodia was accompanied by negligible local histological changes appearing as flattening of the epithelium above the plasmodium.

**Remarks.** The *Myxobolus cyprinicola* species detected from the lamina propria of the intestine differs from the four species (*Myxobolus cyprini*, *M. dispar*, *M. basilamellaris* and *M. intrachondrealis*) reported from Hungary from common carp by its shape and tissue

location. In frontal view the spores of *M. cyprinicola* are typically regular ellipsoidal, while those of *M. cyprini* and *M. basillamellaris* are rather oval in shape. The spore of *M. cyprinicola* contains a large wedge-shaped intercapsular process, while in the other three species this process is relatively small. *M. cyprinicola* can be distinguished from the species *M. intrachondrealis* bearing the greatest resemblance to the former by its well-developed iodophilous vacuole in frontal view and by its thick sutures protruding at the anterior and posterior ends of the much wider spores in lateral view. As far as the intrapiscine location is concerned, by its occurrence in the lamina propria of the intestinal wall *M. cyprinicola* is well distinguishable from the typical gill-parasitic species *M. dispar* and *M. basillamellaris* and from the muscle parasite *M. cyprini*. Of the species occurring in other cyprinids, *M. muelleri* bears the closest resemblance to *M. cyprinicola* which, however, differs from the former by its more elongated elliptical shape.

## DISCUSSION

The number of recorded *Myxobolus* species occurring in the common carp is extremely large. The majority of them have been described and identified as parasites of the Far-Eastern carp subspecies, *Cyprinus carpio haematopterus*. While the majority of the above species are obviously included in the parasite fauna of the common carp as a result of erroneous identification, it is unquestionable that even the accurately identified *Myxobolus* parasites of the Far-Eastern carp subspecies exceed in number those parasitising the European carp subspecies.

The common occurrence of three *Myxobolus* species, *M. cyprini*, *M. dispar* and *M. basillamellaris*, in Hungarian carp farms has long been known (Molnár 1979, Molnár and Kovács-Gayer 1985). Recently *M. intrachondrealis* has been added to these species (Molnár 2000). Unpublished data also suggest that the above parasites occur also in common carp living in natural waters of Hungary. Of the numerous species recorded from common carp, in Czechoslovakia Dyková and Lom (1988) detected *M. cyprini*, *M. cyprinicola*, *M. dispar* and *M. basillamellaris*, and additionally the species *M. encephalicus* (Mulsow, 1911), *M. muelleri* Bütschli, 1882 and *M. oviformis* Thélohan, 1892. The species *M. cyprinicola*, only recently found in Hungary but known to occur in Europe for a long time, appears to

be a rarely occurring parasite causing infection of low prevalence, which explains why it has escaped the researchers' attention so far. Obviously, many researchers mistake this parasite for *M. cyprini*, which until the study of Molnár and Kovács-Gayer (1985) had been regarded as species parasitising also the gut. After the disruption of muscle cells the spores of *M. cyprini* become scattered all over the body and are often excreted through the gut. At such times large masses of spores can be detected from the intestinal mucus, embedded in so-called yellow bodies. It seems probable that the much less frequent *M. cyprinicola* infection often passes unnoticed. The gut relatively rarely serves as a typical location for the different *Myxobolus* species. It is likely that technical books (Shulman 1966, Chen and Ma 1998) erroneously indicate the intestine as a location of infection for several species, and has only been recorded as such due to the fact that spores are excreted via the gut. Despite the above probability there are several examples of the infection of *Myxobolus*-plasmodia in the intestinal wall. Masoumian *et al.* (1996) detected rather large plasmodia of *M. nodulointestinalis* from the muscular layer of the gut of *Barbus sharpeyi*, while in the book edited by Pan *et al.* (1990) the plasmodia of *M. artus* were depicted in a location fully resembling that of *M. cyprinicola* described here. Although the plasmodia of *M. cyprinicola* were indisputably located in the lamina propria of the intestinal wall, i.e. in the connective tissue, it cannot be stated that this parasite shows affinity to the connective tissue. Rather, the capillaries found around the plasmodia indicate that the parasite started developing within a capillary of the lamina propria. In this regard, despite its unquestionable gut wall location *M. cyprinicola* more closely resembles the better studied species that commence their development within the capillaries of the gill lamellae (Dyková and Lom 1978, Molnár 2002). In view of its low prevalence and negligible intensity of infection, *M. cyprinicola* can be considered a rare species of low pathogenicity, which, however, may have diagnostic importance.

Major changes in the identification of Myxosporea species can be anticipated in the near future. Species descriptions based merely on the morphological characteristics of spores are inadequate, and besides the determination of the typical host, host groups and intrapiscine locations, molecular biological identification will also become indispensable. For instance, for *M. cyprinicola* Donec and Shulman (1984) listed, besides the typical host common carp, 10 other cyprinids and also a *Nemacheilus* species as hosts. It seems likely that in the

future, following accurate redescription of the different *Myxobolus* species, determination of their organ and tissue specificity, and DNA analysis, many parasites currently regarded as valid species will prove to be synonyms. At the same time, it is also probable that species erroneously identified as synonyms from taxonomically distant hosts based upon the morphological similarity of the spores will prove to represent separate species and add to the number of existing *Myxobolus* species.

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## What Contributes to Daughter Cells Separation during Cytokinesis of *Amoeba proteus*

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**Summary.** Before the furrow formation the non-polar general contraction resulting in cell de-adhesion and spherulation, and the following relaxation leading to re-adhesion, flattening and spreading, are both necessary prerequisites of a successful cytokinesis in *A. proteus*. The bipartition begins by sudden generation of two divergent cytoplasmic streamings at the late anaphase, always before the formation of furrow. All these facts fit better with the polar relaxation model than with the equatorial contraction model of initiating the fission of amoeba. After formation of the furrow the contractile ring gradually constricts the cytoplasmic connection between daughter cells. Endoplasm flow in the connection bridge is no more bipolar but irregularly reversing; it compensates hydrostatic pressure differences between daughter cells. The final break of the connection is explained by its stretching because of the disparate locomotor activities on both sides of the furrow and owing to the cytoskeleton disassembly inside the connecting bridge.

**Key words:** *Amoeba proteus*, cell adhesion, cell motility, cytokinesis.

### INTRODUCTION

The large majority of Protists have a well pronounced motor polarity expressed by differentiation of anterior and posterior body poles. This poses a problem of the mode of cell division. The fission either may be perpendicular to the body axis (as in ciliates), or longitudinal (as usual in flagellates). Amoebae have resolved this problem differently, by the loss of motor polarity and a transient halt of locomotion. *Amoeba proteus* ceases moving, contracts and rounds up prior to cytokinesis

(cf. Figs 1, 4). However, motility of the two future daughter cells is restored long before they definitely separate. It raises the question whether this final step of cytokinesis is still effected by constriction forces originating locally in the division furrow, as in metazoan eggs and non-motile cells, or is it completed by traction forces generated at some distance from the two sides of the furrow, i.e., by both prospective daughter cells tending to move in different directions. This second mechanism should normally necessitate a bipolar adhesion to the substratum to pull the mother cell in two.

As a matter of fact, in the earliest experimental studies of the cytokinesis of *A. proteus* (e.g. Chalkley 1935, 1951) the pulling force resulting from locomotion of daughter cells was considered as the principal factor

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of the cell division in amoeba. The last study of cytokinesis in this species (Rappaport and Rappaport 1986) concluded, in contrast, that "the constriction activity in the furrow region resembles that of metazoan cells...", whereas "attachment and locomotion... were not essential for cytokinesis, and their involvement is restricted to the final parting of the cytoplasmic thread that connects the daughter cells." These statements are probably partly correct, however they need re-examination, because observations of amoebae strongly flattened under a layer of Halocarbon oil, made by Rappaport and Rappaport (1986), are not conclusive as far as the role of attachment to the substratum and of the natural flattening and spreading of cells at the early stages of cytokinesis are concerned.

In our experiments amoebae were examined in chambers about 100  $\mu\text{m}$  deep, free to attach or not, and to spherulate or spread on the substratum. Besides recording the course of cytokinesis in living specimens in a differential interference contrast microscope (DIC), the presence and distribution of adhesive organelles: minipodia and rosette contacts described recently by us (Grębecki *et al.* 2001), were examined in a scanning electron microscope (SEM) in amoebae fixed at different stages of division, and the accumulation of F-actin in the division furrow was demonstrated in a confocal laser scanning microscope (CLSM) after fluorescent phalloidin staining.

## MATERIALS AND METHODS

*Amoeba proteus* cells (strain C<sub>v</sub>), were grown at 20 $\pm$ 1°C in glass culture dishes with Pringsheim medium. They were fed twice a week with *Tetrahymena pyriformis* and the division spheres were collected one day after feeding. The observation chambers were bordered with parafilm strips which kept the cover slip about 100  $\mu\text{m}$  over the slide, thus allowing the dividing amoebae freely attach to or detach from the substratum. The course of cytokinesis was recorded *in vivo* in a Biolar microscope (PZO, Warsaw) equipped with DIC optics of Pluta system and coupled with a C2400 Hamamatsu camera and NV8051 Panasonic time-lapse recorder adjusted to 8x time compression.

The samples for SEM were fixed in 3.5% paraformaldehyde with 0.5% acrolein, dehydrated through a graded series of ethanol and acetone, dried by the CO<sub>2</sub> critical point method, and coated with carbon and gold. They were examined in a Jeol 1200 EX transmission electron microscope with an ASID 19 scanning attachment, operating at 80 KV. Other samples, after the same fixation, were stained with 1% phalloidin labelled with fluorescein isothiocyanate (Sigma, St. Louis) and examined for F-actin in an Olympus FV-500 confocal laser scanning microscope.

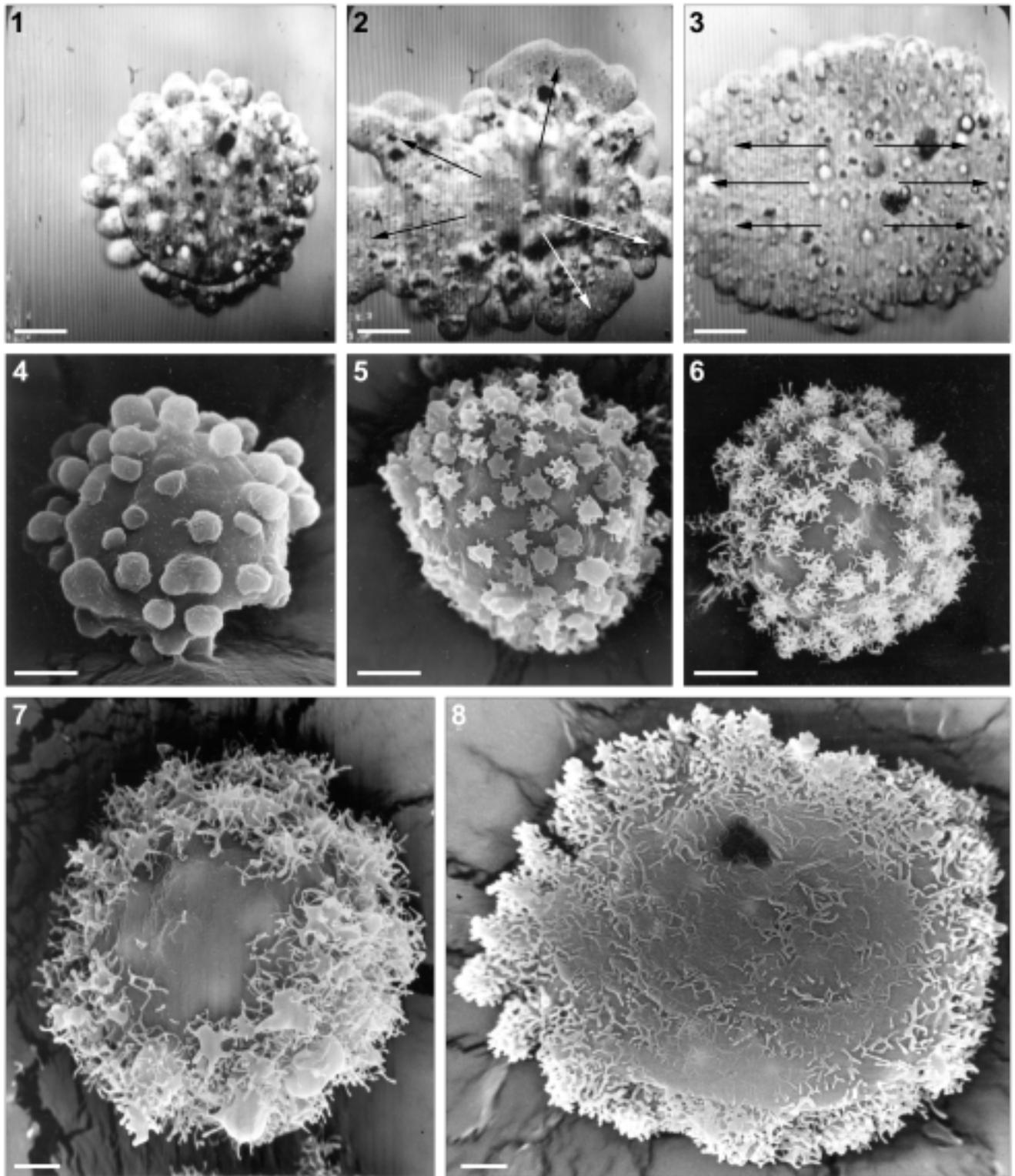
## RESULTS AND DISCUSSION

The division spheres formed in prophase (Fig. 1) freely float in the medium, or so loosely contact with the substratum that they become easily detached by any slight flow of the culture medium, or spontaneously. This is consistent with the absence of any adhesive organelles (such as minipodia) on their surface examined by SEM (Fig. 4). There is no endoplasmic flow inside the spheres, but the cytoplasmic inclusions (seen in Figs 1, 9) show intense erratic movements, independently one from another and never form at least locally co-ordinated streamings. It proves that at this stage the cell is incapable of building any hydrostatic pressure gradient, and means that the usual polarity of peripheral contraction has been lost.

In fact, the circular contour of the cell body appeared in DIC *in vivo* (Figs 1, 9) as a layer much more optically dense than in the locomoting amoebae. In the fixed division spheres stained with FITC-phalloidin CLSM revealed (Fig. 10) accumulation of F-actin in the cortical zone (and in the perinuclear region). Some division spheres, fixed for SEM, were mechanically injured with a microneedle before coating with carbon and gold; in many cases that exposed their submembrane structure to view (Fig. 12). This method always revealed a very extensive development of the three-dimensional network of microfilaments under the surface of division spheres (Fig. 13), which in high magnification (Fig. 14) look identical to F-actin meshworks frequently demonstrated in the literature.

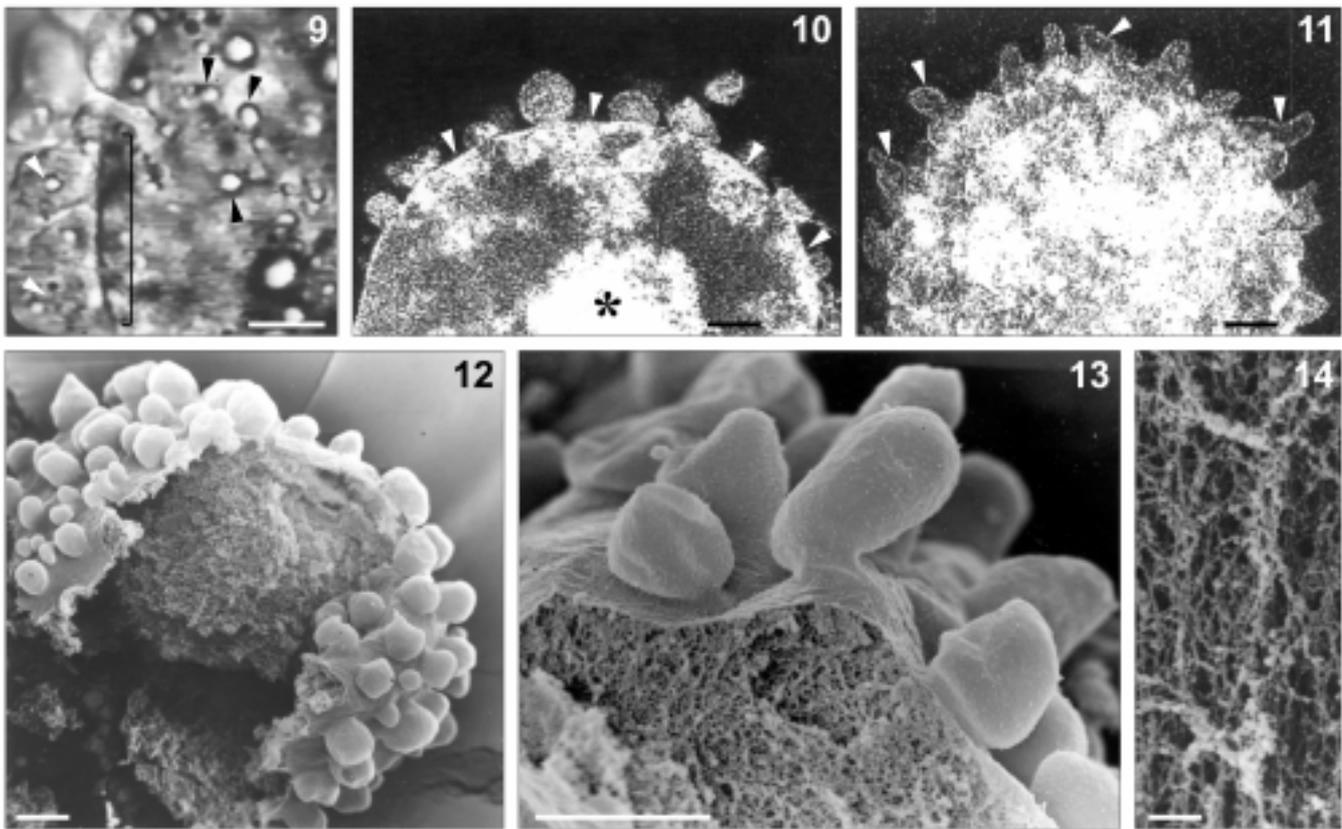
Contraction of this cortical network produces tightly packed bulbous protuberances on the surface of division spheres. These protuberances, however, neither are homologous to the blebs nor to the caps of locomotor pseudopods of moving amoebae and of some motile tissue cells which contain optically empty hyaloplasm (cf. for example Grębecki 1990, Keller and Egli 1998). The protrusions of the division spheres are, in contrast, full of cytoplasmic inclusions (Fig. 9) and are surrounded by F-actin layer (Fig. 11).

The excessive accumulation of F-actin in the cell cortex, uniform squeezing of protuberances around the whole surface, lack of hydrostatic pressure gradients inside, absence of adhesive organelles and deficiency of attachment, all-together strongly suggest that the cell division of amoeba is preceded by a supernormal and uniform cortical contraction. It fits well with the view that not the equatorial over-contraction, but the



**Figs 1-3.** The same cell of *Amoeba proteus* recorded in DIC from the prophase to the late anaphase. 1 - contracted, non-adhering division sphere without endoplasmic flow; 2 - centrifugal streamings and arisal of adhesive pseudopods (arrows) lead to cell spreading; 3 - bipolar flow (arrows) elongates the cell to elliptic shape. Scale bars - 20  $\mu$ m

**Figs 4-8.** Minipodia and rosette contacts on the surface of prophase-to-anaphase cells examined in SEM. 4 - absence of adhesive organelles in an early division sphere (corresponds with Fig. 1); 5, 6 - development of minipodia and rosette contacts; 7 - marginal re-arrangement of adhesive organelles during cell spreading; 8 - adhesive organelles at the stage of bipolar streaming and cell elongation (corresponds with Fig. 3). Scale bars - 20  $\mu$ m



**Fig. 9.** High magnification of a fragment of division sphere in DIC (corresponding with Fig. 1); note elevated optical density of cell cortex (brace), and cytoplasmic particles in the cell body (black arrowheads at right) and in bulbous protuberances of the surface (white arrowheads at left). Scale bar - 10  $\mu$ m

**Figs 10, 11.** F-actin staining with FITC-phalloidin in division spheres examined in CLSM; **10** - note F-actin accumulation in the cortex (arrowheads) and around the nucleus (asterisk); **11** - actin sheets around surface protuberances. Scale bars - 10  $\mu$ m

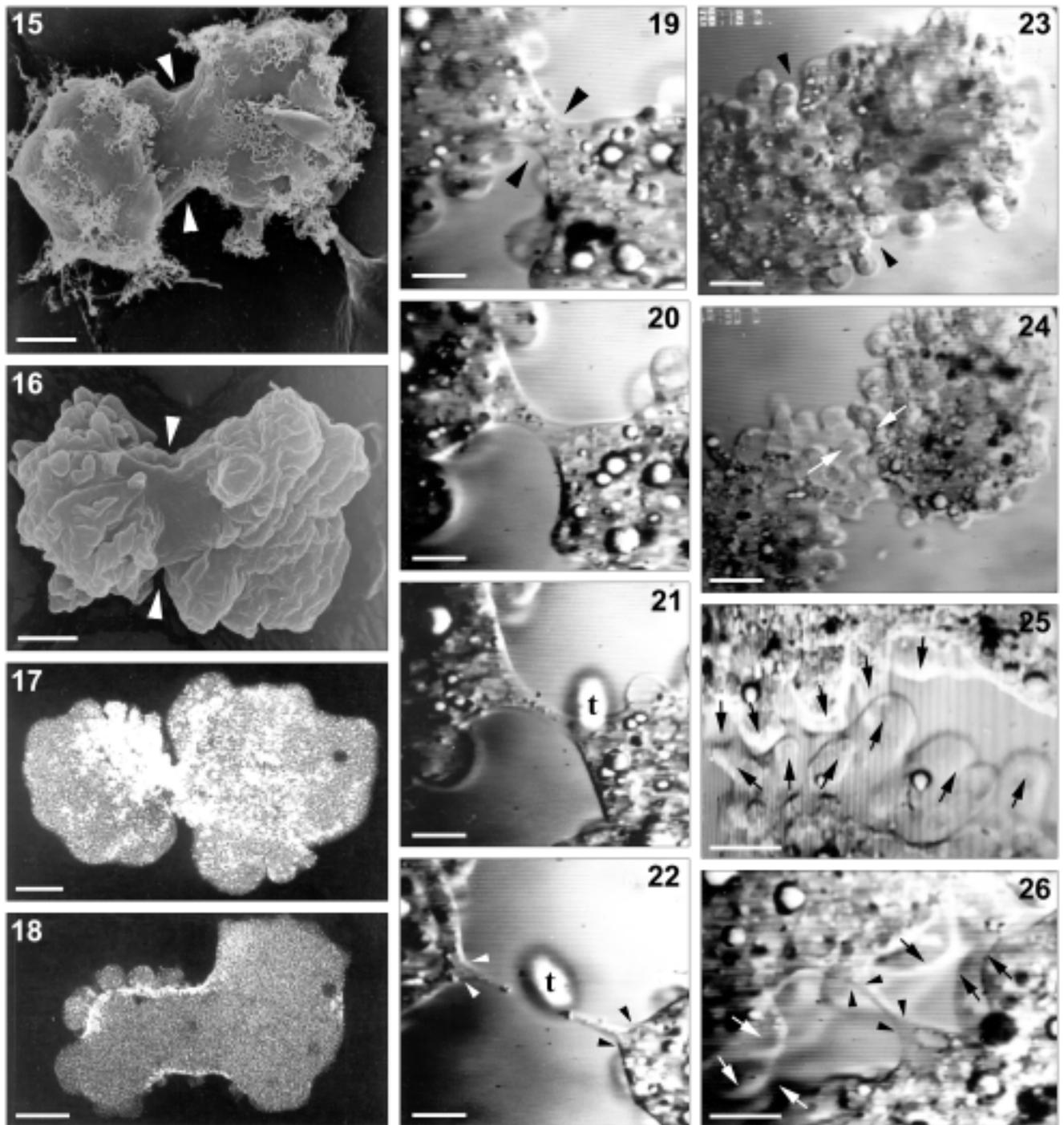
**Figs 12-14.** Extensive sheet of cortical microfilaments seen in SEM in the division spheres injured with a microneedle. **12** - general view of an injured specimen; **13** - the microfilaments layer beneath the cell surface; **14** - high magnification of the microfilaments network. Scale bars - 10  $\mu$ m in 12, 13, and 2  $\mu$ m in 14.

polar relaxation is needed to start cytokinesis. This concept, repeatedly appearing in the past (e.g. Chalkley 1935, Wolpert 1960) as well as more recently (e.g. White and Borisy 1983, Bray and White 1989, Grębecki 1994), seems to be especially well applicable to the case of *A. proteus* in which the whole contractile cortex creates tension, but the locomotion is initiated and controlled by frontal relaxation (Grębecki 1981, 1990, 1994).

The next event, beginning probably in the late metaphase, is the reconstruction of adhesive organelles. SEM reveals that the bulbous protuberances of the division spheres start producing microextensions (Fig. 5) which gradually achieve (Fig. 6) the size and shape of minipodia grouped in adhesive rosettes (identical with those described by us in the attached and moving interphasal amoebae: Grębecki *et al.* 2001). It is worthy to note that Sanger and Sanger (1980) described the

disappearance and reconstruction of "microvilli", similar to our "minipodia", during cytokinesis of PtK<sub>2</sub> cells of epithelial origin.

After this stage, in amoebae recorded *in vivo*, the chaotic particle movements become suddenly (within 1-2 min) ordered into a few local centrifugal streamings, and several flat lobose pseudopodia appear and spread over the glass (Fig. 2). This is the typical behaviour of *A. proteus* re-adhering to the substratum (Kołodziejczyk *et al.* 1995). Re-adhesion leads to cell spreading, so that the division spheres become flat disks (compare the optical cross-sections of the same amoeba in Figs 1 and 2). Examination in SEM shows at this stage minipodia and rosette contacts arranged along disk edges (Fig. 7). As we have suggested earlier (Grębecki *et al.* 2001), marginally distributed adhesive organelles contribute to further cell spreading and flattening (compare Fig. 7



**Figs 15, 16.** SEM pictures of amoebae during formation of the furrow (arrowheads). **15** - the usual bipolar distribution of minipodia and rosette contacts; **16** - the exceptional case of furrowing amoeba without any adhesive organelles in view. Scale bars - 20  $\mu$ m

**Figs 17, 18.** CLSM pictures of amoebae stained with FITC-phalloidin during formation of the furrow. **17** - general view of F-actin aggregation toward the furrow; **18** - thin longitudinal optical section through the furrow shows peripheral actin; bodies of the future daughter cells are not seen (they were out of the section plane). Scale bars - 20  $\mu$ m in 17, and 10  $\mu$ m in 18.

**Figs 19-22.** DIC records of the separation of substratum-attached daughter cells from the stage of a deep furrow (arrowheads in **19**), through gradual narrowing of the connecting bridge (**20, 21**) up to its final break out (between arrowheads in **22**); t - is a *Tetrahymena* which accidentally entered into the field. Scale bars - 20  $\mu$ m

**Figs 23-26.** DIC records of amoebae dividing without attachment to the substratum. **23** - the furrow is masked by pseudopods (between arrowheads); **24, 25** - general view and a higher magnification of pushing one daughter cell against another by growing pseudopods (arrows); **26** - a connecting bridge (between small arrowheads) distended by the neighbour pseudopods (arrows). Scale bars - 20  $\mu$ m

with 8). The three DIC records of the same cell shown in Figs 1-3 demonstrate that its cross-sectional area increased about 3.5 times. In this respect the sequence of Figs 4-8 looks equally impressive, but it is less exact, since it is composed of different specimens which might originally vary in size.

Summarizing all what happens before the furrow formation, we conclude that the non-polar general contraction resulting in cell detachment and spherulation, as well as the following relaxation leading to cell re-adhesion, flattening and spreading, are both necessary prerequisites of a successful cytokinesis in *A. proteus*.

The bipartition of the mother cell begins in amoeba by a dramatic change of intracellular streaming pattern at the late anaphase. Suddenly, the disparate centrifugal endoplasmic streamings become strictly bipolar and the round disks quickly turn into the oval shape (Figs 3, 8). We should strongly stress that two steady streamings oriented exactly in two opposite directions occur, during the whole cytokinesis of *A. proteus*, only at this brief moment, and that this happens before the formation of furrow. Again, it fits better with the polar relaxation model than with the equatorial contraction model of initiating the fission of amoeba.

The time-lapse records in DIC show that during formation of the furrow both divided cell halves generate streamings and extend or retract pseudopods in all directions, and the motor activity of each of them is independent of another. In the furrow region the endoplasm does not steadily flow in two opposite directions from the middle, as it should be expected, but performs shuttle movements reversing at irregular periods. It means that this flow compensates the oscillating pressure differences between both future daughter cells, which are greater than the pressure gradient created by contraction in the furrow.

The motor activity of the prospective daughter cells is consistent with the observation that during formation of the furrow almost all amoebae remain attached to the substratum, and with the demonstration by SEM (Fig. 15) that adhesive minipodia and rosette contacts are common at both opposite cell halves, but scarce or absent at the furrow region. In whole our material we found just one case of amoeba devoid of adhesive organelles during furrow formation (Fig. 16). *Dictyostelium discoideum* may divide even in suspension, but if its cytokinesis is examined by reflection interference microscopy on a solid surface the cell-substratum contact also appears most extensive and

stable under the future daughter cells (see pictures in: Weber *et al.* 1999). If fibroblasts are in this respect similar to amoebae, then the wrinkling of silicone elastic substratum during their division, which was presented as a specific result of the contraction in the furrow (Burton and Taylor 1997), could rather result from the traction exerted between the adhesion sites under the bodies of both future cells.

The equatorial constriction certainly results from actomyosin contraction in the furrow. In the cortex of amoebae, however, a contraction always leads to gradual disintegration of the F-actin network (contraction-solation coupling: Taylor and Fenchheimer 1982). Therefore, the outflow of depolymerized actin from the furrow must be compensated by the centripetal F-actin import along the cell periphery (cortical flow theories: Bray and White 1989, Grębecki 1994). Such cortical transport of F-actin and myosin II toward the furrow was well studied in the dividing *Dictyostelium* amoebae (Kitanishi-Yumura and Fukui 1989, Fukui and Inoué 1991, Yumura and Fukui 1998, Yumura 2001). Strangely, the presence of F-actin in the division furrow of *A. proteus*, although it is obviously expectable, has never been shown. Its aggregation toward the furrow is demonstrated in Fig. 17, and a thin optical section running exactly along the furrow region is presented in Fig. 18.

After formation of the furrow and before final separation (Figs 19-22) the dividing amoebae usually were still attached to the substratum. We have found in whole our material recorded *in vivo* over 50 cases of cytokinesis completed by the adhering cells, but only 4 cases of division accomplished by amoebae which have spontaneously de-adhered. Possibly, the furrowing amoeba without adhesive organelles found in SEM (Fig. 16) belonged to the same category of cells. Moreover, the non-attached amoebae needed up to 1 hour to disconnect, while the well adhering ones achieved it in the limits of 10-20 minutes. Without adhesion both future daughter cells are spherical and extend short pseudopods in all directions and in the three dimensions (Figs 23, 24). Some of these pseudopods produced by the two cells may meet and push against each other (Fig. 25) and then mechanically disrupt the slender connecting bridge situated between them (Fig. 26). This mechanism, proposed very long ago (Chalkley 1935, Liesche 1938), sufficiently explains how the dividing amoebae experimentally detached from the substratum (Rappaport and Rappaport 1986) or spontaneously de-adhering (the present observations) can complete cytokinesis by

separation in two. It must not be, however, forgotten that in *A. proteus* the cell division without adhesion is exceptional.

Normally the daughter cells of *A. proteus*, still linked by a cytoplasmic strand, firmly adhere to the substratum and try to move in various directions. These are random movements not co-ordinated between the two future amoebae. Their exact opposition capable of breaking the connecting bridge up (as in Figs 19-22) can be achieved only by chance, and it may take several minutes until it happens. In contrast to some early ideas (constriction ring as "tail organizer" for the new cells: Goldacre and Lorch 1950), the furrow region changing into a connecting bridge is never wrinkled as the tail of an interphasal amoeba, but smooth and apparently stretched by daughter cells moving farther and farther apart (Figs 19-21). Breaking up of the connection is immediately followed by the elastic recoil of both parts, proving that the bridge was not contracting but distended by pulling in opposite directions (compare Fig. 21 with 22).

At least three factors may contribute to the initial constriction and the final rupture of the bridge which connects the two parts of a dividing *A. proteus*: (1) function of the contractile ring in the furrow, (2) stretching the connecting bridge by opposite locomotor activities on both sides of the furrow, (3) cytoskeleton disassembly inside the connecting bridge.

(1) The contractile ring certainly plays a major role in the first phase by gradually narrowing the lumen of the connection, but it cannot cut it in two by constriction, since it acts from the inside, not from the outside of this cytoplasmic tube; this argument applies to amoebae as well as to all other motile and non-motile cells.

(2) At the final separation phase the connection is broken, in amoebae, by independent motor activities of future daughter cells, *i.e.*, by pulling when they move on a solid substratum, or by pushing one against another when they are in suspension; this mechanism may operate in the cells capable of amoeboid movements, but not in non-motile cells.

(3) The coupling of contraction with solation and the disintegration of actomyosin network in moving amoebae, which were mentioned above, may lead with the elapse of time to exhaustion of the cytoskeletal material in the connecting bridge, what should help or provoke breaking up this last link between daughter cells; gradual disassembly of the contractile ring was observed during cleavage of sea urchin eggs (Schroeder 1972) and in dividing HeLa cells (Maupin and Pollard 1986); this

factor may probably be significant in all types of motile and non-motile cells.

For the time being the speculations about factors of the final separation of daughter cells in *A. proteus* may be in the best way concluded by quotation of the words of Robinson and Spudich (2000) that "the molecular control of this late step is only beginning to be uncovered and promises to bring many more surprises."

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## Distribution of Species of the *Paramecium aurelia* Complex in Israel

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**Summary.** The presence of *P. primaurelia*, *P. biaurelia*, *P. tetraurelia*, and *P. octaurelia* of the *Paramecium aurelia* complex was revealed in Israel. The last species was recorded for the first time in Asia.

**Key words:** ciliate biogeography, *Paramecium*, *P. aurelia* species complex.

### INTRODUCTION

At present 15 species of the *Paramecium aurelia* complex are known world-wide (Sonneborn 1975, Aufderheide *et al.* 1983). Some species are cosmopolitan (*P. primaurelia*, *P. biaurelia*, *P. tetraurelia*, and *P. sexaurelia*), while others have been found only in single localities (e.g., *P. quadecaurelia*). Studies concerning their distribution, however, were carried out unevenly in different areas. For instance, many collections were made first in North America (mainly in the USA, cf Sonneborn 1975) and later in Europe in numerous habitats (cf Przyboś 1998, Przyboś and Fokin 2000).

The sampling in Asia was carried out in Japan, Russia (Far East), Vietnam, Thailand, India, Turkmenia, Georgia, Turkey, Lebanon, and Israel but all samplings were rather accidental. The presence of the following species of the *P. aurelia* complex was recorded in Asia: *P. biaurelia* (14 habitats), *P. primaurelia* (9 habitats), *P. tetraurelia* (5 habitats), *P. sexaurelia* (4 habitats), and *P. novaurelia* (1 habitat) (cf Przyboś and Fokin 2000, 2001).

Studies on the occurrence of species of the *P. aurelia* complex in Israel (Przyboś 1995, Przyboś and Fokin 1999) revealed the presence of *P. primaurelia* in the River Jordan at Qasr el-Yehud near of Jericho, *P. tetraurelia* in the Sea of Galilee at Tabgha (Przyboś 1995), and again *P. primaurelia* in Jerusalem (channel) (Przyboś and Fokin 1999). However, both papers were based only on occasionally collected samples. As "Israel is distinguished by an extraordinary biotic diversity, due to the combination of its geographical position at a

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**Table 1.** Occurrence of species of the *Paramecium aurelia* complex, *Paramecium multimicronucleatum* and *P. caudatum* in the studied sampling places in Israel

Locality	Habitat / pH of water	Species of the <i>Paramecium aurelia</i> complex	Other <i>Paramecium</i> species
Kiryat Motzkin	Na'aman Canal / pH = 7.0		<i>P. multimicronucleatum</i>
Ein Afek	Fish pond No. 1 / pH = 7.5	<i>P. tetraurelia</i>	
Ein Afek	Fish pond No. 2 / pH = 7.4	<i>P. octaurelia</i>	
Na'aman Canal	Water canal / pH = 7.1		<i>P. multimicronucleatum</i>
Neighbourhood of Akko	Canal connecting fish ponds / pH = 7.7		<i>P. multimicronucleatum</i>
Nahal Alon, Carmel National Park, Mt. Carmel	Fish pond / pH = 6.8		<i>P. caudatum</i> , <i>P. multimicronucleatum</i>
Neighbourhood of fortress Atlit	Fish pond / pH = 7.1		<i>P. multimicronucleatum</i>
Gid'ona	Fish pond / pH = 7.1		<i>P. multimicronucleatum</i>
Neighborhood of the Sheikh Hussein Bridge	Fish pond / pH = 7.4		<i>P. multimicronucleatum</i>
Yehudiya Junction	Fish pond / pH = 7.1	<i>P. primaurelia</i>	<i>P. multimicronucleatum</i>
Neighbourhood of Neot Mordechai	Fish pond / pH = 7.4		<i>P. multimicronucleatum</i>
Dan	Tributary of Jordan River / pH = 7.1	<i>P. biaurelia</i>	
Nahal Guvta	Stream / pH = 6.8		<i>P. caudatum</i> , <i>P. multimicronucleatum</i>

continental crossroads, its great physical variety, and rich paleobiological history" (Yom-Tov and Tchernov 1988), it seemed worth while to make there more intensive investigations on the distribution and occurrence of the *P. aurelia* spp. complex in that country. It seemed probable that the species of the *P. aurelia* complex that have not been observed in Israel before would be found here. The present paper reports the results of studies on the occurrence of the *P. aurelia* spp. based on material collected in many places in Israel.

## MATERIALS AND METHODS

The water samples (45 ml) with plankton organisms were collected in April 2001 from 29 sampling places, 24 of which situated in the northern part of Israel, and 5 on the territory of the Negev Desert.

The water was taken from the surface and the littoral part of the water bodies. Ambient temperature and the pH and water temperature were also measured during sampling. Lettuce medium inoculated with *Enterobacter aerogenes* (Sonneborn 1970) was used for cultivation of paramecia.

*Paramecium* species were identified on the basis of analysis of the type and number of their micronuclei (Vivier 1974) on the slides stained by acetocarmine or by Feulgen reaction (after fixation and hydrolysis, cf Przyboś 1978).

Identification of the established clones of the *P. aurelia* spp. complex was carried out according to Sonneborn (1950, 1970). Species of the complex were determined by mating the investigated reactive (mature for conjugation) clones with the reactive mating types of the standard strains. The following standard strains were used: strain 90 of *P. primaurelia* (Pennsylvania, USA), the strain from Rieff, Scotland, of *P. biaurelia*, the strain from Sydney, Australia of *P. tetraurelia*, and the strain 138 of *P. octaurelia* (Florida, USA). The species were determined on the basis of 95-100% conjugation between the complementary mating types of the examined clones with the appropriate standard ones.

## RESULTS AND DISCUSSION

The presence of *P. primaurelia*, *P. biaurelia*, *P. tetraurelia*, and *P. octaurelia* was revealed in Israel by the present study (Table 1).

*Paramecium primaurelia* (seven clones) was identified in the sample (pH 7.1) collected from a pond at Yehudiya Junction, situated east of the Sea of Galilee (Jordan Valley). *P. multimicronucleatum* appeared in the same sample.

*Paramecium biaurelia* (three clones) was found in the sample (pH 7.1) collected from a tributary of the upper River Jordan in Dan (Upper Galilee, Valley). It is the first record of the species in Israel. *P. biaurelia* was found earlier in the adjoining Lebanon (Sonneborn 1974).

*Paramecium tetraurelia* (three clones) was identified in the sample (pH 7.5) collected at Ein Afek (east of Haifa, Northern Coastal Plain) from a pond (designated as No. 1). It appeared together with *P. multimicronucleatum*.

*Paramecium octaurelia* (three clones) was identified in the sample (pH 7.4) collected from a pond designated as no 2 at Ein Afek. It is the first record of its presence in Asia. Previously, *P. octaurelia* was known mainly from the USA but was also recorded in Panama and Uganda (Sonneborn 1975). In Sonneborn's opinion "This species is ... common in the tropical and subtropical Americas and may also be common around the world". The present studies showed that Sonneborn's supposition was correct. *P. octaurelia* was also recorded in Europe by Stoeck and Schmidt (1998) in a water sample collected in southwest Germany. The present findings of *P. octaurelia* in Israel modify our knowledge concerning the range of the species in the world. However, it is difficult to determine the conditions specifically suitable for the appearance of the species.

Among the species recorded at present in Israel *P. primaurelia*, *P. biaurelia*, and *P. tetraurelia* are cosmopolitan (Sonneborn 1975).

The species of the *P. aurelia* complex appeared mainly in the northern part of Israel (Northern Coastal Plain and Jordan Valley) in waters characterized by

neutral pH, ambient and water temperatures during samplings being 22 to 25°C.

As concerns the other *Paramecium* species, *P. multimicronucleatum* was recorded in 10 sampling places, in two of them with *P. caudatum*. *Paramecium* spp. appeared in 13 out of 29 places, mainly in ponds and canals connected with fishponds.

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