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Review Article

Role of Lipids and Fatty Acids in Stress Tolerance in Cyanobacteria

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Summary. Lipids are the most effective source of storage energy, function as insulators of delicate internal organs and hormones and play an important role as the structural constituents of most of the cellular membranes. They also have a vital role in tolerance to several physiological stressors in a variety of organisms including cyanobacteria. The mechanism of desiccation tolerance relies on phospholipid bilayers which are stabilized during water stress by sugars, especially by trehalose. Unsaturation of fatty acids also counteracts water or salt stress. Hydrogen atoms adjacent to olefinic bonds are susceptible to oxidative attack. Lipids are rich in these bonds and are a primary target for oxidative reactions. Lipid oxidation is problematic as enzymes do not control many oxidative chemical reactions and some of the products of the attack are highly reactive species that modify proteins and DNA. This review deals with the role of lipids and fatty acids in stress tolerance in cyanobacteria.

Key words: cyanobacteria, desiccation, fatty acids, lipids, salinity, temperature stress.

INTRODUCTION

Cyanobacteria are gram-negative photoautotrophic prokaryotes having ´higher plant-type‘ oxygenic photosynthesis (Stewart 1980, Sinha and Häder 1996a). Certain cyanobacteria differentiate a small fraction of their cells into heterocysts, the site of aerobic nitrogen fixation. The significant role of these N_2-fixing microorganisms in improving the fertility of wetlands such as rice paddy fields, at the sole expense of photosynthetic energy produced on their own, is well documented (Sinha and Häder 1996a; Sinha *et al*. 1998a; Vaishampayan *et al*. 1998, 2001).

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The cyanobacteria such as *Spirulina* and *Nostoc* have been used as a source of protein and vitamin for humans and animals (Ciferri 1983, Kay 1991, Gao 1998, Takenaka *et al*. 1998). *Spirulina* has an unusually high protein (up to 70 % of the dry weight) content for photosynthetic organisms and is being also used as a source of natural colorants in food, and as a dietary supplement (Kay 1991). *Nostoc flagelliforme* and *Nostoc commune* is considered a delicacy in China (Gao 1998, Takenaka *et al*. 1998) and Philippines (Martinez 1988) respectively. The availability of powerful genetic techniques allow the biotechnological application of cyanobacteria to produce specific products, to biodegrade organic pollutants in surface waters, to control mosquitoes and for many different other purposes (Koksharova and Wolk 2002). The medicinal value of cyanobacteria was appreciated as early as 1500 BC,

when strains of *Nostoc* were used to treat gout, fistula and several forms of cancer (Pietra 1990). The very high incidence of novel, biologically active compounds isolated so far indicates that cyanobacteria are a rich source of potentially useful natural products (Moore 1996). Over 40 different Nostocales species, the majority of which are *Anabaena* and *Nostoc* spp. produce over 120 natural products (secondary metabolites) having activities such as anti-HIV, anticancer, antifungal, antimalarial and antimicrobial. Cyanovirin (CV-N, cyanovirin-N), a 101 amino acid protein extracted from *Nostoc ellipsosporum* was found to have potent activity against all immunodeficiency viruses such as HIV-1, M-and T-tropic stains of HIV-1, HIV-2, SIV (simian) and FIV (feline) (for a review see Burja *et al*. 2001).

Complete genomic sequences have been obtained for the unicellular cyanobacterium, *Synechocystis* sp. strain PCC 6803 (3.57 Mb plus sequenced plasmids) and the filamentous, heterocyst-forming cyanobacterium, *Anabaena* spp. strain PCC 7120 (6.41 Mb plus sequenced plasmids) (Kaneko and Tabata 1997, Kaneko *et al*. 2001a; see http://www.kazusa.or.jp/cyano/). Extensive sequence data are available for two ecotypes (MED4, a high light adapted ecotype from the Mediterranean Sea, and MIT 9313, a low light adapted ecotype from the Gulf Stream) of the unicellular cyanobacterium *Prochlorococcus* (http://www.jgi.doe.gov/JGI-microbial/html/prochlorococcus/prochloro-pickastrain.html), *Nostoc punctiforme* strain PCC 73102 (ATCC 29133) (http://www.jgi.doe.gov/JGI-microbial/html/nostoc/nostoc homepage.html), *Gloeobacter* and several strains of *Synechococcus* (Bryant *et al*. 2001, Holtman *et al*. 2001, Kaneko *et al*. 2001b). Genomic sequence data provide the opportunity for global monitoring of changes in genetic expression at transcriptional and translational levels in response to varying environmental conditions. The available genomic sequence data may be extremely useful in identifying regulatory and structural genes (Ochoa de Alda and Houmard 2000, Zhulin 2000), investigating molecular mechanisms of natural genetic transformation (Yoshihara *et al*. 2001) and analyzing evolutionary events (Herdman *et al*. 2000, Rujan and Martin 2001).

Lipids are esters of fatty acids and alcohols that comprise a large group of structurally distinct organic compounds including fats, waxes, phospholipids, glycolipids etc. Cyanobacteria may contain significant quantities of lipids (fats and oil) with compositions similar to those of vegetable oils. The lipids of some cyanobacterial species are also rich in essential fatty acids such as the C_{18} linoleic (18:2ω6) and y-linolenic (18:3ω3) acids and their C_{20} derivatives, eicosapentaenoic acids (20:5ω3) and arachidonic acid (20:4ω6). These fatty acids are essential components of the diet of humans and animals and are becoming important feed additives in aquaculture (Borowitzka 1988). The lipids of cyanobacteria are generally esters of glycerol and fatty acids (Table 1). They may be either saturated or unsaturated. Some of the filamentous cyanobacteria tend to have large quantities (25 to 60 % of the total) of polyunsaturated fatty acids (Parker *et al*. 1967, Holton and Blecker 1972, Kenyon *et al*. 1972). A few cyanobacterial strains, which show facultative anoxygenic CO_2 photoassimilation with sulphite as electron donor, lack polyunsaturated fatty acids in their lipids (Oren *et al*. 1985).

The cosmopolitan distribution of cyanobacteria indicates that they can cope with a wide spectrum of global environmental stresses such as heat, cold, desiccation, salinity, nitrogen starvation, photo-oxidation, anaerobiosis and osmotic stress etc. (Fay 1992, Tandeau de Marsac and Houmard 1993, Sinha and Häder 1996b). They have developed a number of mechanisms by which cyanobacteria defend themselves against environmental stressors. Important among them are the production of photoprotective compounds such as mycosporine-like amino acids (MAAs) and scytonemin (Sinha *et al*. 1998b, 1999a, b, 2001), enzymes such as superoxide dismutase, catalases and peroxidases (Burton and Ingold 1984, Canini *et al*. 2001), repair of DNA damage (Sinha and Häder 2002) and synthesis of shock proteins (Borbély and Surányi 1988, Bhagwat and Apte 1989, Sinha and Häder 1996b). In this review, we discuss only the role of lipids and fatty acids in stress tolerance in cyanobacteria.

STRUCTURE OF LIPIDS

Triglycerides are the most common storage lipids and may constitute up to 80 % of the total lipid fraction in cyanobacteria (Klyachko-Gurvich 1974, Tornabene *et al*. 1983). Besides triglycerides, the other major lipids are sulphoquinovosyl diglycerides (SQDG), monogalactosyl diglyceride (MGDG), digalactosyl diglyceride (DGDG) and phosphatidyl glycerol (PG). These four major lipids (Fig. 1) can be identified on the basis of their Rf values in TLC, ^{1}H NMR and ^{14}C NMR (Döhler and Datz 1980, Sato and Murata 1981, Piorreck and Pohl 1984, Harwood *et al*. 1988, Singh 2001).

Table 1. Composition of fatty acids in cyanobacteria

Fatty acid	Position of double bond	Percentage of total fatty acids
14:0	-	40
16:0	-	60
16:1	9	50
16:2	6, 9	20
16:2	9, 12	50*
17:1	-	10
18:0	-	30
18:1	9	40
18:2	9, 12	40
18:3	6, 9, 12	30
18:3	9, 12, 15	40
18:4	6, 9, 12, 15	30
20:1	11	10

*Present only in few strains of cyanobacteria. References: Kates and Volcani 1966; Harrington *et al*. 1970; Wood 1974; Mercer and Davies 1975; Paoletti *et al*. 1976a,b; Yurieva *et al*. 1984; Borowitzka 1988

ROLE OF LIPIDS AND FATTY ACIDS IN DESICCATION TOLERANCE

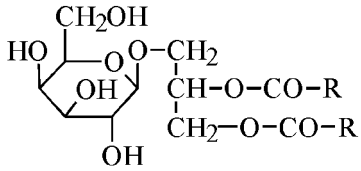
Water is most essential for life. Removal of water from a cell creates a severe, often lethal stress. Desiccation-tolerant cells implement structural, physiological and molecular mechanisms to survive acute water deficit. These mechanisms, and the components and pathways, which facilitate them, are poorly understood in cyanobacteria (Potts 2001). Mechanisms, which maintain the structural integrity of membranes, appear to be of importance. Some sugars, particularly trehalose, prevent damage from dehydration not only by inhibiting fusion between adjacent membrane vesicles during drying, but also by maintaining membrane lipids in the fluid phase in the absence of water (Crowe *et al*. 1987, 1992). Water molecules are critical components of the reaction mechanism; they contribute to the stability of proteins, DNA and lipids. Water may have played a determinative role in the origin and evolution of the genetic code (Wolfenden *et al*. 1979, Potts 1994).

Some desiccation-tolerant cells accumulate large amounts of either one or both of the disaccharides trehalose and sucrose (Crowe *et al*. 1992). Such observations have led to the conclusion that these disaccharides are effective at protecting enzymes during both freeze-drying and air-drying. Recent evidence show that the P=O stretch of the phospholipid increases in frequency by about 30 cm^{-1} when the protein is dried without trehalose but is decreases to or below the frequency of hydrated P=O when the protein is dried with trehalose. Molecular modelling shows that trehalose can fit between the phosphate of adjacent phospholipids (Rudolph *et al*. 1990). At low trehalose/lipid ratios trehalose is not available to bind water thus showing a direct interaction between the sugar and lipid.

When the membranes are isolated, free radicals cause fatty acids deesterification from phospholipids. Free fatty acids typically accumulate in desiccation sensitive cells during aging and are a cause of reduced membrane integrity. The respiratory rate prior to desiccation correlates well with the number of free radicals in the dry state, which suggests that the curtailment of the respiratory metabolism prior to rehydration may be essential for the retention of membrane integrity and desiccation tolerance (Hoekstra 1993). The imibition of viable, dry cells may result in extensive leakage and death, particularly when it occurs at low temperature, because rehydration may involve a reverse phase change of membrane lipids from the gel to liquid-crystal phase which occurs in the presence of water (Crowe *et al*. 1987).

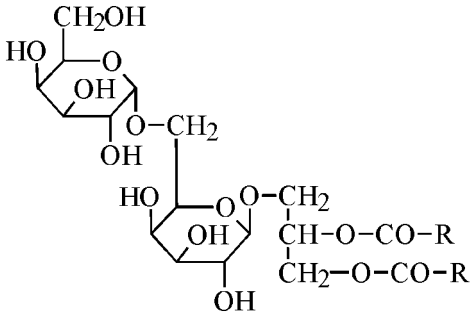
Alteration in the lipid content of membranes of an organism is of major importance in response to environmental stresses (Olie and Potts 1986, Ritter and Yopp 1993). Maintenance of membrane integrity in anhydrobiotic organisms represents a central mechanism of desiccation tolerance (Carpenter and Crowe 1989, Crowe and Crowe 1992a). The role of membrane fluidity and lipid composition on survival of bacteria at extreme temperatures, salinity and drying has been reported (Russell and Fukunga 1990, Oliver *et al*. 1998). Almost 60 % of the total phospholipids in the purified cytoplasmic membrane of *N. commune* UTEX 584 was found to be 20:3ω3 fatty acid (Olie and Potts 1986). Rehydration of dry mats of *Scytonema geitleri* resulted in a slight increase in the amount of total lipids (Singh 2001). Trehalose can stabilize membranes (Crowe and Crowe 1986, 1992a,b; Crowe *et al*. 1987; Leslie *et al*. 1994). Membranes dried without trehalose undergo vesicle fusion, change in morphology and loss of calcium transport activity upon subsequent rehydration (Crowe *et al*. 1992). Oliver *et al*. (1998) developed the water replacement hypothesis to describe how the non-reducing sugar trehalose protects cells, membranes, proteins, and nucleic acids when they are dried. Trehalose seems to depress the phase transition temperature of the dry lipids after desiccation and maintains them in the liquid-crystal state (Crowe *et al*. 1992, Leslie *et al*. 1994).

(a) Monogalactosyldiacylglycerol (MGDG)



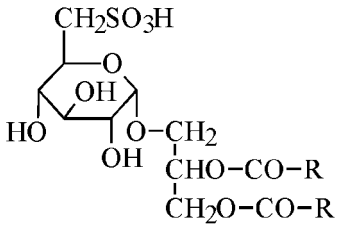
1,2-di-O-acyl-3-O-β-D-galactopyranosyl-sn-glycerol

(b) Digalactosyldiacylglycerol (DGDG)



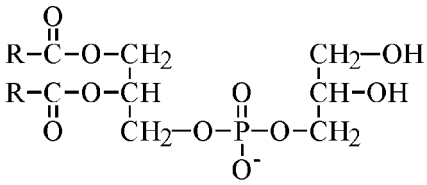
1,2-di-O-acyl-3-O[-α-D-galactopyranosyl-(1'-6')-
O-β-D-galactopyranosyl]-sn-glycerol

(c) Sulfoquinovosyl-diacylglycerol (SQDG)



6-sulfo-α-D-quinovopyranosyl-(1-43')-1',2'-diacyl-sn-glycerol

(d) Phosphatidyl glycerol (PG)



3-sn-phosphatidyl-1'-sn-glycerol

Fig.1. Structural formula based on ^{13}C and ^{1}H NMR of lipids of *Scytonema geitleri* as separated on silica gel TLC

NMR measurements of dry mixtures of trehalose and 1,2-dipalmitoyl-*sn*-phosphatidylcholine (DPPC) indicate that the sugar is in close proximity to the hydrophilic region from the phosphate head group to the interfacial regions (Lee *et al*. 1986). The eight hydroxyls on each trehalose are all available for hydrogen bonding to the phosphate and carbonyl groups of the lipids. The sugar is thought to occupy some space between the lipid molecules.

A number of desiccation tolerant cyanobacteria have highly pigmented sheaths. One pigment that is unique and restricted to only a few cyanobacteria is the yellow-brown lipid-soluble pigment scytonemin. It is an optically inactive dimeric pigment located in the extracellular polysaccharide sheath with a molecular mass of 544 Da and a structure based on indolic and phenolic subunits (Proteau *et al*. 1993). Scytonemin with absorption maximum at 386 nm (but also absorbs significantly at 252, 278, and 300 nm) has been proposed to serve as an ultraviolet (UV) sunscreen (Sinha *et al*. 1998b, 1999b). Once synthesized, scytonemin remains highly stable and carries out its screening activity without further metabolic investment from the cell. Rapid photodegradation of scytonemin does not occur which is evidenced by its long persistence in terrestrial cyanobacterial crusts or dried mats. This strategy may be invaluable to several scytonemin containing cyanobacteria inhabiting harsh habitats and subject to regular cycles of desiccation and rewetting and must survive long periods of metabolic inactivity (Sinha *et al*. 1999b). Thus the mechanism of desiccation tolerance by lipids and fatty acids in cyanobacteria seems to be complex interactions, which will not be easily resolved through genetic analysis (Potts 1999).

ROLE OF LIPIDS AND FATTY ACIDS IN TOLERANCE TO SALT-INDUCED DAMAGE IN CYANOBACTERIA

There are many environmental factors that limit growth and productivity of microorganisms; salt stress is one of them. The mechanisms of the hyperosmotic stress-induced and the salt stress-induced inactivation of the photosynthetic machinery, particularly the oxygen evolving machinery of the photosystem II complex, have been investigated in *Synechococcus* sp. PCC 7942 (Allakhverdiev *et al*. 2000a,b; 2001). Hyperosmotic stress due to 1 M sorbitol induces the efflux of water through water channels and reduces the volume of cells by more than 50 %. This loss of water from the cytosol might be expected to increase the intracellular concentration of salts and leads to the rapid but reversible inactivation of the oxygen evolving machinery (Allakhverdiev *et al*. 2000b). Salt stress due to 0.5 M NaCl has both osmotic and ionic effects (Allakhverdiev *et al*. 2000a). The osmotic effect due to 0.5 M NaCl is not as strong as the effect of 1 M sorbitol and inactivates reversibly the photolytic water splitting. The ionic effect of 0.5 M NaCl is caused by the influx of Na^+ ions through K^+ (Na^+) channels and the resultant increase in the intracellular concentration of Na^+ ions and counterpart anions that are mostly Cl^- ions (Allakhverdiev *et al*. 2000a). These changes result in the irreversible inactivation of the oxygen evolving machinery. As a consequence salt stress appears to be much more damaging to the oxygen evolving machinery than osmotic stress. Cyanobacteria have several kinds of mechanisms that allow them to acclimate to salt stress. For example, the inducible synthesis of compatible solutes such as sucrose is synthesized in salt-sensitive strains of cyanobacteria such as *Synechococcus* (Mackay *et al*. 1984, Reed *et al*. 1986, Joset *et al*. 1996, Hagemann and Erdmann 1997); glucosylglycerol is synthesized in strains with intermediary tolerance such as *Synechocystis* sp. PCC 6803 (Hagemann *et al*. 1987, 2001; Erdmann *et al*. 1992; Joset *et al*. 1996; Hagemann and Erdmann 1997; Mikkat and Hagemann 2000); glycinebetaine is synthesized in salt tolerant *Synechococcus* sp. PCC 7418 (Mackay *et al*. 1984, Reed *et al*. 1986, Joset *et al*. 1996, Hagemann and Erdmann 1997). Direct evidence for the ability of these compatible solutes to protect the cyanobacterial cells may be seen from studies of transgenic systems (Deshnium *et al*. 1995, 1997; Ishitani *et al*. 1995; Nakamura *et al*. 1997). Genes encoding a substrate-binding protein (ggtB) and two integral membrane proteins (ggtC and ggtD) of the binding-protein-dependent ABC transporter for osmoprotective compound glucosylglycerol have been identified in the genome of *Synechocystis* sp. PCC 6803. These genes are clustered on the chromosome about 220 kb away from the previously identified *ggtA* gene, which encodes the ATP-binding protein of this transport system (Mikkat and Hagemann 2000). Changes in matrix water potential (a term generally applied to considerations of water interactions at surfaces and interfaces; Nobel 1983) have been reported to affect total lipids. Total lipids have been shown to be decreased on lowering of the matrix water potential in a cyanobacterium *Scytonema geitleri*. The maximum decrease was observed on

- 2.8 megaPascals (MPa). There was no apparent change between - 14.5 and - 21 MPa (Singh 2001).

Many reports have suggested that lipids might be involved in the protection against salt stress (Huflejt *et al*. 1990, Khamutov *et al*. 1990, Ritter and Yopp 1993). When photosynthetic organisms are exposed to salt stress, the fatty acids of membrane lipids are desaturated. Allakhverdiev *et al*. (2001) have used targeted mutagenesis to alter genes for fatty acid desaturases in *Synechocystis* sp. PCC 6803, and they have produced strains with decreased levels of unsaturated fatty acids in their membrane lipids (Tasaka *et al*. 1996) as well as decreased the tolerance to salt (Allakhverdiev *et al*. 1999). Their results demonstrate that an increase in the unsaturation of fatty acids in membrane lipids enhances the tolerance to salt stress of the photosynthetic and Na^+/H^+ antiport systems of *Synechococcus*. Wild type cells are more sensitive to NaCl and less able to recover from its effects than *desA*$^+$ cells. It can be explained by the following four mechanisms. (i) The activity of water channels is responsible for the sorbitol-induced inactivation (Allakhverdiev *et al*. 2000b) and the rapid phase of the NaCl-induced inactivation (Allakhverdiev *et al*. 2000a). Therefore, it is quite possible that their activity might be affected by the unsaturation of membrane lipids or by changes in the fluidity of the membrane, (ii) K^+ (Na^+) channels, as well as the water channels, are located in the plasma membrane, and their activities might be depressed by the unsaturation of fatty acids of membrane lipids (Allakhverdiev *et al*. 2000b, 2001), (iii) The Na^+/H^+ antiport system, consisting of Na^+/H^+ antiporter(s) and H^+ ATPase(s), is also located in the plasma membrane. The unsaturation of fatty acids in membrane lipids might activate the Na^+/H^+antiport system *via* enhanced fluidity of the membrane with resultant protection of PSII and PSI activities (Blumwald *et al*. 1984, Padan and Schuldiner 1994, Allakhverdiev *et al*. 2001). The activities of several membrane bound enzymes are known to be affected by changes in membrane fluidity (Kates *et al*. 1984, Kamada *et al*. 1995). (iv) The unsaturation of fatty acids might stimulate the synthesis of the Na^+/H^+ antiporter(s) and/or H^+ ATPase(s). The increased density in the membrane of these components of the antiport system might result in a decrease in the concentration of Na^+ in the cytosol, which would tend to protect PSII and PSI against NaCl-induced inactivation and to accelerate the recovery of PSII and PSI activities (Allakhverdiev *et al*. 2001).

DESATURATION OF MEMBRANE LIPIDS FAVORS CYANOBACTERIA TO ACCLIMATIZE IN LOW TEMPERATURE

The environmental factors compel organisms to acclimatize to the external conditions. The physical properties of a biological membrane depend on the fatty acid composition of its component membrane lipids. Unsaturated fatty acids are essential constituents of polar glycerolipids of biological membranes and the unsaturation level of membrane lipids is important in controlling the fluidity of membranes (Chapman 1975).

Cyanobacteria respond to a decrease in ambient growth temperature by desaturating the fatty acids of membrane lipids to compensate for the decrease in membrane fluidity at low temperatures (Murata and Nishida 1987). Fatty acid desaturases are the enzymes that introduce the double bonds into the hydrocarbon chains of fatty acids, and thus these enzymes play an important role during the process of cold acclimation of cyanobacteria (Sato and Murata 1980, 1981, 1982; Wada and Murata 1990). The unsaturation of fatty acids occurs without *de novo* synthesis of fatty acids during low temperature acclimation of cyanobacterial cells (Sato and Murata 1981, Wada and Murata 1990). Most of the cyanobacterial desaturases are intrinsic membrane proteins that act on acyl-lipid substrates. It has been demonstrated that the unsaturation of membrane lipids is essential for low temperature tolerance of cyanobacteria by genetic manipulation of the *desA* (Δ12 desaturase) gene, which was isolated from the transformable cyanobacterium *Synechocystis* sp. strain PCC 6803 (Wada *et al*. 1990, 1992, 1994; Gombos *et al*. 1992, 1994; Sakamoto *et al*. 1998). In *Synechocystis* sp. strain PCC 6803, the mRNA level for the *desA* gene, which was shown to be regulated in response to temperature (Los *et al*. 1993, 1997), and *desB* (ω3 or Δ15 desaturase) transcripts accumulated in cells grown below 26°C (Sakamoto *et al*. 1994). Chemical hydrogenation of the cytoplasmic membrane stimulated accumulation of the *desA* transcripts, implying that a change in membrane fluidity might be the primary signal for the onset of expression of the desaturase genes (Vigh *et al*. 1993). It has been suggested that the accumulation of *desA* transcripts at low temperatures might be explained by an acceleration of transcription and by stabilization of the *desA* mRNA (Los and Murata 1994). It should be stressed that cold-sensitive *Synechocystis* with monounsaturated fatty acids only become cold-tolerant

by introduction of the gene for Δ12 desaturase that allows cells to synthesize diunsaturated fatty acids (Wada *et al*. 1990). On the other hand, directed mutations of desaturases in cold-tolerant *Synechocystis* that lead to production of monounsaturated fatty acids make this strain cold-sensitive (Tasaka *et al*. 1996). However, the temperature-sensing mechanism(s) and the control mechanism(s) regulating the expression of the desaturase genes are still unknown.

LIPID DESATURATION: REGULATION AND CHARACTERIZATION OF GENE EXPRESSION AS A FUNCTION OF TEMPERATURE

The unicellular marine cyanobacterium *Synechococcus* sp. strain PCC 7002 is classified as member of Group 2, based upon the fatty acid composition of its lipids and their pattern of desaturation (Murata *et al*. 1992). This cyanobacterium synthesizes lipids containing C_{18} fatty acids with none, one, two or three double bonds at the Δ9, Δ12, and *w*3 or Δ15 positions at the *sn*-1 position and C_{16} fatty acids containing none or one double bond at the Δ9 position of the *sn*-2 fatty acid (Murata and Wada 1995). Double bonds in the *sn*-1 fatty acid are added sequentially starting with desaturation at the Δ9 position and proceeding to the *w*3 position. Three desaturases, DesC (Δ9 desaturase) DesA (Δ12 desaturase) DesB (*w*3 or Δ15 desaturase) are responsible for the conversion of stearate to α-linoleate. Desaturation at the Δ9 and Δ12 positions occurs both at 34°C and at 22°C, while desaturation at the *w*3 (or Δ15) position only occurs in cells grown at low temperatures (Murata *et al*. 1992). Sakamoto and Bryant (1997) isolated and characterized the DesB (*w*3 desaturase) and DesC (Δ9 desaturase) genes from *Synechococcus* sp. strain PCC 7002 and studied the patterns of expression of the three desaturase genes in response to change in ambient temperature. They also demonstrate that changes in the stabilities of mRNAs for the desaturase genes contribute the overall regulation of the desaturase gene expression. The study of Sakamoto and Bryant (1997) revealed that transcription of the three desaturase genes in *Synechococcus* sp. strain PCC 7002 is differently regulated in response to changes in temperature in terms of mRNA synthesis and mRNA stabilization. Moreover, based upon a kinetic analysis with a time resolution of 5 min, they demonstrate that accumulation of mRNAs from these genes occurs very quickly during the process of the acclimation to low temperature. It has been proposed that specific temperature-sensor and signal transduction mechanisms might be involved in the regulation of the desaturase genes during cold acclimation of cyanobacteria (Murata and Wada 1995). However, it is possible that changes in the transcriptional and translational activities at lower temperatures as well as inherent differences in mRNA stability might have a direct influence on the up-regulation of the desaturase genes immediately following a temperature shift-down. However, the rates of mRNA synthesis from the *desA*, *desB* and *desC* genes as a function of temperature remain to be examined, and the mechanism(s) responsible for the transient increase of mRNA synthesis from the *desC* genes has not yet been identified.

ROLE OF LIPIDS IN TOLERANCE TO HIGH LIGHT-INDUCED PHOTOINHIBITION

Exposure of cyanobacteria to high PAR (photosynthetically active radiation) or UV radiation leads to photoinhibition of photosynthesis thereby limiting the efficient fixation of light energy (Han *et al*. 2001, Nishiyama *et al*. 2001). Photoinhibition occurs due to two basic mechanisms: (i) photoinduced, nonphotochemical quenching of excitation energy and (ii) photoinduced damage to the photosynthetic machinery (Krause 1988). In cyanobacterial photosynthesis, the nonphotochemical quenching particularly measured by O_2 evolution is not induced by light, indicating that the photoinhibition is mainly due to the photoinduced damage to the photosynthetic machinery. The molecular mechanism of photoinhibition revealed that the light-induced damage is caused by inactivation of the D1 protein of the PSII complex (Aro *et al*. 1993, Kanervo *et al*. 1993, Tyystjärvi *et al*. 2001). The damaged D1 protein is degraded proteolytically leaving the PSII complex depleted of the D1 protein. In the recovery process the precursor of the D1 protein is synthesized *de novo*, incorporated into the PSII complex, and then processed to yield the active D1 protein, with resultant generation of the active PSII complex (Andersson *et al*. 1992). The extent of the photoinhibition depends on the balance between the inactivation of the PSII complex and the recovery of the complex from the inactivated state (Gombos *et al*. 1994). Transformation of the cyanobacterium *Synechococcus* sp. PCC 7942 with the *desA* gene for a Δ12 desaturase have been reported to increase the unsaturation of membrane lipids and thereby enhance the tolerance of cyanobacterium to high light

(Gombos *et al*. 1997). These findings demonstrate that the ability of membrane lipids to desaturate fatty acids is important for the photosynthetic organisms to tolerate high light stress, by accelerating the synthesis of the D1 protein *de novo*.

LIPID PEROXIDATION

Oxidants and free radicals are deleterious in many ways. The organisms employ numerous approaches to block their production or limit their damage. Hydrogen atoms adjacent to olefinic bonds are susceptible to oxidative attack and especially those between unconjugated olefinic bonds. Lipids are rich in these bonds and thus are primary targets for oxidative reactions. Lipids oxidation is problematic as many oxidative chemical reactions are not controlled and constrained by enzymes and may show exponential reaction rates. Some of the products of the attack are highly reactive species that modify proteins and DNA (McIntyre *et al*. 1999). Lipid peroxidation has been shown to increase under drought conditions. Generally, in organisms undergoing a stress response, the enzymes of the Halliwell-Asada pathway and their main substrates have relatively higher activities and levels than those encountered under normal conditions (Elstner and Osswald 1994, Foyer *et al*. 1994). Hydroperoxides and MDA were often considered as indicators of membrane damage (Hagege *et al*. 1990a, b). Hydroperoxides are the initial products of lipid oxidation and usually account for the majority of bound oxygen measured by the peroxide value. MDA and a variety of aldehydes have long been recognized as secondary products derived from the degradation of lipid hydroperoxides.

Reactive oxygen species (ROS) such as O_2^-, H_2O_2 and $^{\cdot}OH$ are highly toxic to cells. Cellular antioxidant enzymes, and the free radical scavengers normally protect a cell from toxic effects of the ROS. There are reports that a decrease in PUFA (polyunsaturated fatty acid) content coincides with the increased levels of MDA in response to high osmotic stress. These responses, which are temporarily associated with an increase in electrolyte leakage, suggest that in fact water stress induces damage at the cellular and subcellular membrane levels *via* lipid peroxidation (Asada 1992, Aziz and Larher 1998). Cell membranes, which are structurally made up of large amounts of PUFA, are highly susceptible to oxidative attack and consequently changes in membrane fluidity, permeability, and cellular metabolic functions (Bandopadhyay *et al*. 1999).

CONCLUSIONS

Most cyanobacteria are a common source of a wide range of fats, oils, hydrocarbons and sterols with potential not only as a renewable source of liquid fuels but also for the production of a range of pharmacologically and industrially important products. The application of cyanobacteria in the production of these latter compounds is only just being explored and their importance has yet to be determined. New developments in the chemical industries, particularly in the area of converting natural products to industrial feedstocks, will further enhance the range of commercially important products synthesized by cyanobacteria.

In cyanobacterial cells, lipids are typically found only in the membranes. Hence the increased desaturation of lipids at low temperature must represent an environmentally triggered acclimation to improve membrane functionally at low temperature. Earlier studies have suggested that irreversible damage to cyanobacterial cells in the dark is initiated at low temperature by a phase separation of plasma membrane lipids. The phase separation of thylakoid membrane lipids occurs at a higher temperature. In most cyanobacteria, phase separation of thylakoid membrane lipids causes reversible loss of photosynthetic activity, and this depression of photosynthetic activity is reversed when cells are returned to their growth temperature. Increased desaturation of the lipids of the thylakoid membrane might be less important in the overall acclimation of cells to low temperature than desaturation of the lipids of the plasma membrane. The uptake of nutrients has an important role of the plasma membrane. It has been proposed that the decreased rate of the nutrient uptake from the environment could be the rate-limiting step for growth of cyanobacteria at low temperature. All three enzymes for nitrate assimilation are associated with membranes: the nitrate transporter is integrated in the plasma membrane, and nitrate and nitrite reductases are components of the thylakoid membranes. It is possible that membrane lipid unsaturation protects these enzymes from inactivation at low temperature. Covalent modification with lipids is a common feature of many membrane associated proteins, and the acyl groups function to anchor such proteins to membranes.

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PROTOZOOLOGICA

A Century of Research on the Amoeboflagellate Genus *Naegleria*

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Summary. The amoeboflagellate genus *Naegleria* contains pathogenic and nonpathogenic species. As most species are morphologically indistinguishable, species are defined and identified by molecular methods. For routine identification, isoenzyme analyses are performed. For the description of a new species, sequences of ribosomal DNA are increasingly used and the analyses of these sequences also allow us to define the phylogenetic relationships between species and strains. In the present monograph 27 *Naegleria* lineages are discussed and identified as separate species. Using molecular methods, *Naegleria* spp. have been identified which either form dividing flagellates or which do not form flagellates at all, thus contradicting the accepted definition of the genus. *Willaertia*, which forms dividing flagellates, is the genus that is the closest relative of the genus *Naegleria*. The genus *Naegleria* has some particularities in its molecular biology, such as circular ribosomal DNA plasmids, group I introns in the small and large subunit ribosomal DNA, and an unusual pyrophosphate-dependant phosphofructokinase. The phylogeny of the *Naegleria* spp. is compared to the situation concerning the other genera of the familyVahlkampfiidae. Also discussed is the state of affairs concerning species designation based on phylogeny in the genus *Acanthamoeba*, another free-living amoeba with species pathogenic to man.

Key words: *Acanthamoeba*, *Balamuthia*, dividing flagellates, group I introns, *Hartmannella*, *Naegleria pagei* sp. n., *N. pringsheimi* sp. n., *N. tihangensis* sp. n., non-flagellating, phylogeny, ribosomal DNA, *Vahlkampfia*.

Abbreviations used: AP - acid phosphatase, ATCC - American Type Culture Collection, bp - basepairs, CCAP - Culture Collection of Algae and Protozoa, CSF - cerebrospinal fluid, EMBL - European Molecular Biology Laboratory, IC - intracerebral, IN - intranasal, ITS - internal transcribed spacer, LSU - large subunit, mt - mitochondrial, NACM - *Naegleria* amoebae cytopathogenic material, NRS - non-ribosomal sequence, ORF - open reading frame, PAM - primary amoebic meningoencephalitis, PE - propionyl esterase, PCR - polymerase chain reaction, PPi-PFK - pyrophosphate-dependant phosphofructokinase, RAPD - random amplified polymorphic DNA, rDNA - ribosomal DNA, RFLP - restriction fragment length polymorphism, SSU - small subunit.

INTRODUCTION

A century ago Schardinger (1899) discovered an *Amoeba. lobosa* that could transform into a flagellate stage, and called it *Amoeba gruberi*. The genus name *Naegleria* was suggested much later by Alexeieff (1912).

Before 1970 *Naegleria* was studied mainly as a model for transformation because the amoebae easily transform into flagellates (Willmer 1956, Chang 1958, Fulton and Dingle 1967). However, the genus attracted much more attention, especially from the biomedical world, when it was found that some *Naegleria* isolates cause a fatal brain infection, primary amoebic meningoencephalitis (PAM) in humans. The infection almost invariably results in death. The *Naegleria* isolates that

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cause PAM were given species status and named *N. fowleri*, after Malcolm Fowler who first recognized the disease in Australia (Carter 1970). Cases of PAM were soon afterwards detected all over the world. The most recent review on the diseases produced by *N. fowleri* and other opportunistic free-living amoebae, belonging to the genera *Acanthamoeba* and *Balamuthia*, can be found in Martinez and Visvesvara (1997).

Between October 1970 and October 1972 four PAM cases were diagnosed in Belgium in children aged between 11 and 14 years old (Hermanne *et al*. 1973). All cases were diagnosed around the city of Antwerp. Although *N. fowleri* was never isolated from the swimming pools where the four children had been swimming before becoming ill, it was presumed that these swimming pools were implicated. Because the swimming pools are filled with water from the drinking water suppliers, the latter were accused of introducing the pathogenic amoeba into the swimming pools. Therefore, the Belgian water distributors decided to have the water of their network investigated for the presence of *N. fowleri*. This is where my involvement with *Naegleria* started. As a young biologist, with no experience in protozoa at all, I was hired to work on the grant that was assigned to a university laboratory, that also had no experience in protozoology whatsoever.

It was quite a coincidence that a new case of PAM was diagnosed in Belgium (Van Den Driessche *et al*.1973) only six months after I started to investigate methods for the identification and isolation of *N. fowleri*. Not only was the timing useful, the place where the 14 year-old boy probably became infected gave a clue to the ecology of the pathogenic *N. fowleri*. The deceased boy had been swimming in a brook that received cooling water from a metallurgical factory, and we were able to demonstrate the presence of *N. fowleri* in that water (De Jonckheere *et al*. 1975). Therefore, an investigation was started on the presence of *N. fowleri* in warm water discharges of different industries in Belgium. This investigation demonstrated that cooling waters were indeed the places where this pathogen could proliferate (De Jonckheere and van de Voorde 1977a). In addition, the absence of *N. fowleri* in drinking water and swimming pools in Belgium was also subsequently proven (De Jonckheere 1979a). This is in contrast to the situation in other parts of the world where higher annual water temperatures are prevalent and the presence of *N. fowleri* in drinking water is not uncommon (South Australia is the most notorious example Dorsch *et al*. 1983).

In the attempts to isolate *N. fowleri*, many different *Naegleria* strains were isolated that have properties that did not fit the descriptions of either *N. gruberi* (non-pathogenic) or *N. fowleri* (pathogenic), the only two species described at that time. At the time of writing this monograph, 20 *Naegleria* spp. have been fully described. Three strains are given species status here, and a few more descriptions are in preparation (Table 1).

It is mostly due to the use of molecular biology techniques that species descriptions are possible in a genus where morphology is not discriminative. However, this latter statement may now have to be reconsidered. Until recently all vahlkampfiids with dividing flagellates had been classified in genera other than *Naegleria*. It has been found that some amoeboflagellates whose flagellates can divide (Dobson *et al*. 1993, B. Robinson personal communication) are in fact *Naegleria* spp. (De Jonckheere and Brown 1995, 1999b). In addition there are two *Naegleria* strains that do not form flagellates under laboratory conditions (De Jonckheere *et al*. 2001), and a few *N. fowleri* strains from one location in France have never formed flagellates. A *Naegleria* strain also exists that fails to form cysts, but this seems to be due to the presence of a bacterial parasite (Michel *et al*. 2000). Infection of other *Naegleria* strains with the parasite impaired their capacity to form cysts. This bacteria did not interfere with the transformation to the flagellate stage. As cyst morphology is informative for identifying amoeboflagellate genera, and because the bacteria also infects other genera of amoebae, the investigation of whether the originally-infected amoeboflagellate does indeed belong to the genus *Naegleria* is recommended.

In 1988 the definition of the *Naegleria* genus was: these are vahlkampfiids whose flagellate stage normally has two flagella, lacks a cytostome, and does not divide. The cysts have plugged pores through which the amoeba excysts (Page 1988). Although the statement about the plugs in the cysts remains valid the rest of the definition of *Naegleria* should be emended as follows: these are vahlkampfiid amoebae with a temporary flagellate stage in most species, but lacking or difficult to induce in some species and in individual strains of others. Where present, the flagellate stage lacks a cytostome, is usually biflagellate and incapable of division. In at least two lineages, flagellates initially have four flagella and divide once to

Table 1. Species of the genus *Naegleria*

Species	author, year	Max. °C	Flagellates	EMBL*
N. gruberi	Schardinger, 1899, emend. De Jonckheere, this paper	39	+	M18732
N. fowleri	Carter, 1970	45	+	U80059
N. jadini	Willaert and Le Ray, 1973	35	+	-
N. lovaniensis	Stevens, De Jonckheere and Willaert, 1980	45	+	U80062
N. australiensis	De Jonckheere, 1981	42	+	U80058
N. italica	De Jonckheere, Pernin, Scaglia and Michel, 1984	42	+	U80060
N. andersoni	De Jonckheere, 1988	40	+	U80057
N. jamiesoni	De Jonckheere, 1988	42	+	U80062
N. clarki	De Jonckheere, 1994	37	+	-
N. galeacystis	De Jonckheere, 1994	35	+	-
N. minor	De Jonckheere and Brown, 1995	38	divide	X93224
N. pussardi	Pernin and De Jonckheere, 1996	41	+	-
N. carteri	Dobson, Robinson and Rowan-Kelly, 1997	45	+	Y10189
N. morganensis	Dobson, Robinson and Rowan-Kelly, 1997	44	+	Y10188
N. niuginensis	Dobson, Robinson and Rowan-Kelly, 1997	45	+	Y10186
N. sturti	Dobson, Robinson and Rowan-Kelly, 1997	44	+	Y10185
N. robinsoni	De Jonckheere and Brown, 1999	38	divide	AJ237786
N. fultoni	De Jonckheere, Brown, Dobson, Robinson and Pernin, 2001	35	+	AJ243440
N. chilensis	De Jonckheere, Brown, Dobson, Robinson and Pernin, 2001	30	-	AJ243442
N. indonesiensis	De Jonckheere, Brown, Dobson, Robinson and Pernin, 2001	38	-	AJ243441
N. tihangensis	De Jonckheere, this paper	42	+	-
N. pringsheimi	De Jonckheere, this paper	37	+	-
N. pagei	De Jonckheere, this paper	37	+	-
N. philippinensis	In preparation	40	+	NA
WA variant *N. lovaniensis*	In preparation	45	+	Y10187
NG597	In preparation	42	+	Y10184
antarctic *Naegleria* sp.	In preparation	<30	+	ND

* - EMBL accession N° of SSUrDNA
- - not at EMBL, but partial sequences have been published (De Jonckheere 1994a, Pernin and De Jonckheere 1996)
NA - not available yet
ND - not done (DNA could not be isolated because of poor growth)

form typical biflagellate cells. *Naegleria* can only be identified to species level by biochemical and molecular techniques.

MATERIALS AND METHODS

Culture

Established cultures of *Naegleria* strains are grown either monoxenically on non-nutrient agar plates with *Escherichia coli* (Page 1988) or axenically in a liquid medium (De Jonckheere 1977).

Isoenzyme analysis

Protein extracts are prepared by adding 0.25% Triton X-100 to amoebae concentrated by centrifugation. The suspensions are frozen and thawned several times to make the amoebae burst. For isoenzyme analyses the proteins are separated by agarose gel isoelectric focusing (De Jonckheere 1982a) or cellulose acetate electrophoresis (Robinson *et al*. 1992), and the bands of enzyme activity were visualized according to procedures published by these authors.

DNA sequence analysis

DNA is extracted from cell pellets using either a phenol-chloroform-isoamyl alcohol method or a guanidium thiocyanate-sarkosyl method (Pitcher *et al*. 1989). The small subunit ribosomal DNA (SSU rDNA), large subunit ribosomal DNA (LSU rDNA) and the internal transcribed spacer (ITS) regions, including the 5.8S rDNA, are amplified using primers and polymerase chain reaction (PCR) conditions described by De Jonckheere (1994a, 1998). In preparation for sequencing PCR products were treated with exonuclease I and shrimp alkaline phosphatase for 15 min. at 37°C. After inactivating these enzymes by heating at 80°C for 15 min., the PCR products were sequenced using the Sequenase PCR product sequencing kit (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England) using either [^{32}P] -dATP or [^{33}P] -dATP. SSU rDNA and ITS amplification and conserved internal primers were used (De Jonckheere 1994a, 1998). Approximately 800 basepairs (bp)

between two conserved Pst I sites within the *Naegleria* SSU rDNA were sequenced and used for phylogenetic analysis (De Jonckheere 1994a). Sequences of group I introns in the SSU and LSU rDNA are determined using internal rDNA and group I intron primers (De Jonckheere 1993). The nucleotide sequence data reported in this paper are available in the European Molecular Biology Laboratory (EMBL) nucleotide sequence database and the accession numbers are indicated at each species description.

Phylogenetic analysis

The DNA sequences are aligned by eye using the Eyeball Sequence Editor (ESEE) (Cabot and Beckenbach 1989). Phylogenetic trees are constructed from the aligned sequences using the DNAPARS (parsimony), DNADIST (distance matrix), NEIGHBOR (Neighbor joining and UPGMA), FITCH (Fitch-Margoliash), KITCH (Fitch-Margoliash with evolutionary clock) and SEQBOOT (bootstrapping) programs of the PHYLIP (version 3.572c) package (Felsenstein 1989). For phylogenetic analyses of proteins the PROTPARS and PROTDIST programs of the same package are used.

MOLECULAR BIOLOGY OF THE GENUS *NAEGLERIA*

Chromosomes and ploidy

Naegleria has an intranuclear mitosis, called promitosis, following the classical pattern of chromosome separation, but the chromosomes are too small to be counted by conventional histological techniques (Fulton 1970). However, it has been possible to enumerate the chromosomes with the use of pulsed field gel electrophoresis. The number of chromosomes and their size differ between species and even between strains of the same species. Two strains of *N. gruberi sensu lato* have 23 chromosomes, but the size of some chromosomes differ (Clark *et al*. 1990). These two strains are considered now to belong to two different species, *N. gruberi sensu stricto* and *N. pringsheimi* (see below). Within the species *N. fowleri* differences in number and size of chromosomes are observed with different isolates (De Jonckheere 1989).

The ploidy of the *Naegleria* genome is still not known. The sum of the chromosome sizes (approximately 19 Mb) does not equal the expected genome size (approximately 104 Mb), which indicates that *Naegleria* might be polyploid (Clark 1990). It has been demonstrated that differences in ploidy exist between strains (Fulton 1993), and isoenzyme studies of *Naegleria* spp. usually imply diploidy (Cariou and Pernin 1987, Adams *et al*. 1989). Isoenzyme studies also reveal that genetic exchange occurs in *N. lovaniensis* but not in other species (Pernin *et al*. 1992). Of course, genetic recombination does not mean sexuality, which involves meiosis to form monoploid cells, and karyogamy. It has been argued that the flagellates of *Naegleria* are gametes (Fulton 1993) and the fact that in some *Naegleria* spp. the flagellates divide once (De Jonckheere and Brown 1995) could be in support of monoploidy formation. However, meiosis in *Naegleria* has not been proven experimentally.

rDNA plasmid

In *N. gruberi*, the rRNA genes are carried exclusively on a 14-kp circular plasmid, and each plasmid contains only one rDNA repeat unit (Clark and Cross 1987). The number of rDNA circles per cell was estimated to be 4,000. This circular plasmid is a general feature of the rDNA genes in all the vahlkampfiids (Clark and Cross 1988a). The length of the rDNA plasmid varies according to the species and strain investigated. It is not known whether different numbers of rDNA repeats per plasmid, as was found in the anaerobic *Entamoeba histolytica* (Bhattacharya *et al*. 1998), contribute to the plasmid length differences. Length differences in the ribosomal genes themselves are mainly due to the presence of group I introns. The SSU rDNA of several species carry these introns (De Jonckheere 1994b). Length differences in the ITS1 and/or ITS2 also contribute to repeat unit size variability (De Jonckheere 1998). In a few *Naegleria* strains, group I introns are also present in the LSU rDNA (De Jonckheere and Brown 1998a, 2001).

The rDNA plasmid of *N. gruberi* strain EG_B has been completely sequenced. The molecule is 13,996 bp in length with an overall G+C content of 40.7% (Mullican, J. C. Molecular characterization and complete sequence analysis of the extrachromosomal ribosomal DNA element in *Naegleria gruberi.* Ph D thesis. The graduate College in the University of Nebraska, Omaha, Nebraska, USA:1-163,1995). A putative open reading frame (ORF) for a heat shock protein is detected in the non-ribosomal sequence (NRS) of the *N. gruberi* plasmid (Mullican and Tracy 1993). No similarities were observed in the NRS between *N. fowleri* (strain LEE) and *N. gruberi* (strain NG_B).

Another plasmid has been detected in *N. minor* (De Jonckheere and Brown 1995). The function of this 6.0 kb plasmid is unknown but it seems not to be involved in flagellate division as it is not found in *N. robinsoni*, currently the only other *Naegleria* species with dividing flagellates.

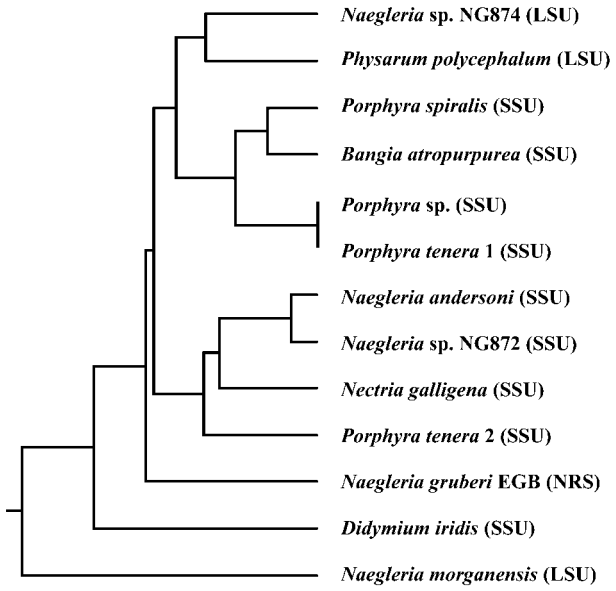

Fig. 1. Phylogenetic tree inferred from the amino acid alignments of the His-Cys box in the ORF of the group I introns

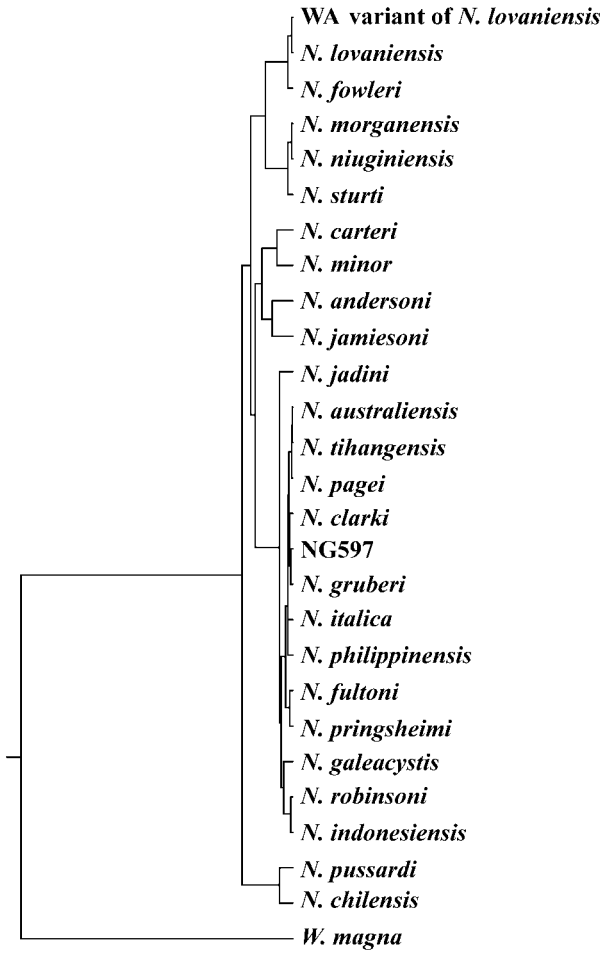

Fig. 2. Phylogenetic tree inferred from partial SSU rDNA of species in the genus *Naegleria* and its closest relative *Willaertia magna*

Group I introns

Group I introns are catalytic RNA molecules that occur within transcribed sequences and are able to self-excise. The group I intron in the SSU rDNA of *Naegleria* spp. is a twintron (Einvik *et al*. 1998), consisting of two distinct ribozymes (catalytic RNAs) and an ORF encoding a homing endonuclease with a His-Cys box (Johansen *et al*. 1993). Endonucleases with His-Cys boxes are uncommon (Johansen *et al*. 1997). A similar twintron has only been found in the myxomycete *Didymium* (Einvik *et al*. 1998). In one *Naegleria* lineage the twintron has lost the ribozyme that carries the endonuclease (De Jonckheere and Brown 1994). The group I introns in the LSU rDNA of *Naegleria* either carry an endonuclease or do not (De Jonckheere and Brown 1998a, 2001).

In the genus *Naegleria* the group I intron seems to be transferred vertically in the SSU rDNA (De Jonckheere 1994b) and horizontally in the LSU rDNA (De Jonckheere and Brown 1998a, 2001). From this it is inferred that the SSU rDNA group I intron was acquired in an ancestral state and lost in most of the *Naegleria* spp. In all described *Naegleria* spp. with a group I intron in the SSU rDNA, the presence of this intron is a property of the species. Only the WA variants of *N. lovaniensis* could be exception to this rule. An ORF with approximately 30% identity to the ORF in the SSU rDNA group I intron of *N. pringsheimi* has been found in the NRS of the rDNA plasmid of strain EG_B of *N. gruberi* (Mullican, J. C. Molecular characterization and complete sequence analysis of the extrachromosomal ribosomal DNA element in *Naegleria gruberi*. Ph D thesis. The graduate College in the University of Nebraska, Omaha, Nebraska, USA: 1-163, 1995). It is trancriptionally silent and may be a remnant of the group I intron that was lost from the SSU rDNA of *N. gruberi*. The His-Cys box is still present in the ORF in strain EG_B, but comparison with His-Cys boxes in the SSU rDNA introns of other *Naegleria* spp. shows it is phylogenetic distinct (Fig. 1).

It may have evolved faster because it is silent and thus under different constraint, or it was introduced horizontally from another organism.

Standard introns

Standard introns are not prevalent in any of the structural genes sequenced to date in *Naegleria*, except for two introns present in the calcineurin B gene (EMBL accession N° U04380). The introns are 258 and 55 bp long, respectively, and flanked by characteristic splice junction sequences (Remillard *et al*. 1995). *Naegleria* might be the earliest branching eukaryote known to contain canonical introns in only one gene. Together with the fact that this is the first gene encoding calcineurin B found in an organism other than metazoa and yeast, one wonders whether this gene might have been acquired by horizontal transmission.

mtDNA

Recently, the circular 49,843 bp mitochondrial (mt) DNA of strain NEG-M of *N. gruberi sensu stricto* has been sequenced and the sequence is available at EMBL under accession N° AF288092. Non-coding sequences amount to only 8%. Two genes have been identified that have previously only been detected in jakobid mtDNA (Lang *et al*. 1999). The *Naegleria* mtDNA seems to share an evolutionary history with that of the jakobids, even though the mtDNA in the latter is linear. It will be interesting to see whether the *Naegleria* mtDNA show other peculiarities, such as RNA editing as found in transfer RNAs in mtDNA of *Acanthamoeba* (Lonergan and Gray 1993).

Pyrophosphate-dependant phosphofructokinase

From isoenzyme studies it is known that several glycolytic enzymes are present in *Naegleria*. Few have been studied in detail probably because it has been assumed that these enzymes are rather similar in all eukaryotes. However, *Naegleria* seems to be one of the eukaryotes that have a pyrophosphate-dependant phosphofructokinase (PPi-PFK), and shares this property with the amoeba *Entamoeba*, the flagellates *Trichomonas, Tritrichomonas* and *Giardia*, the ciliate *Isotricha* and the apicomplexa *Toxoplasma, Eimeria* and *Cryptosporidium*. The *Naegleria* PPi-PFK is regulated by AMP, a property that distinguishes it from all of its counterparts, which are either unregulated or stimulated by fructose 2,6-biphosphate (Mertens *et al*. 1993). The enzyme was investigated both in *N. fowleri* and in a strain of *N. gruberi sensu lato*. The cloned PPi-PFK gene of *N. fowleri* has been sequenced (EMBL accession N° U11733) and the expressed protein had the same properties (Wessberg *et al*. 1995) as the native protein.

OVERVIEW OF SPECIES

Fulton (1993) has argued strongly against naming too many *Naegleria* spp. He reasoned that we might end up with "more than two-dozen species of *Naegleria*, many identifiable only by isoenzyme patterns, a level of obscurity so wonderful that everyone would wisely ignore those of us who worked on these impossibly classified organisms". Fulton used *N. gruberi* as an example of a species that is an easily recognizable species by conventional criteria. Unfortunately, he did not say what these criteria were, but at the same time he admited that *N. gruberi* is a polyglot assembly of diverse isoenzyme types. Page (1988) called *N. gruberi* "intolerably heterogeneous on detailed study". It is this heterogeneity of a single species that will scare people away from studying the organism, unless everybody works on the same strain, i.e. EG.

The heterogeneity in *N. gruberi* is really not so surprising. Until recently, most *Naegleria* isolates that didn't fit into the few described *Naegleria* spp. were called *N. gruberi*. Fortunately, new isolates which are not further typed are now called *Naegleria* sp. instead of *N. gruberi*.

Several investigations have indicated that *N. gruberi* is in fact a complex of at least 10 species (De Jonckheere 1982a, Adams *et al*. 1989, Clark *et al*. 1989, Pernin and Cariou 1989, Robinson *et al*. 1992). *N. gruberi* strains fall into distinct clusters, separated by genetic distances similar to those separating the better-characterised taxa *N. fowleri, N. lovaniensis, N. jadini, N. australiensis, N. italica, N. andersoni* and *N. jamiesoni* (Robinson *et al*. 1992). Allozymes and rDNA sequences give the same conclusions for delineating *Naegleria* spp. (Dobson *et al*. 1997, De Jonckheere and Brown 1997). In the present monograph, the four clusters that have been recognized previously in *N. gruberi* (Clark *et al*. 1989), are given separate species status. Clearly new species names are needed rather than lumping everything under *N. gruberi*.

There is a good reason for naming new species. When the species name is given one knows already some of the characteristics of the strain under study, for

example its pathogenicity, thermotolerance, capacity to transform into flagellates and whether the latter are able to divide. I strongly support the notion that, apart from the species name, the strain identification number should always be given (Fulton 1993) as interstrain differences exist. It is obvious that different clonotypes can evolve (Fulton 1993), but these clonotypes never form different clusters in allozymes-derived trees. Slight allozyme differences between strains of the same species have been demonstrated in *N. fowleri* (Pernin *et al*. 1985, De Jonckheere 1987, Adams *et al*. 1989), *N. lovaniensis* (De Jonckheere 1982a, Pernin *et al*. 1985, Adams *et al*. 1989, Robinson *et al*.1992), *N. australienis* (De Jonckheere *et al*. 1984, Pernin *et al*. 1985, Adams *et al*. 1989, Robinson 1992), *N. andersoni* (De Jonckheere 1988a, Robinson 1992), *N. jamiesoni* (De Jonckheere 1988a, Robinson 1992), *N. sturti* and *N. carteri* (Dobson *et al*. 1997), but these are much smaller than those between different species and therefore, creating a subspecies would be invalid (see Rivera *et al*. 1990, De Jonckheere 1994a).

Since 1994 I have based species delineation on SSU rDNA sequences (De Jonckheere 1994a) because not only are the differences easier to quantify, but they also allow more distantly-related organisms to be compared than when using isoenzyme differences. SSU rDNA sequences have been used for delineating species of other protists such as *Giardia* and *Cryptosporidium* (Thompson *et al*. 2000, Xiao *et al*. 2000). rDNA sequencing provides unambiguous data which is reproducible between laboratories. However, sequencing is also prone to errors. Generally, small sequencing errors will not influence the phylogenetic identification of a strain under study to a high degree, but if errors become numerous they could influence species identifications, especially in cluster 5 (Fig. 2).

Restriction fragment length polymorphism (RFLP) analysis of PCR amplified ribosomal DNA, or riboprinting, produces similar phylogenetic trees as SSU rDNA sequencing (De Jonckheere 1994c) and constitutes less work. However, the latter is less accurate because in riboprinting less information is obtained. Also the treebuilding can be biased by the presence of group I introns (De Jonckheere 1994b). Riboprinting the LSU rDNA is preferred (De Jonckheere 1994c) as in the genus *Naegleria* group I introns are found infrequently in the LSU rDNA (De Jonckheere and Brown 1998a, 2001) compared to the SSU rDNA. Sequence analysis of ITS1, 5.8S rDNA and ITS2 has confirmed the species delineation and also aids finetuning the descripions (De Jonckheere 1998).

Eight species descriptions in the *Naegleria* genus are based on only one isolate, while different strains of three species came from the same place. The "one strain" species are *N. jadini*, *N. pussardi*, *N. niuginensis*, *N. galeacystis*, *N. minor*, *N. robinsoni*, *N. chilensis* and *N. indonesiensis*. The three species with strains from only one place are *N. italica*, *N. morganensis* and *N. fultoni*. Therefore, the variability within these species is unknown. For all these species, data on the rDNA sequences, especially ITS sequences, from different strains are needed to assess the variability within the species. A recent isolate of *N. italica* from Australia shows indeed ITS2 variation in this species (Henderson *et al*. 2001). Until now, only *N. fowleri* has been thoroughly studied, and a great deal of variability in ITS1 sequence was detected (De Jonckheere 1998).

Thermophilic species

The genus *Naegleria* comprises pathogenic and nonpathogenic species. All pathogenic species are thermophilic but not all thermophilic species are pathogens. Thermophilic is defined here as the ability to grow at 40°C or higher. It is important to know one is working with a pathogenic species as special precautions have to be taken.

It had been observed that *N. fowleri* can grow at temperatures up to 45°C while nonpathogenic *Naegleria* spp. did not tolerate such a high temperature (Griffin 1972). This approach was used in the routine testing of water samples for *N. fowleri* where concentrated water samples were incubated at temperatures between 42°C and 45°C in an attempt to suppress the growth of other amoebae. When using this isolation method it was soon realized that more *Naegleria* strains than those belonging to *N. fowleri* can grow at these high temperatures. There are currently 12 named thermophilic *Naegleria* spp. (Table 1). The term thermophilic is arbitrarily chosen with some using the borderline of 42°C (Robinson *et al*. 1992) instead of 40°C (De Jonckheere 1988a). In addition, differences in thermophilic growth temperatures have been noted for the same strain (see below).

Pathogenic thermophilic species

Besides the human pathogen *N. fowleri*, also *N. australiensis* and *N. italica* kill experimental ani-

mals. However, no human infection due to these two other pathogenic *Naegleria* spp. has been diagnosed to date. Although the amoeba was found not to be responsible for the disease, *N. lovaniensis* has been found as a contaminant in the cerebrospinal fluid (CSF) of a patient in Mexico (Rivera *et al.* 1989).

About 15 years ago a *Naegleria* was isolated from human CSF. It will be described as a new species. (see *N. philippinensis*). I have tested the pathogenicity of the latter isolate recently in experimental animals and no mice were killed. Therefore, it is important to discuss virulence and pathogenicity tests. In some of the species that are described as nonpathogenic, positive mouse tests have been reported.

In some of the pathogenicity tests with nonpathogenic variants of *N. fowleri*, later described as *N. lovaniensis*, one out of five mice died after intranasal (IN) inoculation but amoebae could not be recovered from the brain (De Jonckheere and van de Voorde 1977b). Virulence could not be induced in these strains, in contrast to strains of *N. fowleri* with attenuated virulence (De Jonckheere 1979b), and it was concluded *N. lovaniensis* is a nonpathogenic species. In the USA a *Naegleria* isolated at an incubation temperature of 42°C killed one mouse out of three and was identified on this basis as being *N. australiensis* (John and Howard 1995). However, this isolate (EPA-741) was later identified as *N. lovaniensis* by immunofluorescence (John *et al.* 1998) and this identity was confirmed by isoenzyme analysis (De Jonckheere unpublished). This isolate should not be considered a pathogenic strain of *N. lovaniensis*, not only because only one mouse died, but because it also died after one day (John and Howard 1995), which is too short a time after IN instillation for the amoeba to be the cause of death. In addition, subsequent inoculations with isolate EPA-741 did not kill mice (John *et al.* 1998). As a result, *N. lovaniensis* should still be considered a nonpathogenic species.

Also one mouse died after the first intracerebral (IC) inoculation with the type strain of *N. jadini*, but subsequent attempts to prove pathogenicity of the strain remained unsuccesful. It was concluded that the presumed pathogenicity was probably due to contaminating bacteria and fungi, which is highly possible after IC inoculation (Willaert and Le Ray 1973).

Two mice each out of six were killed after IN inoculation with strains RU30 and RU42 (Jamieson J. A. Studies of amoebae of the genus *Naegleria*. Master of Science thesis, Adelaide, 1-48, 1975). These *Naegleria* isolates were later described as *N. clarki*. Subsequent attempts with amoebae isolated from the mouse brain continued to kill some mice with strain RU30, but the results didn't show an increased virulence as found for strain PP397, which was later described as pathogenic *N. australiensis*. Pathogenicity tests with strains RU30 and RU42 appeared to remain negative on later attempts (Willaert 1976) and it was concluded *N. clarki* is a nonpathogenic species (De Jonckheere 1994a), although *N. clarki* is closely related to the pathogenic *N. italica*.

Some *Naegleria* isolates from a swimming pool in Czechoslovakia did kill a few mice soon after isolation, but lost that capacity upon subculture (Kadlec 1981). From the serological tests it can be deduced that some might have been *N. lovaniensis* but that some of the isolates belonged to other species.

Because of the difficulty in interpreting some of the pathogenicity results the Culture Collection of Algae and Protozoa (CCAP) decided to put a warning of "possible pathogen" on all *Naegleria* spp. At the American Type Culture Collection (ATCC) a public health permit is neceassary only for *N. fowleri*, *N. australiensis* and *N. italica.*

Naegleria fowleri Carter, 1970

Both in the USA and in Australia reports were published in 1968 on the isolation of a *Naegleria* sp. from human CSF which was different from all the known *N. gruberi* strains (Butt *et al*. 1968, Callicott *et al*. 1968, Culbertson *et al*. 1968, Carter 1968). At that time, pathogenicity had only been proven experimentally in free-living amoebae belonging to the genera *Hartmannella - Acanthamoeba*, so similar cases had been previously attributed to *Hartmannella - Acanthamoeba*. At that time confusion existed over the genera *Hartmannella* and *Acanthamoeba*. In all older literature where *Hartmannella* is indicated as a human pathogen it probably refers to *Acanthamoeba*. Because the amoebae could not be isolated from CSF of these first human cases they could not be studied in detail.

In 1970 Carter distinguished the human pathogenic *Naegleria* from the common *N. gruberi* and named it after M. Fowler who first described the disease in Australia (Carter 1970). This species distinction was based exclusively on morphological differences and the pathogenicity of the isolates. The separate species status of the pathogen was later confirmed using serological,

biochemical and molecular techniques. The human pathogenic *Naegleria* sp. has also been called *N. aerobia* (Singh and Das 1970) but the use of the name, mainly in India, has been abandoned. The junior synonym *N. invadens* (Chang 1971) has also disappeared rapidly from the literature.

The disease PAM occurs worldwide and very few people survive an infection with *N. fowleri*. Only Amphotericin B seems to be really effective in curing the disease if given at a very early stage of the infection. The best documented cases of succesful treatment occurred in California (Seidel *et al*. 1982) and in Australia (Anderson and Jamieson 1972). There are some additional reports from Hong Kong, Thailand, Italy, England and Mexico, but there is doubt whether these patients really suffered from PAM.

Mice are the preferred animals for testing the pathogenicity of *Naegleria* isolates. Although some differences in susceptibility to *N. fowleri* infections are observed between different mice breeds, the age of mice is more important (De Jonckheere 1979b). The amoebae preferentially migrate to the brain where they multiply to high numbers while eating the tissue. However, in experimental infections of mice pneumonitis and hepatitis are also observed with masses of amoebae present in the tissues. Splenitis occurs frequently, while amoebae are often found in glomular capillaries as well (Carter 1970). However, kidney and liver transplants from a 11 year old donor who had died of a *N. fowleri* infection did not induce infection in three different organ recipients (Kramer *et al*. 1997). Also, guinea pigs (Philips 1974), old world monkeys (Wong *et al*. 1975), sheep (Simpson *et al*. 1982), rabbits (Smego and Durack 1984), squirrels, cotton rats and muskrats (John and Hoppe 1990) are susceptible to experimental infection with *N. fowleri*. On the other hand, cottontail rabbits, opposums and raccoons seem to be not susceptible (John and Hoppe 1990). A natural *N. fowleri* infection has been detected in a South American tapir (Lozano-Alarcon *et al*. 1997).

Upon isolation pathogenic strains of *N. fowleri* are highly virulent, but tend to lose virulence after longterm axenic culturing (Wong *et al*. 1977, De Jonckheere 1979b). In reports of *N. fowleri* strains with low virulence immediately after isolation (Kadlec 1981) the isolates were probably strains of *N. australiensis* because, contrary to *N. fowleri* strains, they were difficult to axenise and their temperature optimum decreased rapidly after isolation. On the other hand, *N. italica* was first reported as being most probably an isolate of *N. fowleri* (Scaglia *et al*. 1983). Contrary to other *Naegleria* spp., strains of *N. fowleri* adapt easily to an axenic medium, which makes this a quick method for seperating this pathogen from other *Naegleria* spp. (De Jonckheere 1977). Other *Naegleria* spp. can be adapted only slowly to axenic growth. A chemically-defined medium has been described in which *N. fowleri* can be cultured (Nerad *et al*. 1983).

Most strains of *N. fowleri* transform into flagellates, but several strains isolated in France could never be induced to transform (De Jonckheere *et al*. 2001). There has been a report of dividing *N. fowleri* flagellates in a brain infection (Clavel *et al*. 1996), however, the isolated strain turned out not to be a *Naegleria* sp. but some kind of *Platyamoeba* and is considered to be a contaminant (De Jonckheere and Brown unpublished). The photographs of the organism that was purportedly present in the CSF of this case, showed the morphology and the typical axostyle of a *Trichomonas* sp. (De Jonckheere unpublished), which explains why the flagellates were seen to divide. In the meantime, another case of *Trichomonas* meningoencephalitis has been reported (Okamota *et al*. 1998).

Restriction endonuclease digestion of whole-cell DNA (De Jonckheere 1988a) reveals differences between *N. fowleri* strains from different places, differences that are confirmed by isoenzymes, random amplified polymrphic DNA (RAPD) and ITS sequences analyses. The most clearcut divide is found between the Australian-Asian strains and those from other regions. A point mutation is found in the conserved 5.8S rDNA of these two different *N. fowleri* lineages (De Jonckheere 1998). Further differentiations of *N. fowleri* strains can be made by using the different lengths found in the ITS1 (Table 2) (De Jonckheere 1998, Pélandakis *et al*. 2000).

Other variations in *N. fowleri* strains have been observed. While DNA RFLP made similar distinctions between strains of different continents, a *N. fowleri* isolate from a surviving patient showed the most different DNA RFLP (De Jonckheere 1987), and the strain showed swellings of the flagella that looked like paddles, not found in any other *Naegleria* strain (John *et al*. 1991).

The ITS1, 5.8S rDNA and ITS2 sequences of different *N. fowleri* strains are available at EMBL under accession numbers X96561 till X96567, while the SSU

Table 2. Lenght in bp of ITS1, 5.8S rDNA and ITS2 in different *Naegleria* spp.

Cluster	Species	Strain	ITS1	5.8S rDNA	ITS2	Total	EMBL
1	*N. fowleri*	KUL	86	175	106	367	X96561
		LEE	86	175	106	367	X96562
		M4E	142	175	106	423	X96563
		AR12	42	175	106	323	X96564
		Northcott	84	175	106	365	X96565
		Mst1	84	175	106	365	X96566
		J/16/1/42E	84	175	106	365	X96567
	N. lovaniensis	Aq/9/1/45D	41	175	103	319	X96568
		F9	41	175	103	319	X96569
	WA variant	NG872	36	175	103	314	Y10191
2	*N. morganensis*	NG236	34	175	226	435	Y10192
	N. sturti	NG334	35	175	118	328	Y10195
	N. niuginiensis	NG427	46	175	117	338	Y10193
3	*N. carteri*	NG055	34	174	100	308	Y10197
	N. minor	WTO43	30	175	528	738	X96577
4	*N. andersoni*	A2	35	174	100	309	X96572
	N. jamiesoni	T56E	34	174	100	308	X96570
5	*N. australiensis*	PP397	33	175	100	308	X96573
		LSR34	33	175	100	308	AJ132034 [b]
	N. philippinensis	RJTM	33	175	105	313	-
	N. italica	AB-T-F3	33	175	162	370	X96574
	N. clarki	RU30	33	175	201	409	X96575
	N. fultoni	NG885	33	175	106	314	AJ243445
	Naegleria sp.	NG597	33	175	115	323	Y10194
	N. gruberi	EG_B	33	175	114	322	[a]
		AUD1	33	175	114	322	AJ132031[b]
		DRI	33	175	114	322	-
	N. tihangensis	T2A	33	175	102	310	-
		NG202	33	175	102	310	-
	N. pringsheimi	CCAP1518/1D	33	175	134	342	-
	N. pagei	CCAP1518/1E	33	175	165	373	-
		CCAP1518/1F	33	175	153	361	AJ132022 [b]
	N. jadini	O400	35	175	>519	>729	X96576
subcluster	*N. galeacystis*	AV500	33	175	181	389	X96578
	N. indonesiensis	NG945	33	175	182	390	AJ243444
	N. robinsoni	NG944	33	175	176	384	AJ237787
6	*N. pussardi*	EDF258	38	174	92	304	X96571
	N. chilensis	NG946	152	175	129	456	AJ243443

Results are from my laboratory except for [a] (Mullican 1995) and [b] (Pélandakis *et al*. 2000)

rDNA sequence of strain MCM is under number U80059. The partial SSU rDNA sequence of strain KUL has been published (De Jonckheere 1994a).

The type strain Nf66 of *N. fowleri* is available from ATCC (N° 30214) together with other *N. fowleri* isolates from all over the world.

Naegleria australiensis De Jonckheere, 1981

The type strain of *N. australiensis* (PP397) was originally isolated from water in Australia (Jamieson J. A. Studies of amoebae of the genus *Naegleria*. Master of Science thesis, Adelaide, 1-48, 1975). It is virulent for

Table 3. Summary on the presence and length (in nt) of group I introns in the nuclear rDNA of the genus *Naegleria*

Cluster	Species	SSU rDNA	EMBL	LSU rDNA	EMBL
1	*N. fowleri*	-		-	
	N. lovaniensis	-		-	
	WA variant NG874	+ (+ ORF) [a]	-	869 (+ ORF)*	AJ271406
	WA variant NG872	1318 (+ ORF)	AJ001399	474 (- ORF)	AJ001316
	WA variant NG881	-		+	
	WA variant NG876	-		-	
2	*N. morganensis*	-		389 (- ORF) $	AJ001314
				919 (+ ORF)	AJ001315
	N. sturti	-		-	
	N. niuginensis	-		-	
3	*N. carteri*	+1324(+ ORF)	Y10190	-	
	N. minor	-		-	
4	*N. andersoni*	1309 (+ ORF)	X78280	-	
	N. jamiesoni	1307 (+ ORF)	X78279	-	
5	*N. australiensis*	-		-	
	N. philippinensis	1297 (+ ORF)	-	-	
	N. italica	1319 (+ ORF)	X78277	-	
	N. clarki	1305 (+ ORF)	X78281	-	
	N. fultoni	-		-	
	N. gruberi	-		-	
	N. tihangensis	-		-	
	N. pringsheimi	1316 (+ ORF)	X78278	-	
	N. pagei	-		-	
	N. jadini	-		-	
	Naegleria sp. NG597	375 (- ORF)	X79070	-	
subcluster	*N. galeacystis*	-		-	
	N. indonesiensis	-		-	
	N. robinsoni	-		-	
6	*N. pussardi*	-		-	
	N. chilensis	-		-	

* - at slighty different location from in NG872, $ - different group I introns at different locations in the same strain, [a] - present but sequence not determined

mice but the virulence is lower than that of *N. fowlei*. Generally strains of *N. australiensis* kill fewer animals and require a longer incubation time (De Jonckheere *et al*. 1983a). Moreover, *N. australiensis* loses virulence more quickly than *N. fowleri* in axenic culture (De Jonckheere 1981), probably due to this lower virulence. Therefore, a negative virulence test does not indicate the strain under investigation does not belong to *N. australiensis*. The species differs antigenically (De Jonckheere 1981) from *N. fowleri* and can be separated by allozyme (De Jonckheere 1982a) and DNA (De Jonckheere 1994a) analyses. Intraspecies differences in allozyme and whole rDNA plasmid restriction patterns are observed and the latter indicated that this species might have a European origin (Clark *et al*. 1989). However, the ITS1, 5.8S rDNA and ITS2 sequences of strain PP397 from Australia (De Jonckheere 1998) are exactly the same as those of strain LSR34 from France (Pélandakis *et al*. 2000). Therefore, the difference in whole rDNA plasmid restriction patterns observed between those two strains must be due to sequence differences in the NRS of the plasmid.

The maximum temperature tolerated for growth by this species is 42°C, which is 3°C lower than for *N. fowleri*. Strains of *N. australiensis* occur worldwide in warm waters and a strain of *N. australiensis* has been isolated from the brain of a fish (Dykova *et al*. 2001) but they have never been isolated from a human being. This species is found very frequently in cooling waters in Belgium (De Jonckheere unpublished) because the incubation of concentrated water samples at 44°C allows the isolation of species that only tolerate 42°C. The exact reason for this is unknown, but it could be that the strains are adapted to higher temperatures in

the environment or because the interior of the agar plates does not attain 44°C during the short incubation time for isolation. It has been reported that the upper temperature tolerance limits of described *Naegleria* spp. vary narrowly (<2°C) between many isolates (Robinson *et al* 1996a). Some strains of *N. fowleri* reported to have low virulence upon isolation, with a temperature optimum that rapidly decreases after isolation (see above) and which also do not adapt readily to axenic growth (Kadlec 1981) might actually belong to the species *N. australiensis*.

The ITS1, 5.8S rDNA and ITS2 sequences of strain PP397 and LSR34 are available at EMBL under accession number X06573 and AJ132034 respectively, while the SSU rDNA sequence of strain PP397 is under U80058. The partial SSU rDNA sequence of strain PP397 has also been published (De Jonckheere 1994a).

Strains of *N. australiensis* were adapted to axenic growth. A chemically-defined medium has been described in which *N. australiensis* can be cultured. This is a modification of the one in which *N. fowleri* and *N. lovaniensis* are grown (Nerad *et al*. 1983). The type strain PP397 is available from ATCC (N° 30958), as are three additional strains from France, Australia and the USA, respectively. The strain from the USA was isolated from kidney tissue of a goldfish and submited to my laboratory for typing (De Jonckheere unpublished).

Naegleria italica De Jonckheere, Pernin, Scaglia and Michel, 1984

When *N. italica* was isolated for the first time it was reported as probably being a strain of *N. fowleri* (Scaglia *et al*. 1983). Because of high crossreaction with antiserum against *N. australiensis* it was subsequently described as a subspecies of the latter (De Jonckheere *et al*. 1984). However, allozyme studies suggested that the subspecies deserved species level rank (Adams *et al*. 1989), and the genetic distance between the SSU rDNA sequences of the subspecies are comparable to those between other *Naegleria* spp. (De Jonckheere 1994a). Therefore the subspecies of *N. australiensis* were given species status. In contrast with *N. australiensis* (Table 3), *N. italica* has a group I intron in the SSU rDNA (De Jonckheere 1993). Although the pathological findings in mice are the same as for *N. australiensis*, *N. italica* is much more virulent.

The maximum temperature tolerated for growth by *N. italica* is 42°C and strains of *N. italica* have been adapted to axenic growth. Until recently, this species has never been isolated from anywhere other than the spa in Italy where it was originally found and continued to be detected on subsequent occasions (Scaglia *et al*. 1987). In February 2000, two strains isolated from an artificial water body in Western Australia have been identified as *N. italica* on the basis of nearly identical allozyme profiles (Henderson *et al*. 2001). The 5.8S rDNA with flanking ITS sequences gave 98% identity with the published sequence for *N. italica*. The australian strains have the same insert in the ITS2 that is typical for *N. italica*, but it is in this stretch that the sequence divergence with the typestrain is observed. Based upon the extensive sampling for *Naegleria* spp. in Australia, *N. italica* seems to be a rare organism on that continent as well.

The ITS1, 5.8S rDNA and ITS2 sequences of strain AB-T-F3 are available at EMBL under accession number X96574, while the partial SSU rDNA sequence is under U80060. The sequence of the SSU rDNA group I intron of strain AB-T-F3 is available at EMBL under accession number X78277. The partial SSU rDNA sequence of strain AB-T-F3 has also been published (De Jonckheere 1994a).

The type strain AB-T-F3, relabeled SWL NG-073, is available from ATCC (N° 50347).

Nonpathogenic thermophilic species

Most *Naegleria* strains isolated from the environment are nonpathogenic. It is important to identify them and find out whether they could be indicators for the presence of the pathogenic species.

Naegleria lovaniensis Stevens, De Jonckheere and Willaert, 1980

This species was described initially as a nonpathogenic variant of *N. fowleri* (De Jonckheere and van de Voorde 1977b) because the amoebae reacted postively with antiserum against the pathogenic *N. fowleri*, yet these isolates were not pathogenic. There have been some reports on positive pathogenicity tests with *N. lovaniensis* strains, but the amoebae were probably not the cause of death (see above). More detailed studies showed that, although antigenically being the closest relative, *N. lovaniensis* differs from *N. fowleri* in many more aspects than by virulence alone. Therefore, it was given separate species status (Stevens *et al*. 1980). Also, in phylogenetic analysis based on SSU rDNA sequences, *N. lovaniensis* is the closest relative of *N. fowleri* (De Jonckheere 1994a). In contrast to

N. fowleri, no length variation was found in the ITS1 of five *N. lovaniensis* strains investigated (De Jonckheere 1998).

Strains of *N. lovaniensis* have been found worldwide leading to the hypothesis of a common origin and recent dispersion throughout the world (Pernin *et al*. 1992). The *N. lovaniensis tarasca* subspecies and purepecha variant of *N. lovaniensis* from Mexico (Rivera *et al*. 1990) fall within the variability of zymograms observed in the species and, therefore, are invalid names (De Jonckheere 1994a). The particular nucleolar morphology in these Mexican strains had been observed repeatedly in one natural population of *N. lovaniensis* in Australia. Evidence has been presented for genetic recombination in *N. lovaniensis* (Cariou and Pernin 1987, Pernin *et al*. 1992) but genetic exchange could not be proven in other *Naegleria* spp. investigated, such as *N. fowleri*, *N. australiensis* and *N. gruberi sensu lato* (Pernin and Cariou 1997).

A fast method to separate *N. lovaniensis* from *N. fowleri* isolates is the use of a liquid axenic medium, in which only *N. fowleri* grows immediately to high numbers, while *N. lovaniensis* needs a lot of time to adapt (De Jonckheere 1977). Also, other *Naegleria* spp. can be grown in this medium but only after long adaptation, by each week decanting the medium and adding fresh medium to the tube. A chemically-defined medium has been described in which *N. lovaniensis* can be cultured; a property it shares with *N. fowleri* (Nerad *et al*. 1983).

The ITS1, 5.8S rDNA and ITS2 sequences of strains Aq/9/1/45D and F9 are available at EMBL under accession numbers X96568 and X96569, while the SSU rDNA sequence of strain C-0490 is under U80062. The partial SSU rDNA sequence of strain Aq/9/1/45D has also been published (De Jonckheere 1994a).

Strains LvH1 (30811),TS (30569) and K-1 (30467) of *N. lovaniensis* are present in ATCC although they had originally been sumitted under different species names. The three strains were isolated in Belgium, the USA and Australia, respectively.

Naegleria andersoni De Jonckheere, 1988

Naegleria andersoni is a thermophilic species defined on the basis of isoenzyme patterns and rDNA sequences (De Jonckheere 1988a). It is closely-related to *N. jamiesoni*, which was originally described as a subspecies of *N. andersoni*. A group I intron is found in the SSU rDNA of six strains investigated of both species (De Jonckheere 1993). The combined ITS1, 5.8S rDNA and ITS2 PCR product of both species have a similar length in all six strains (De Jonckheere 1998) but the sequence of only one strain of each species was determined. The ITS1 seems to be 1 bp longer in *N. andersoni* than in *N. jamiesoni* (Table 2), but in the combined ITS1, 5.8S rDNA and ITS2, 17 bp differences exist between the two species. The 5.8S rDNA in both *N. andersoni* and *N. jamiesoni* is 1 bp shorter than in the majority of *Naegleria* spp., a character they share with *N. carteri* and *N. pussardi*.

Strains of *N. andersoni* were first isolated in Australia (strains A2 and PPMFB6 in Willaert 1976) and later in Belgium from water associated with imported fish from Malawi, Singapore, Nigeria and Brazil (De Jonckheere 1988a). Strains of *N. andersoni* have been isolated again in Australia from an aquarium and from the public water supply (Robinson *et al*. 1992) and in Japan from industrial cooling water (De Jonckheere *et al*. 1991). This species has not been detected in cooling waters in Belgium, probably because the upper temperature limit for *N. andersoni* is 40°C to 41°C, while the samples are incubated at 44°C. Strains of *N. andersoni* are sometimes isolated from surface water in Australia where different incubation temperatures are used for isolating *Naegleria* spp. (Robinson personal communication). Strains A2 and PPMFB6 were shown to be nonpathogenic in experimental animals (Willaert 1976).

The ITS1, 5.8S rDNA and ITS2 sequences of strain A2 are available at EMBL under accession number X96572, while the SSU rDNA sequence of strain PPMFB6 is under U80057. The partial SSU rDNA sequence of strain A2 has also been published (De Jonckheere 1994a). The sequence of the SSU rDNA group I intron of strain A2 is available at EMBL under accession number X78280.

Strains of *N. andersoni* have been adapted to axenic growth. Type strain Aq/4/1H is available from CCAP under accession N° 1518/16.

Naegleria jamiesoni De Jonckheere, 1988

This species was originally described as a subspecies of *N. andersoni* on the basis of similarities in isoenzyme and DNA restriction patterns (De Jonckheere 1988a). However, allozyme studies suggested that the subspecies could be regarded as separate species (Robinson *et al*. 1992). Also, the difference in the SSU rDNA sequence between the two subspecies far exceeded those found between some described *Naegleria* spp. Therefore, the subspecies were given species status, but they do form a cluster in phylogenetic trees (De

Jonckheere 1994a). As with its closest relative, *N. andersoni*, *N. jamiesoni* has a group I intron in the SSU rDNA (Table 3). Strains of this species were originally isolated in Belgium from water associated with imported fish from Malawi and Singapore. This species is found sporadically in cooling waters in Belgium (De Jonckheere unpublished) and in the environment in Australia (Robinson personal communication). Incubating the samples at 44°C apparently allows species to be isolated which have a maximum temperature tolerance of 42°C (see also *N. australiensis*).

The ITS1, 5.8S rDNA and ITS2 sequences of strain T56E are available at EMBL under accession number X96570, while the SSU rDNA sequence is under U80061. The partial SSU rDNA sequence of strain T56E has also been published (De Jonckheere 1994a). The sequence of the SSU rDNA group I intron of strain T56E is available at EMBL under accession number X78279.

Although strains of *N. jamiesoni* have been adapted to axenic growth there is currently no strain available at either ATCC or CCAP.

Naegleria pussardi Pernin and De Jonckheere, 1996

Based on allozyme and SSU rDNA sequences, the species *N. pussardi* appeared to be the most distantly-related *Naegleria* sp. (Pernin and De Jonckheere 1996). It is one of the few *Naegleria* spp. that shows a morphological particularity; during promitosis the nucleolus tends to fragment in an unequal way in prophase. The description of this species is based on one single isolate (EDF258) from river water in France. The maximum temperature tolerated for growth is 41°C. Therefore, as for *N. jamiesoni*, there is little chance that it will be isolated while incubating water samples at 44°C for *N. fowleri* detection. In a tree based on 5.8S rDNA and ITS sequences strain NG260 (allozyme cluster B in Robinson *et al*. 1992) clusters with *N. pussardi* (Pélandakis *et al*. 2000). The 5.8S rDNA is 174 bp long, which is the same as in the typestrain EDF258. The ITS1 is, however, two bp shorter in the typestrain of *N. pussardi*. Also the maximum growth temperature is only 37°C for strain NG260.

The ITS1, 5.8S rDNA and ITS2 sequences of strain EDF258 are available at EMBL under accession number X96571. The partial SSU rDNA sequence of strain EDF258 has been published (Pernin and De Jonckheere 1996).

The typestrain EDF258 is available from ATCC (N° 50564) and another strain (VA-1) that was reclassified from *Mastocystis marylandensis* to *N. pussardi* is also available from ATCC (N° 50652). It is not certain whether this strain was reclassified on the basis of its morphological particularity or on the basis of isoenzyme analysis.

Naegleria carteri Dobson, Robinson and Rowan-Kelly, 1997

The differentiation of the species *N. carteri* was based on allozyme studies of nine strains isolated from different parts in Australia. Strains of this species were also isolated from Sri Lanka (Dobson *et al*. 1997). Slight differences in allozymes are detected between different strains of *N. carteri*, and it shares with *N. fowleri* and *N. lovaniensis* the capacity to grow at 45°C (Dobson *et al*. 1997). The validity of the separate species status was confirmed by rDNA sequence analysis of reference strain NG055 (De Jonckheere and Brown 1997). In phylogenetic trees based on SSU rDNA sequences *N. carteri* forms a cluster with *N. minor*, which is known for the capacity of its flagellates to divide. The strain of *N. carteri* investigated (NG055) has a group I intron in the SSU rDNA. *N. carteri* is one of the four *Naegleria* spp. in which the 5.8S rDNA is one bp shorter (Table 2).

The ITS1, 5.8S rDNA and ITS2 sequences of strain NG055 are available at EMBL under accession number Y10197, the partial SSU rDNA sequence under Y10189, and the SSU rDNA group I intron under Y10190.

Strains of *N. carteri* have never been grown axenically and there is currently no strain of *N. carteri* available at either ATCC or CCAP.

Naegleria morganensis Dobson, Robinson and Rowan-Kelly, 1997

The differentiation of the species *N. morganensis* was based on allozyme studies of four strains isolated from the River Murray in South Australia. It grows at 44°C (Dobson *et al*. 1997), and the validity of the separate species status is confirmed by rDNA sequence analysis of the typestrain NG236 (De Jonckheere and Brown 1997). In phylogenetic trees based on SSU rDNA sequences *N. morganensis* forms a cluster with two other *Naegleria* spp. that grow at 44-45°C, *N. niuginensis* and *N. sturti*. In this cluster, *N. morganensis* is the only described species with group I introns in the LSU rDNA (De Jonckheere and Brown 1998a). Actually, it has two group I introns in the LSU rDNA, one with and one without an ORF (Table 3). The description of the only other species known to have group I introns in the LSU rDNA, is in preparation

(see WA variant of *N. lovaniensis* in preparation). The only other strains known to belong to the *N. morganensis* lineage were isolated from the same location as the type strain. One of these other strains (NG258) was found to generate the same length LSU rDNA PCR product as NG236, indicating the presence of the same two introns as in the type strain. When isolating other strains of *N. morganensis* in the future, they should be investigated for the presence of group I introns in the LSU rDNA as these introns are quite unusual. The type strain of *N. morganensis* has no group I intron in the SSU rDNA (Table 3).

The ITS1, 5.8S rDNA and ITS2 sequences of strain NG236 are available at EMBL under accession number Y10192, while the partial SSU rDNA sequence is under Y10188. The sequence of the two group I introns in the LSU rDNA of strain NG236 are available at EMBL under accession numbers AJ001314 and AJ001315 respectively.

The type strain NG236 of *N. morganensis* is available from ATCC (N° 50351) and strain NG237 from CCAP (N° 1518/22).

Naegleria niuginensis Dobson, Robinson and Rowan-Kelly, 1997

The differentiation of the species *N. niuginensis* was based on allozyme studies of only one strain (NG427) isolated from lake sediment in New Guinea. It shares with *N. fowleri* and *N. lovaniensis* the capacity to grow at 45°C (Dobson *et al*. 1997). The validity of the separate species status is confirmed by rDNA sequence analysis of the type strain (De Jonckheere and Brown 1997).

The ITS1, 5.8S rDNA and ITS2 sequences of strain NG427 are available at EMBL under accession number Y10193, while the partial SSU rDNA sequence is under Y10186.

The only known strain (Dobson *et al*. 1997) of *N. niuginensis* is currently unavailable at either ATCC or CCAP.

Naegleria sturti Dobson, Robinson and Rowan-Kelly, 1997

The differentiation of the species *N. sturti* was based on allozyme studies of four strains isolated from water in Australia and Asia. Strains of *N. sturti* grow at 44°C (Dobson *et al*. 1997). The validity of the separate species status is confirmed by rDNA sequences of reference strain NG334 (De Jonckheere and Brown 1997). Differences in allozymes detected between different strains divides the species into two subgroups (Dobson *et al*. 1997). The allozyme differences between strains NG334/NG390 and NG221/277, respectively, are intermediate and in the same order as those between *N. australiensis* and *N. tihangensis*. The latter was previously called "sister species" of *N. australiensis* (Adams *et al*. 1989), later the spa variant of *N. australiensis* (Robinson, B. Protozoology. State Water Laboratory. Engineering and Water Supply Department. South Australia. Protozoology. Report No. 39, 1992), more recently a subgroup of *N. australiensis* (Dobson *et al*. 1997) and is given species status in the present monograph (see *N. tihangensis*). Therefore, it is possible that *N. sturti* comprises actually two different species. Because the SSU rDNA sequence of strain NG334 has been determined to substantiate the establishment of *N. sturti*, sequencing the SSU rDNA of strain NG277, which is available from ATCC, will resolve this issue.

The ITS1, 5.8S rDNA and ITS2 sequences of strain NG334 are available at EMBL under accession number Y10195, while the partial SSU rDNA sequence is under Y10185.

Only strain NG277 of *N. sturti* is available from ATCC (N° 50356).

Naegleria tihangensis sp. n.

The *N. gruberi* complex (Clark *et al*. 1989) is divided in this monograph into four different species. The second cluster of the *N. gruberi* complex contains strains that were isolated from Belgium (De Jonckheere *et al*. 1983b) and Mexico (De Jonckheere unpublished) in 1980 and 1983, respectively, while trying to isolate *N. fowleri* at 44°C. Therefore, the isolates are thermophilic but the maximum temperature tolerated is only 42° C. The Belgian strains came from a fish farm that used the cooling water from a nuclear power station, while the Mexican strains were isolated from geothermal water. The cluster is given species status based on SSU rDNA (De Jonckheere 1994a) and ITS DNA (Table 2) sequence analyses. The SSU rDNA of strain T2A of *N. tihangensis* differs only in one bp (0.125%) from that of strain NG202 within the 800 bp sequenced (De Jonckheere unpublished). This base substitution is in the highly variable loop 17 of the secondary structure. The ITS1, ITS2 and 5.8S rDNA of the two strains are identical (De Jonckheere unpublished). Also the LSU riboprints had been proven to be identical (Brown and De Jonckheere 1997). Strain NG202 is a representative of what was first called a "sister species" of

N. australiensis (Adams *et al*. 1989), later the spa variant of *N. australiensis* (Robinson, B. Protozoology. State Water Laboratory. Engineering and Water Supply Department. South Australia. Protozoology. Report No. 39, 1992), and more recently a subgroup of *N. australiensis* (Dobson *et al*. 1997). Therefore, the "sister species", spa variant, or subgroup of *N. australiensis* belongs to *N. tihangensis*. The latter species name refers to the location of the power plant in Tihange, Belgium, from which cooling water strains of this species were isolated. Strains of *N. tihangensis* are isolated frequently during attempts to isolate *N. fowleri* in Australia (Robinson, B. Protozoology. State Water Laboratory. Engineering and Water Supply Department. South Australia. Protozoology. Report No. 39, 1992) and Belgium (De Jonckheere unpublished). Strain EDF145, isolated in France, (Pernin and De Jonckheere 1996) has the same acid phosphatase (AP) and propionyl esterase (PE) isoenzymes, as well as an identical partial SSU rDNA sequence, and belongs, therefore, to this new species, *N. tihangensis.*

Although the original Belgian and Mexican strains are no longer available the species can still be identified based on the partial SSU rDNA sequences of strain T2A that has been published (De Jonckheere 1994a).

Strain NG202 from Australia is available from CCAP (N° 1518/21).

Nonthermophilic species

Naegleria gruberi, the first *Naegleria* sp. described turned out to be a large species complex (De Jonckheere 1982a, Adams *et al*. 1989, Clark *et al*. 1989, Pernin and Cariou 1989, Robinson *et al*. 1992).

The flagellates of two non-thermophilic species, *N. minor* and *N. robinsoni*, have the capacity to divide. The amoebae of two species, *N. chilenis* and *N. indonesiensis* could not be induced to transform into flagellates (De Jonckheere *et al*. 2001).

A note on N. gruberi sensu lato. The four clusters distinguished previously by rDNA RFLP plasmid typing (Clark *et al*. 1989) in *N. gruberi sensu lato* are given the status of species in the present paper (Table 4). One of the clusters retains the species name *gruberi.* Strains belonging to cluster 2 are considered thermophilic as they were isolated at 44°C (De Jonckheere *et al*. 1983b). They are named *N. tihangensis* (see above) and correspond to what was first called a "sister species" of *N. australiensis* (Adams *et al*. 1989), and later the spa variant of *N. australiensis* (Robinson, B. Protozoology. State Water Laboratory. Engineering and Water Supply Department. South Australia. Protozoology. Report No. 39, 1992). More recently they have been considered a subgroup of *N. australiensis* (Dobson *et al*. 1997). It is not unexpectedly that a thermophilic cluster is found within the *N. gruberi sensu lato* as other thermophilic species are found to branch in the midst of the complex (Clark *et al*. 1989, Robinson *et al*. 1992, De Jonckheere 1994a). Two other clusters in *N. gruberi sensu lato* are named *N. pringsheimi* and *N. pagei*, respectively.

With allozymes it was demonstrated that strains assigned to *N. gruberi sensu lato* consist of at least 10 species (Adams *et al*. 1989). Although some of these clusters correspond to presently described species, more new species descriptions might be expected (Table 5). Strains of some of these unnamed allozyme clusters are available from ATCC and can thus be studied by anyone interested. I stated previously (De Jonckheere 1994a) that it would be preferable to study all the other allozyme groups before giving species rank to the riboprint clusters in *N. gruberi*, but it seems the latter will not be achieved soon. Therefore, I decide to upgrade those that have been studied most extensively.

Over the years some doubts have developed over the authenticity of certain CCAP strains asigned to *N. gruberi sensu lato*. Page suspected that strain CCAP 1518/1E might have been transposed and allozymes of the strain were indeed found to be identical to the ones of the Pringsheim strains (De Jonckheere 1987, Adams *et al*. 1989). This is the reason why CCAP decided not to supply strains CCAP 1518/1A, 1B, 1C, 1D, 1E and 1S anymore, but a strain called CCAP 1518/1X (Table 4), which would correspond to *N. pringsheimi* (Brown personal communication). I have done an in depth analysis of this problem by looking at my old lab notes and corespondence with CCAP. I have used strains CCAP 1518/1E and CCAP 1518/1F as references in comparisons of isoenzyme patterns (De Jonckheere 1982a, De Jonckheere *et al*. 1984) and unpublished identifications of Belgian and Mexican *Naegleria* isolates in 1984 and 1985. In these studies CCAP 1518/1E was different from CCAP 1518/1F, but also from CCAP 1518/1D, one the Pringsheim strains. In 1986 (De Jonckheere 1987), the isoenzyme pattern of CCAP 1518/1E had suddenly changed and was identical to those of the Pringsheim strains CCAP 1518/1A, 1C, 1D and 1S. However, in the same publication the DNA RFLP of the latter strains were identical to each other, but that of CCAP 1518/1E did not correspond to it. In my correspondence I noticed that all the CCAP 1518 strains

Table 4. Strains of *N. gruberi sensu lato*

Strain	Max. °C	Analysis	Species
EG and descendants	39	allozyme, SSUrDNA, ITS, riboprints	*N. gruberi sensu stricto*
AUD1	(?)	riboprints, ITS, allozyme	*N. gruberi sensu stricto*
DRI	38	RFLP, SSU rDNA, riboprints, ITS, allozyme	*N. gruberi sensu stricto*
CCAP1518/1A(<1986)	37	riboprints	*N. pringsheimi*
CCAP1518/1C(<1986)	37	riboprints	*N. pringsheimi*
CCAP1518/1D(<1986)	37	riboprints, SSU rDNA	*N. pringsheimi*
CCAP1518/1S(<1986)	37	riboprints	*N. pringsheimi*
CCAP1518/1X	37	riboprints	*N. pringsheimi*
BL (contaminant)	(?)	riboprints	*N. pringsheimi*
CCAP1518/1E(<1986)	37	riboprints, SSUrDNA, allozyme	*N. pagei*
CCAP1518/1F	39	riboprints, ITS, allozyme	*N. pagei* (?)
CCAP1518/1G	(?)	allozyme	(?)
CCAP1518/7	37	riboprints, allozyme	*N. pagei*
Philar	(?)	riboprints, allozyme	*N. pagei*
B14	(?)	riboprints, allozyme	*N. pagei*
B26	(?)	riboprints, allozyme	*N. pagei*
B27	(?)	riboprints	*N. pagei*
R6a	(?)	allozyme	*N. pagei*
113/1	(?)	riboprints	*N. galeacystis*
T2A	42	riboprints, SSU rDNA, ITS	*N. tihangensis*
T2S	42	riboprints	*N. tihangensis*
Mx6J	42	riboprints	*N. tihangensis*
Mx6G	42	riboprints	*N. tihangensis*
NG202	42	SSU rDNA, ITS	*N. tihangensis*

used in this last isoenzyme analysis had been sent to me from CCAP on September 1, 1986, while the DNA of CCAP 1518/1E and CCAP 1518/1F had been prepared from the strains CCAP 1518/1E and CCAP 1518/1F that I had already in my laboratory before that. So it turned out that a transposition of strains had occurred during the difficult process that preceeded the transition of the CCAP collection from Cambridge to Windermere. Strains of CCAP 1518/1E used in different laboratories might therefore not be the original strain if obtained after 1985. It is comforting to know that all DNA studies I have done with this strain were, and still are, with the DNA from the original CCAP 1518/1E strain. The mislabeled strain was sent to me on September 1986, while the first publication in which I used the DNA of the original CCAP 1518/1E was submitted on August 2, 1986 (De Jonckheere 1987). Also the isoenzyme studies I published before 1987 were performed with the original CCAP 1518/1E strain.

Because of change in cyst diameter over a 10 year period Page mentioned that a possible transposition of labels for CCAP 1518/1E and CCAP 1518/1F might have happened already between 1964/1965 and 1974 (Page 1975). However, a transposition at that time can be excluded, as both strains still appeared to be different (De Jonckheere 1982a, De Jonckheere *et al*. 1984). Thus the change in cyst diameter must have been caused by changes over the years in culture, the second possible explanation (Page 1975).

In the older studies most emphasis was given to the identification of pathogenic and thermophilic strains, while it was only stated that there was an extreme heterogeneity in strains of *N. gruberi sensu lato*. However, serious consideration was given to the mesophilic *Naegleria* in Australia (Adams *et al*. 1989, Robinson *et al*. 1992). In examining my published studies and unpublished isoenzyme gels I can conclude a few things on the strains of *N. gruberi sensu lato* (Table 4). According to allozyme results strain R6a belongs to *N. pagei*, while CCAP 1518/1F might not belong to it. Strain CCAP 1518/1G would still belong to an un-named species other than the four into which *N. gruberi sensu*

lato is here divided Robinson *et al*. 1992, De Jonckheere unpublished).

Naegleria gruberi Schardinger, 1899

This is the first described species of *Naegleria* (Schardinger 1899), and was originally described as *Amoeba gruberi*, but later redefined as a separate genus (Alexeieff 1912, Calkins 1913). The whole history of how the genus *Naegleria* was born has been described in detail (Fulton 1970). Until the description of *N. fowleri* in 1970, all amoeboflagellates that conformed to the genus *Naegleria* were assigned to the species *gruberi.* Due to newer techniques that were introduced for more rapid identification of the pathogen *N. fowleri*, it was realised that there is an enormous diversity within the strains that were catalogued as *N. gruberi*. By using rDNA analyses it was possible to group *N. gruberi* strains into four clusters (Clark *et al*. 1989). The first cluster contains strain EG, and its descendants, that are used in different laboratories as the model for differentiation from amoebae to flagellates (Fulton 1993). In the absence of any type material, it was decided to retain the species name *gruberi sensu stricto* for strains that cluster with this strain EG and its descendants. Strain EG was originally isolated in the early sixties by F. Schuster in California from an eucalyptus grove, hence its designation EG (Fulton 1993). The first SSU rDNA sequence from the genus *Naegleria* (EMBL accession N° M18732) was obtained (Clark and Cross 1988b) from strain NEG-M, a descendant of strain EG, which has a ploidy double that of the original strain (Fulton 1993). The same descendant was used to determine the full sequence of the mtDNA (Lang *et al*. 1999). For obtaining the full sequence of the plasmid containing the rDNA (Mullican and Tracy 1993) strain NG_B was used, the EG strain kept by Balamuth. The EG strain that contains virus-like particles is called EG_S (Fulton 1993).

It was mentioned that strain NEG, a clonal strain of EG, will grow at up to 40-41°C with a maximum rate at about 33°C (Fulton 1970). Since the temperature optimum for each species is approximately 4°C lower than the upper temperature limit (Robinson *et al*. 1996a), the latter temperature is probably around 37°C for *N. gruberi*, otherwise it would be a thermophilic species. Strain ATCC 30544, corresponding to Fulton's strain NEG was reported to tolerate a maximum temperature of 39°C (Robinson *et al*. 1992). It has also been reported that strains can decrease their maximum temperature tolerance after maintenance in the laboratory for a certain amount of time (Dobson *et al*. 1997). Different culture conditions could also explain the difference in maximum temperature noted between strain CCAP 1518/1E (see *N. pagei*) from CCAP and that from ATCC (Robinson *et al*. 1992). A maximum temperature tolerance of 38°C is observed (Brown personal communication) with strain DRI, which belongs to *N. gruberi sensu stricto* (see below).

Naegleria amoebae cytopathogenic material (NACM) kills various cell cultures (Dunnebacke and Walen 1999) and was isolated from strain EG_S, the descendant of strain EG with virus-like particles. NACM is a small acidic protein, that resists inactivation by irradiation, nucleases, and a number of proteases, while it is inactivated by proteinase K and at elevated temperatures.

The reduced species *N. gruberi sensu stricto* still has a worldwide distribution. Other strains belonging to the emended *N. gruberi* species are strains AUD1 isolated from a swimming pool filter in France and strain DRI isolated from a water station drain on a golf course in Australia (Table 4). I have sequenced part of the SSU rDNA of strain DRI (De Jonckheere 1994a) and there is only one bp difference with that published for strain NEG-M. This indel is thought to be probably a sequencing error in strain NEG-M, as the deletion is a nucleotide that is conserved not only in all other *Naegleria* spp., but in all eukaryotes. In the sequence published for strain EG_B (Mullican 1995), another descendant of strain EG, there is, indeed, no such deletion (I made the same sequencing error in the SSU rDNA of *N. minor* strain WT043). The combined ITS1, 5.8S rDNA and ITS2 PCR product has the same length in strains AUD1, DRI and EG_B (De Jonckheere 1998). The sequences of these areas in strain EG_B (Mullican, J. C. Molecular characterization and complete sequence analysis of the extrachromosomal ribosomal DNA elemant in *Naegleria gruberi*. Ph D thesis. The graduate College in the University of Nebraska, Omaha, Nebraska, USA:1-163, 1995) in strain AUD1 (Pélandakis *et al*. 2000) and DRI are identical (Table 2). Strains that cluster in allozyme analysis with strain NEG of *N. gruberi sensu stricto* have been isolated from Australia, Japan, Germany and Ireland (Robinson *et al*. 1992). These isolates have all an upper temperature tolerance for growth of 38-39°C.

The ITS1, 5.8S rDNA and ITS2 sequences of strain EG_B have been determined (Mullican, J. C. Molecular characterization and complete sequence analysis of the extrachromosomal ribosomal DNA elemant in *Naegleria gruberi*. Ph D thesis. The graduate College in the University of Nebraska, Omaha, Nebraska, USA: 1-163, 1995) while those of strain AUD1 are available at

EMBL under accession number AJ132031. These sequences of strain DRI are in Table 2. The partial SSU rDNA sequence of strain NEG-M is available at EMBL under accession number M18732 while that of strain DRI has been published (De Jonckheere 1994a).

Strain DRI is currently available from CCAP under accession number 1518/17. Strain EG and descendants of this strain, NEG, NEG-M and EG_S are availabale from ATCC under accession numbers 30311, 30233, 30224 and 30540, respectively.

Naegleria pringsheimi sp. n.

The third cluster observed by Clark *et al.* (1989) in the *N. gruberi* complex contains strains isolated before 1950 by Pringsheim, of which CCAP 1518/1D is a representative. The clustering of these strains by DNA analysis was confirmed by allozyme analysis (Adams *et al*. 1989). This cluster is given species status based on SSU rDNA sequences (De Jonckheere 1994a). All five strains of *N. pringsheimi* studied have a group I intron in the SSU rDNA (De Jonckheere 1993) and the combined ITS1, 5.8S rDNA and ITS2 PCR product has the same length in those five strains (De Jonckheere 1989). Strains CCAP 1518/1D and CCAP 1518/1S were reported to grow at a maximum temperature of 37°C (Griffin 1972) as do the other CCAP strains belonging to *N. pringsheimi* (Robinson *et al*. 1992). Strain CCAP 1518/1S came from Singh and could be derived from CCAP 1518/1C, while CCAP 1518/1D came from Fulton with the information that it came to him from Balamuth as strain NB-1, which was derived from a subculture of CCAP 1518/1A (Page 1975). The latter is supported by the fact that CCAP 1518/1A and CCAP 1518/1D both have cysts of the rough type, but CCAP 1518/1S has the angular-smooth cyst type (Page 1975). However, Pussard and Pons (1979) believe that the morphology of the cysts depends too much on the condition of the medium on which they are formed and they found extreme differences in cyst morphology of clonal cultures. According to Page (1975) strain CCAP 1518/1A is the one used by Willmer in his experimental study of amoeba to flagellate transformation published in 1956. According to Pussard and Pons (1979), strain CCAP 1518 that Willaert used corresponds to CCAP 1518/1A or CCAP 1518/1D, which are identical (Page 1975). It is important to know the exact origin and history of a strain as conclusions drawn from using different strains may actually apply to several descendants of the same strain. For example, in a study of cytopathic effect of *N. gruberi sensu lato,* eight strains were used (John and John 1990) but there were probably only three strains with different origin and the others being descendants of strain EG. Riboprinting (Clark *et al*. 1989) demonstrated a slightly higher variation in *N. pringsheimi* strains (cluster 3 of *N. gruberi sensu lato*) than in *N. gruberi sensu stricto* strains (cluster 1 of *N. gruberi sensu lato*).

Strain BL also clusters with the Pringsheim strains, and although strain BL was claimed to have been isolated from a New Zealand case in 1968, the strain turned out to be nonpathogenic and reacts with *N. gruberi* antibodies and not *N. fowleri* antibodies. It is clear that the strain must have been mislabeled at some point and the BL strain that I used (De Jonckheere 1993, 1998) certainly clusters with the Pringsheim strains. Also, allozyme analysis groups strain BL with CCAP 1518/1D (Pernin and Cariou, 1989). On the other hand, strain BL available at ATCC (N° 22758) might be the original *N. fowleri* strain. It was submitted as a *Echinostelium* sp. by Mandal, one of the original authors of the PAM case in New Zealand (Mandal *et al.* 1970). The reason for this assumption is that in a recent study (Pélandakis *et al.* 2000) the ITS and 5.8S rDNA of strain BL correspond to what is found in *N. fowleri* (De Jonckheere 1998). As strain BL in different labs might be a mislabeled strain, except the one at ATCC, it appears that all strains of *N. pringsheimi* known at present are restricted to Europe.

The species *N. pringsheimi* can be identified based on the DNA sequences of the partial SSU rDNA sequence of strain CCAP 1518/1D that has been published (De Jonckheere 1994a). The sequence of the SSU rDNA group I intron of strain CCAP1518/1D is available at EMBL under accession number X78278. The ITS1, 5.8S rDNA and ITS2 sequences of strain CCAP 1518/1D are in Table 2.

Unfortunately, CCAP 1518/1D is no longer available, but CCAP 1518/1A and CCAP 1518/1C are at ATCC under N° 30874 and 30875, respectively.

Naegleria pagei sp. n.

Several strains isolated by F. Page in the USA (of which CCAP 1518/1E is a representative), strain Philar isolated in 1981 from the river Nile in Egypt, and strains isolated in 1980 from different lakes in Belgium, form a fourth cluster in the *N. gruberi* species complex (Clark *et al*. 1989). This cluster is now given species status on the basis of SSU rDNA sequences (De Jonckheere 1994a). The name is given in recognition of the contribution of F. C. Page to the knowledge

Table 5. Identification of clusters defined on the basis of allozymes by Robinson *et al*. (1992)

Cluster	N° of strains	Type strain	Max. °C	Culture collection N°	Group I intron*	Species
A	10	NG257	34	ATCC 50354	-	TD
B	2	NG260	37	ATCC 50355	-	*N. pussardi* (?)
C	2	NG158 (T56E)	42	-	+	*N. jamiesoni*
D	6	NG160 (Aq/4/1H)	40	CCAP 1518/16	+	*N. andersoni*
E	11	NG152 (CCAP 1518/1E)	35	ATCC 30876	-	*N. pagei*
		NG006	36	CCAP 1518/1G	-	TD
F	2	NG608	39	-	ND	TD
G	6	NG282	37	ATCC 50357	+	*N. clarki*
		NG010 (PD)	39	ATCC 30544	ND	idem
H	9	NG206	40	ATCC 50349	-	TD
		NG250	40	ATCC 50352	ND	TD
I	5	NG106	37	CCAP 1518/7	-	*N. pagei*
		NG230	37	ATCC 50350	-	idem
J	2	NG395	40	-	ND	TD
K	2	NG005	40 (39)	CCAP 1518/1F (ATCC 50348)	-	*N. pagei* (?)
L	8	NG008 (NEG)	39	ATCC 30223	-	*N. gruberi*
M	5	NG001	37	CCAP 1518/1A (ATCC 30874)	+	*N. pringsheimi*
N	2	NG408	<30	-	ND	Antarctic *Naegleria* sp.
Gr-3#	2	NG256	37	ATCC 50353	-	TD

* - De Jonckheere and Brown unpublished, # - in Adams *et al*. 1989, ND - not done, TD - to determine

of amoebae and who isolated several of the strains belonging to this species. The species has a worldwide distribution. With riboprinting (Clark *et al*. 1989) *N. pagei* strains (cluster 4 of *N. gruberi sensu lato*) were found to be more variable than *N. pringsheimi* strains (cluster 3 of *N. gruberi sensu lato*). The difference between CCAP 1518/1E and CCAP 1518/1F with the other *N. pagei* strains is greater than that between *N. italica* and *N. clarki* (Clark *et al*. 1989). Of the five strains of *N. pagei* investigated only strain CCAP 1518/1F has a combined ITS1, 5.8S rDNA and ITS2 PCR product that is a little shorter than the other strains (De Jonckheere 1998). Also, the isoenzyme patterns of CCAP 1518/1F do not correspond to that of CCAP 1518/7 (De Jonckheere 1987). Strain CCAP 1518/1F grows at a maximum temperature of 40°C (only slightly according to Pernin and Cariou 1989), while the other strains of *N. pagei* only tolerate 37°C (Robinson *et al*. 1992). The maximum temperature tolerance of strain CCAP 1518/1F has been recently confirmed to be 39°C (Brown personal communication). Severe differences in cyst morphology between strains CCAP 1518/1E and CCAP 1518/1F have been reported (Pussard and Pons 1979) and no cross-reaction was obtained with strain CCAP 1518/1F when antiserum prepared against strain CCAP 1518/1E was used (De Jonckheere *et al*. 1974). Furthermore, in allozyme studies strain CCAP 1518/1F does not cluster with CCAP 1518/7 of *N. pagei* (Robinson *et al*. 1992). Because of difference in ITS2 length, differences in isoenzymes and different maximum temperature tolerance, cyst morphology and antigens, strain CCAP 1518/1F does not really belong to *N. pagei* and needs further investigaton. According to Pernin and Cariou (1989) strain CCAP 1518/1E would not grow at 37°C, but I found in my labnotes of 1974 the strain does grow at this temperature. A discrepancy between the maximum temperature tolerated (Robinson *et al*. 1992) was found between the CCAP 1518/1E strain received

from CCAP and ATCC. See above (*A note on N. gruberi sensu lato*) for information on the CCAP strains. Page suspected that strain CCAP 1518/1E might have been transposed and allozymes for the strain were found to be indeed identical to the ones of the Pringsheim strains (Adams *et al*. 1989). However, the DNA analyses (Clark *et al*. 1989, De Jonckheere 1994a) indicate that they have been performed on the DNA from the original strains before the transposition (see *A note on N. gruberi sensu lato*). These strains have indeed been acquired by me from CCAP in the early seventhies. I even found in my labnotes of 1974 the results of the temperature tolerance tests on these strains, so that I was able to compare these to those published (Adams *et al*. 1989, Robinson *et al*. 1992).

The species *N. pagei* can be identified based on the partial SSU rDNA sequence of strain CCAP 1518/1E that has been published (De Jonckheere 1994a). The ITS1, 5.8S rDNA and ITS2 sequences of strain CCAP 1518/1F are available at EMBL under accession number AJ132022, while that of CCAP 1518/1E are in Table 2.

Strains of *N. pagei* are still available at CCAP (N° 1518/1F and N° 1518/7) and ATCC (N° 30876), but it should be investigated whether the latter corresponds indeed to the original CCAP 1518/1E (see above). Because strain CCAP 1518/1F seems to be the most different strain I propose to use CCAP 1518/7 as the type strain of *N. pagei*.

Naegleria jadini Willaert and Le Ray, 1973

The type strain of *N. jadini* was isolated from a swimming pool in Antwerp, Belgium. Only one strain of this species is known and it was identified by immunoelectrophoretic analysis, a technique not currently employed in species identification (Willaert and Le Ray 1973). The species can be differentiated from other species by isoenzyme analysis, a technique used more frequently in the identification of *Naegleria* isolates (De Jonckheere 1982a). However, *N. jadini* is unlikely to be encountered in routine *Naegleria* analyses as during these isolation procedures high incubation temperatures are used, and *N. jadini* does not grow above 35°C (Robinson *et al*. 1992). However, even the Australian Water Quality Centre which uses different incubation temperatures on samples from all over the world never seems to have encountered this species (Robinson *et al*. 1992).

The type strain of *N. jadini* appears to have one of the longest ITS2 region in the genus *Naegleria* (Table 2). The larger size is due to a number of repeats (De Jonckheere 1998). On the other hand, *N. jadini* has one of the smallest rDNA plasmids reported (Clark *et al*. 1989).

The ITS1, 5.8S rDNA and ITS2 sequences of strain 0.400 are available at EMBL under accession number X96576, while the partial SSU rDNA sequence has been published (De Jonckheere 1994a).

The type strain of *N. jadini* has been adapted to axenic growth. The type strain 0400 is available from ATCC (N° 30900) and from CCAP (N°1518/2).

Naegleria clarki De Jonckheere, 1984

Two *Naegleria* strains from New Zealand were found to form a separate cluster related to *N. italica* in a tree based on rDNA restriction patterns (Clark *et al*. 1989). They have special isoenzyme patterns and total DNA patterns not found in described species (De Jonckheere 1988b). The SSU rDNA of strain RU30 was sequenced and on this basis this cluster was given species status (De Jonckheere 1994a). The two strains of *N. clarki* have a group I intron in the SSU rDNA (De Jonckheere 1993). The two type strains of *N. clarki*, RU30 and RU42, were isolated from water at the Golden Springs in Rotorua, New Zealand (Jamieson J. A. Studies of amoebae of the genus *Naegleria*. Master of Science thesis, Adelaide, 1-48, 1975). When tested for upper temperature limit strain RU30 was found to grow at 37°C (Brown personal communication). Based on allozyme, strains of this species have been identified in water samples from the USA, Australia, Korea, China and Guyana, of which the majority grows at an upper temperature limit of 37°C, a few at 38°C and only one at 40°C (Dobson personal communication). Strains closely related to *N. clarki*, based on SSUrDNA sequences, have been isolated from organs of fish as well (Dykova *et al*. 2001). It is of interest to note that soon after isolation the two strains of *N. clarki* killed some mice after IN instillation (Jamieson J. A. Studies of amoebae of the genus *Naegleria*. Master of Science thesis, Adelaide, 1-48, 1975), but later attempts could not confirm the pathogenicity of this species (Willaert 1976).

The ITS1, 5.8S rDNA and ITS2 sequences of strain RU30 are available at EMBL under accession number X96575, while the partial SSU rDNA sequence has been published (De Jonckheere 1994a). The sequence of the SSU rDNA group I intron of strain RU30 is available at EMBL under accession number X78281.

Strains of *N. clarki* can be grown axenically. Strains RU30 and RU42 are available from CCAP (N° 1518/14 and 1518/15, respectively).

Naegleria galeacystis De Jonckheere, 1994

The strain that was used in different laboratories as the type strain of *Adelphamoeba galeacystis* (ATCC 30294) appears now to be a strain that was mislabelled. Adams *et al*. (1989) already considered ATCC strain 30294 to be a *Naegleria* species. Since this mislabelled strain showed SSU rDNA (De Jonckheere 1994a) and ITS rDNA (De Jonckheere 1998) sequences compatible with the genus *Naegleria*, but different from described *Naegleria* spp., it was given separate species status within the genus and the species name was retained. A strain isolated in South Australia, that conformed to the original morphological description for *A. galeacystis*, turned out to have identical SSU rDNA sequences as *Didascalus thorntoni* (De Jonckheere *et al*. 1997). Thus *Adelphamoeba* is a nonvalid junior name of *Didascalus*. Therefore, it is acceptable to use the species name *galeacystis* for this *Naegleria* sp.

The mislabelling of this isolate probably occurred in Napolitano's laboratory before it was distributed, because the typestrain available from ATCC and submitted by Napolitano, is also a *Naegleria* strain. The close similarity of strain *A. galeacystis* from ATCC with strains of *N. gruberi* had already been observed by studying rDNA restriction patterns (Clark and Cross 1988a). Strain 113/1 from France probably belongs to *N. galeacystis* (Clark *et al*. 1989). I have done a phylogenetic analysis of the partial SSU rDNA published for a *Naegleria* isolated from a necrotic lesion in a reptile (Walochnick *et al*. 1999). The sequence invariably clusters with that from *N. galeacystis*, although the sequence is not identical. The reptile isolate should be further studied for proper identification to species level.

The ITS1, 5.8S rDNA and ITS2 sequences of strain A.V.500 are available at EMBL under accession number X96578, while the partial SSU rDNA sequence has been published (De Jonckheere 1994a).

Strain A.V.500 of *N. galeacystis* has been adapted to axenic growth. Typestrain A.V. 500 is available from ATCC (N° 30294) and from CCAP (1506/1).

Naegleria fultoni De Jonckheere, Brown, Dobson, Robinson and Pernin, 2001

Strains described as *N. fultoni* are different from other *Naegleria* spp. in allozyme pattern (Robinson personal communication), while the SSU rDNA and the ITS1, 5.8S rDNA and ITS2 sequences of the typestrain NG885 are also novel (De Jonckheere *et al*. 2001). Strains of this species grow at a maximum temperature of 35°C and only form flagellates under certain conditions (De Jonckheere *et al*. 2001). Several strains of this new species were isolated from a reservoir in South Australia but, as far as I know, not from other places. Strains of the WA variant of *N. lovaniensis* with a group I intron in the LSU rDNA were also found in this reservoir (see WA variant of *N. lovaniensis* in preparation).

The ITS1, 5.8S rDNA and ITS2 sequences of strain NG885 are available at EMBL under accession number AJ243445, while the partial SSU rDNA sequence is under AJ243440.

Typestrain NG885 of *N. fultoni* is not yet available from either ATCC or CCAP.

Naegleria spp. with dividing flagellates

Although flagellates with different numbers of flagella are seen in different *Naegleria* spp. (Fulton 1970, John *et al*. 1991), the flagellates were never observed to divide although some scanning electron micrographs (John *et al*. 1991) might suggest that they were in the process of division (De Jonckheere and Brown 1995). Recently two *Naegleria* spp. with dividing flagellates have been described, and they belong to different phylogenetic clusters within the genus *Naegleria*.

Naegleria minor De Jonckheere and Brown, 1995

As in *Willaertia magna*, the flagellates of *N. minor* (strain WTO43) have four flagella, but the flagellates of *N. minor* can divide only once and the daughter cells have only two flagella. Strain WTO43 was originally described as a new *Willaertia* sp., i.e. *W. minor* (Dobson *et al*. 1993) but SSU rDNA sequence comparisons showed that the type strain of *W. minor* clusters within the genus *Naegleria* and not with *W. magna*. Therefore, it was renamed *N. minor* (De Jonckheere and Brown 1995). Because of the fact that the flagellates divide only once, it is speculated that the division might be a meiosis, and that the monoploid daughter flagellates might act as gametes. Although this hypothesis has not been proven experimentally, the division of the flagellates would support the idea of the flagellates being gametes which has been proposed earlier (Fulton 1993). There is only one strain known of *N. minor* which was isolated from the Northern Territory in Australia. It is the only

Table 6. Keratitis cases implicating amoebae other than *Acanthamoeba* spp.

Reference	Country	Strains isolated	Year	Contact lens	Age	Sex
Haase and De Jonckheere, unpublished (mentioned in: De Jonckheere and Brown 1998b)	Germany	*Hartmannella* sp.	1989	?	?	F
Kennedy *et al*. 1995	Ireland	*H. vermiformis*	?	soft	17	F
Aitken *et al*. 1996	UK	*Hartmannella* sp. (+ *Vahlkampfia* sp.)	1992	+	24	M
Inoue *et al*. 1998	Japan	*Hartmannella* sp. (+ *Acanthamoeba* sp.)	1994	hard	54	F
Aimard *et al*. 1998.	France	*Hartmannella* sp. (+ *Acanthamoeba* sp.)	?	+	40	F
Dua *et al*. 1998	UK	*Vahlkampfia jugosa* *	1995	soft	38	M
		Naegleria sp.	1997	soft	18	F
Alexandrakis *et al*. 1998	USA	*Vahlkampfia* sp.	?	-	30	M
Belle and De Jonckheere, unpublished	Belgium	*Hartmannella* sp.	1999	-	?	M
Belle and De Jonckheere, unpublished	Belgium	*Vahlkampfia inornata*	2000	+	18	F

* - *Vahlkampfia jugosa* was renamed *Paratetramitus jugosus* (Darbyshire *et al*. 1976) and *Tetramitus jugosus* (Brown and De Jonckheere 1999)

Naegleria strain in which a plasmid has been detected other than the rDNA plasmid (De Jonckheere and Brown 1995). It is not known what this other DNA plasmid is coding for, but it might be a good candidate for DNA-mediated transformation instead of the rDNA plasmid. The strain of *N. minor* has one of the longest ITS2 in the genus *Naegleria* (Table 2) but, contrary to *N. jadini*, this is not due to repeats (De Jonckheere 1998).

The ITS1, 5.8S rDNA and ITS2 sequences of strain WT043 are available at EMBL under accession number X96577, while the SSU rDNA sequence is under X93224.

The strain of *N. minor* grows at a maximum temperature of 38°C and has been adapted to axenic growth. The typestrain WT043 is available from ATCC (N° 50320) and CCAP (N° 1518/18).

Naegleria robinsoni De Jonckheere and Brown, 1999

This species description is based on only one strain. As is the case with *N. minor*, *N. robinsoni* transforms into dividing flagellates with four flagella, and the daughter cells have only two flagella. After this division the flagellates can no longer divide (Robinson unpublished). In phylogenetic trees based on SSU rDNA sequences *N. robinsoni* does not cluster with *N. minor* (De Jonckheere and Brown 1999b), the other *Naegleria* sp. with dividing flagellates. The typestrain, NG944, was isolated from freshwater on Kangaroo Island at the South coast of Australia and grows at a maximum temperature of 38° C, which is the same temperature as for *N. minor*.

The ITS1, 5.8S rDNA and ITS2 sequences of strain NG944 are available at EMBL under accession number AJ237787, while the partial SSU rDNA sequence is under AJ237786.

Type strain NG944 of *N. robinsoni* is available from CCAP (N° 1518/19).

Naegleria spp. in which no transformation to flagellates could be induced

It has been reported that four strains of *N. fowleri* (Cable and John 1986) and four strains of *N. lovaniensis* (John *et al*. 1991) could no longer be induced to transform to flagellates in the laboratory. These strains had

formed flagellates before and this loss of ability to transform is probably due to prolonged axenic cultivation. The capacity to transform was not restored by subculturing on bacteria (John *et al*. 1991). In addition, there are a few *N. fowleri* strains from one location in France that could never be induced to form flagellates since their isolation (Pernin personal communication). In addition there are two *Naegleria* spp., *N. chilensis* and *N. indonesiensis*, that have never formed flagellates under laboratory conditions, even immediately after isolation (Robinson personal communication).

While *N. fultoni* (see above) only forms flagellates under certain conditions most *Naegleria* spp. easily form flagellates soon after isolation (Fulton 1993). In most cases I even donot need to perform a separate transformation test on new *Naegleria* isolates. They tend to form flagellates in the liquid that accumulates at the border between the agar surface and the agar block on which they are transferred for subculturing.

Naegleria chilensis De Jonckheere, Brown, Dobson, Robinson and Pernin, 2001

The type strain of *N. chilensis*, NG946, was isolated from alpine Chile and barely grows at 30°C. The strain fails to form flagellates (Robinson personal communication) but the SSU rDNA shows that it belongs to the genus *Naegleria*. Because of unique allozyme patterns and SSU rDNA sequence differences it is described as a new species (De Jonckheere *et al*. 2001). Only one strain of this species has been isolated. In phylogenetic trees based on SSU rDNA sequences *N. chilensis* clusters with *N. pussardi*, which is the most distantly related species in the genus *Naegleria*.

The type strain of *N. chilensis* has the longest ITS1 ever found in the genus *Naegleria* (Table 2).

The ITS1, 5.8S rDNA and ITS2 sequences of strain NG946 are available at EMBL under accession number AJ243443, while the partial SSU rDNA sequence is under AJ243442.

Type strain NG946 of *N. chilensis* is not yet available from either ATCC or CCAP.

Naegleria indonesiensis De Jonckheere, Brown, Dobson, Robinson and Pernin, 2001

The type strain of *N. indonesiensis*, NG945, was isolated from Bali, Indonesia, and grows at an upper temperature limit of 38° C. This strain also fails to form flagellates (Robinson personal communication) but the SSU rDNA shows that it belongs to the genus *Naegleria*. Because of unique allozyme patterns and SSU rDNA sequence differences it is described as a new species (De Jonckheere *et al*. 2001). Only one strain of this species has been isolated. In phylogenetic trees based on SSU rDNA sequences *N. indonesiensis* clusters with *N. robinsoni*, a species with dividing flagellates.

The ITS1, 5.8S rDNA and ITS2 sequences of strain NG945 are available at EMBL under accession number AJ243444, while the partial SSU rDNA sequence is under AJ243441.

Type strain NG945 of *N. indonesiensis* is not yet available from either ATCC or CCAP.

New *Naegleria* spp. of which the descriptions are in preparation

There are three *Naegleria* groups for which most of the allozyme and rDNA typing has been done and which will be described as new species in the near future. These three are all thermophilic species. Another species description has been long in preparation for strains that were isolated from places at low temperatures, cluster N in Robinson *et al*. (1992). I have been unable to perform DNA typing on it because of difficulties in culturing these strains in the laboratory.

Naegleria philippinensis in preparation

In 1984 a *Naegleria* strain (RJTM) was isolated from the CSF of a young patient diagnosed with encephalitis in the Philippines (Matias, R. R. *Naegleria philippinensis*: characterization based on morphology, surface membrane antigens, isoenzyme patterns and hydrolases. Doctoral Dissertation, University of the Philippines, 1991). Similar strains were isolated from a thermally polluted river and from a heated swimming pool in the Philippines. A *Naegleria* isolate with similar isoenzyme patterns as the Phillippine isolate was recovered from a swimming pool in Hungary (Szénasi *et al*. 1998). The Philippine strain from thermally polluted water was reported to be of low virulence as high numbers of amoebae were needed to kill mice (Simeon *et al*. 1990). I have tested the CSF strain by IN inoculation of mice with similar amounts of amoebae and found it did not cause mortality. On NNE it grows only at a temperature up to aprox. 40°C. Since pathogenic *Naegleria* spp. either grow at 42°C or 45°C, the upper temperature tolerance of strain RJTM would support that it is not a pathogenic species. The positive virulence

tests early on are comparable to those obtained with nonpathogenic species, such as *N. lovaniensis*, *N. jadini* and *N. clarki* (see above).

I was able to analyse the isoenzymes of the CSF isolate only. The AP pattern is the same as for *N. tihangensis* and *N. clarki* but the PE pattern is different. However, differences in PE patterns have been observed in several species while the AP pattern seems to be more specific to the species. The AP isoenzyme pattern of the different *N. philippinensis* strains appear to be the same (Matias, R. R. *Naegleria philippinensis*: characterization based on morphology, surface membrane antigens, isoenzyme patterns and hydrolases. Doctoral Dissertation, University of the Philippines, 1991), but some of these strains may belong to *N. tihangensis*, especially since the latter seem to be very common in the environment. The isolate from Hungary was reported to have similar isoenzyme profiles as the Philippine isolate, but the former is thermophilic (Szénasi *et al*. 1998). Therefore, the Hungarian isolate might be a strain of *N. tihangensis*.

In phylogenetic trees based on SSU rDNA sequences, the CSF isolate of *N. philippinensis* (strain RJTM) clusters within the *N. australiensis* - *N. italica* group (Fig. 2). Between the two PstI sites strain RJTM differs in respectively 14 bp (1.8%) and 16 bp (2.0%) from these two pathogenic species. Strain RJTM has the same 5.8S rDNA sequence as the two pathogenic strains and *N. clarki*. I found the ITS2 to be 105 bp long, but Fontanilla *et al*. (2001) reported that it is 115 bp long in the same isolate. The ITS2 of *N. philippinensis* is 5 nt longer than in *N. australiensis* and 3 nt longer than in *N. tihangensis* (Table 2). In *N. italica* the ITS2 has a long insert. Contrary to *N. australiensis* and *N. tihangensis*, *N. philippinensis* has a group I intron in the SSU rDNA, as is the case in *N. italica* and *N. clarki*. Since group I introns in the SSU rDNA is a property of the species, *N. philippinensis* cannot belong to *N. australiensis* or *N. tihangensis*. However, it can also not belong to *N. italica* and *N. clarki* as the group I introns are quite different. In *N. philippinensis* the group I intron is 1297 bp long while the length in *N. italica* and *N. clarki* is 1319 bp and 1305 bp, respectively (Table 3).

Type strain RJTM of *N. philippinensis* is available from CCAP (N° 1518/20).

The WA variant of *Naegleria lovaniensis* in preparation

At the State Water Laboratory of South Australia several thermophilic *Naegleria* strains with an upper temperature limit of 44°C have been isolated that show allozyme divergence at nearly 30% of loci with *N. lovaniensis* (Robinson and Dobson 2001). These strains are provisionally named WA variant as they appear to be restricted to western and northern Australia. Also, in phylogenetic trees based on SSU rDNA, strain NG872 of WA variant clusters with *N. lovaniensis* (De Jonckheere and Brown 1997). The two differ by only 3 bp (0.5%) in the 800 bp sequenced. Investigation of the SSU rDNA of strain NG872 demonstrated the presence of a group I intron. Moreover, a group I intron is also present in the LSU rDNA of this strain (De Jonckheere and Brown 1998a). This is very uncommon in *Naegleria* spp. as group I introns in the LSU rDNA have only been found in *N. morganensis* and the WA variant of *N. lovaniensis*. A recent study of different isolates belonging to the WA variant of *N. lovaniensis* showed that, in fact, two different group I introns are present in this lineage depending on the strain investigated, and group I introns might be absent from either the LSU rDNA and/or the SSU rDNA of different isolates (De Jonckheere and Brown 2001). While the first detected group I intron in the LSU rDNA has no ORF (De Jonckheere and Brown 1998a), the second one is much longer and encodes an endonuclease (De Jonckheere and Brown 2001). In contrast with the group I intron in the SSU rDNA, which is transmitted vertically (De Jonckheere 1994b), the group I introns in the LSU rDNA are transferred horizontally (De Jonckheere and Brown 1998a, 2001).

The ITS1, 5.8S rDNA and ITS2 sequences of strain NG872 are available at EMBL under accession number Y10191, while the partial SSU rDNA sequence is under Y10187. The sequences of the LSU rDNA group I intron of strain NG872 and NG874 are available at EMBL under accession number AJ001316 and AJ271406, respectively, while the sequence of the SSU rDNA group I intron of strain NG872 is under AJ001399.

No strains of the WA variant of *N. lovaniensis* are available yet from either ATCC or CCAP.

Naegleria sp. NG597 in preparation

The three strains known of this lineage were isolated from Australia, Papua New Guinea and Indonesia, respectively (Dobson personal communication).The clustering of these three strains is based on allozyme studies (Robinson personal communication), and analysis of the SSU rDNA sequence of one strain (NG597) places the lineage close to *N. clarki* and *N. gruberi sensu stricto* (De Jonckheere and Brown 1997). This *Naegleria* sp. is special as it is the only known *Naegleria* lineage that has lost the group I intron ORF (De Jonckheere and Brown 1994), which is part of the twintron present in the SSU rDNA of other intron-bearing *Naegleria* spp. (Einvik *et al*. 1998). The loss of part of the twintron is found in the three different strains investigated of this lineage and the group I introns differ by only three and four bp, respectively, from each other. The NG597 *Naegleria* lineage is another thermophilic *Naegleria* sp. (upper temperature limit of 42°C), but is awaiting proper species description.

The ITS1, 5.8S rDNA and ITS2 sequences of strain NG597 are available at EMBL under accession number Y10194, while the partial SSU rDNA sequence is under Y10184. The sequences of the SSU rDNA group I introns of strains NG434, NG597 and NG650 are available at EMBL under accession numbers X79069 till X79071.

No strains of this lineage are available yet from either ATCC or CCAP.

Antarctic *Naegleria* sp. in preparation

The research group of the State Water Laboratory of South Australia has isolated *Naegleria* strains from Antarctica that only grow at temperatures lower than 30°C. Identical strains have also been reported from the northern hemisphere (Robinson personal communication). By allozyme analysis they form cluster N in Robinson *et al*. (1992), and are the closest relative of *N. jadini*, yet are certainly a different species. I have not been able to determine DNA sequences as the strains are difficult to culture. Therefore, details about their SSU rDNA phylogeny are lacking.

PHYLOGENETIC RELATIONSHIPS

The genus *Naegleria*

Although based on a limited dataset of SSU rDNA sequences, it was already known from 1989 that the evolutionary distance between *N. gruberi sensu lato* and *N. fowleri* was as great as the distance between amphibians and mammals (Baverstock *et al*. 1989). However, the mutational distance of the SSU rDNA might not represent the true evolutionary distance since the rDNA might have evolved at different rates in different organisms. In spite of this, the mutational differences in *Naegleria* between the SSU rDNA seem to be related to other molecular distances, such as allozyme and antigenic differences. Therefore, the use of SSU rDNA sequence differences is a good quantitative method for delineating species. Although I had used RFLP of total DNA to characterise *Naegleria* spp. before (De Jonckheere 1987), I decided in 1994 to use SSU rDNA sequences for differentiating between different *Naegleria* spp. (De Jonckheere 1994a).

The genus that is most closely related to *Naegleria* is *Willaertia*, an amoeboflagellate genus with dividing flagellates (De Jonckheere 1997). As shown above, based on SSU rDNA analysis, the other *Willaertia* sp., *W. minor*, has turned out to be a *Naegleria* sp.

The phylogenetic tree shown in Fig. 2. is based on partial SSU rDNA sequences and different clusters within the genus *Naegleria* can be discerned. It is obvious that the pathogenic species do not cluster, neither do the thermophilic species, nor the species with dividing flagellates, nor the species which do not transform into flagellates. Therefore, it is impossible to group these isolates into different genera based upon a particular behaviour of the flagellates. These new genera would have to include species with typical *Naegleria* characteristics.

The human pathogen *N. fowleri* clusters with *N. lovaniensis* and the WA variant of *N. lovaniensis*. All strains of these three *Naegleria* grow at a maximum temperature of 44-45°C. In this cluster, group I introns are found only in the WA variant of *N. lovaniensis*, in the LSU rDNA as well as in the SSU rDNA (De Jonckheere and Brown 1998a, 2001).

Three other species that grow at up to 44-45°C, *N. morganensis*, *N. niuginensis* and *N. sturti*, form a closely-related cluster. Also in this second cluster, one species (*N. morganensis*) contains group I introns in the LSU rDNA. However, group I introns are not found in the SSU rDNA of this species.

The third cluster contains *N. minor*, one of the two *Naegleria* spp. in which the flagellate stage divides. It is not thermophilic (upper growth temperature limit of 38°C), although the second species in this cluster, *N. carteri*, is thermophilic (upper growth tem-

perature limit of 45°C) and has a group I intron in the SSU rDNA.

In the fourth cluster, both species are less thermophilic (upper growth temperature limits of 40 and 42°C, respectively). They had been described originally as two subspecies, but the phylogenetic analysis of SSU rDNA sequences confirms their independent relationship. In addition, both *N. andersoni* and *N. jamiesoni* contain a group I intron in the SSU rDNA (Table 3).

Except for *N. minor*, the species in the two clusters above have a 5.8S rDNA of 174 bp length (Table 2). All other *Naegleria* spp. have a 5.8S rDNA of 175 bp, except *N. pussardi* in cluster 6 where it is also 174 bp.

Cluster 6 is the most distant cluster in the *Naegleria* tree. The two species in this cluster are also quite different from each other in many respects. While *N. pussardi* grows at a maximum temperature limit of 41°C, *N. chilensis* only grows at a maximum of 30°C. In addition, *N. chilensis* could not be induced to transform into a flagellate stage under laboratory conditions, and in *N. pussardi* the nucleolus behaves differently during prophase than those in other *Naegleria* spp.

The 14 other *Naegleria* spp. form one big cluster 5. In this cluster, *N. galeacystis*, *N. robinsoni* and *N. indonesiensis* could be considered a subcluster although the latter three species have very little in common. *Naegleria robinsoni* is one of the two *Naegleria* spp. in which the flagellate stage can divide, while *N. indonesiensis* could not be induced to transform into flagellates under laboratory conditions. However, the single strains of these three species have a similar upper temperature limit for growth between 35°C and 38°C.

The 11 other species in cluster 5 vary in pathogenicity, temperature tolerance (Table 1) and in the presence of a group I intron in the SSU rDNA (Table 3). The length of the ITS1 of all strains in this big cluster is 33 bp, except for *N. jadini*, in which it is 35 bp (Table 2). Also, in the SSU rDNA phylogeny *N. jadini* seems to be the most distant species in cluster 5 (Fig. 2). Big differences in length of the ITS2 (from 100 to over 500 bp) are observed in species belonging to this cluster. The four different clusters of the *N. gruberi sensu lato* branch within cluster 5 as well (De Jonckheere 1994a). In the present monograph these four lineages of the *N. gruberi* species complex are given species status: *N. gruberi sensu stricto*, *N. tihangensis*, *N. pringsheimi* and *N. pagei*.

It has not been the intention to split the genus into as many different species as possible. As a matter of fact, strains of *N. lovaniensis*, a species that is closely-related to the pathogenic *N. fowleri* in many respects (except that it is not pathogenic) were called at first nonpathogenic *N. fowleri* variants (De Jonckheere and van de Voorde 1977b). The use of molecular biology techniques allowed a much more precise delineation and separation of species. Proper identification of thermophilic *Naegleria* strains is important as it was first thought that all *Naegleria* strains isolated at 45°C were pathogenic *N. fowleri*.

Our strategy of defining species on the basis of SSU rDNA can lead to other proposals than describing more species. This technique can also lead to assembling several genera under one genus name. The situation presently concerning the other vahlkampfiids is an example of this.

Other genera in the Vahlkampfiidae

The genus *Vahlkampfia* has gained notoriety recently because some cases of keratitis have been attributed as being caused by strains of this genus (Aitken *et al*. 1996, Alexandrakis *et al*. 1998, Dua *et al*. 1998) as well as by strains of *Hartmannella* (Kennedy *et al*. 1995, Aitken *et al*. 1996, Aimard *et al*. 1998, Inoue *et al*. 1998) and even by a strain of *Naegleria* (Dua *et al*. 1998) (Table 6). Yet, in none of these keratitis cases is there enough evidence for the isolated strains to be confirmed as the cause of the keratitis infection (De Jonckheere and Brown 1998b, c, d, 1999a). In a recently published study of amoeba strains from keratitis patients in Austria, no proof was found either that the three *Vahlkampfia* and two *Hartmannella* isolates were of clinical relevance (Walochnik *et al*. 2000).

The genus *Vahlkampfia*, as previously defined, consists of species in which the amoebae are unable to transform into flagellates as the common character (Page 1988). When it was found that *V. jugosa* did form flagellates after all, it was described as another genus, *Paratetramitus jugosus* (Darbyshire *et al*. 1976).

Recently we concluded that the original assignment of all vahlkampfiid species which lack a flagellate phase to a single genus was inappropriate as they form different clusters on the basis of SSU rDNA analyses. It was proposed that the seven *Vahlkampfia* spp. should be partioned between four genera (Brown and De Jonckheere 1999): *V. avara* and *V. inornata* retain the genus name *Vahlkampfia*, while *V. aberdonica*, *V. lobospinosa* and *V. enterica* are transferred to the genus *Tetramitus*, together with *Didascalus* and *Paratetramitus*. The difference in SSU rDNA between

Paratetramitus and *Didascalus* is 1 %, while the difference between these and *Tetramitus* amount to 10 %, exactly within the range we found for *Naegleria* spp. The two other *Vahlkampfia* spp. became two new, distantly related genera: *Neovahlkampfia* and *Paravahlkampfia*. Thus, while the genus *Vahlkampfia* was split into different genera, some species of it and other genera were actually assembled in the genus *Tetramitus*. As a result the genus *Tetramitus* contains species that form flagellates, and species that do not form flagellates, as is also now the case in the genus *Naegleria*. In *Vahlkampfia*, the phylogenetic trees based on the whole SSU rDNA molecules were the same as those based on approx. 800 bp of the molecule. We are therefore confident that in *Naegleria* the phylogeny can be based on the approx. 800 bp spanned between the two Pst I sites, typical for the genus, and that it is not necessary to sequence the whole molecule.

The genus *Acanthamoeba*

The genera *Acanthamoeba* and *Balamuthia* do not belong to the Vahlkampfiidae; they are even phylogenetically unrelated. However, it is appropriate to mention the situation in these genera here because in the free-living genera *Acanthamoeba*, *Balamuthia* and *Naegleria* pathogenic species occur and they are, therefore, studied in the same laboratories. In the genus *Acanthamoeba* a SSU rDNA analysis has also been performed using the whole SSU rDNA sequence (Stothard *et al*. 1998). The situation in *Acanthamoeba* was more confused by the fact that many different species had been described already purely on the basis of morphology before biochemical and molecular typing started, which is in contrast to *Naegleria*. It turned out that different morphological species of *Acanthamoeba* cluster in sequence type 4. Although Stothard *et al.* (1998) are in favor of the proposal that each cluster should be equated with a single species, they prefer to call them sequence types until strains have been included of all the other *Acanthamoeba* spp. that were not sequenced yet. As is the case in several *Naegleria* spp., some *Acanthamoeba* sequence types are represented by only one isolate. Only in some cases *Acanthamoeba* sequence types did correspond to one described species, such as sequence type 5, which was shown to be a homogeneous group of strains of *A. lenticulata*. In sequence type four, 24 out of 25 *Acanthamoeba* keratitis isolates cluster. The other keratitis strain is a sequence type 3 (*A. griffini*). Recently, a keratitis isolate turned out to belong to sequence type 11 (Walochnik *et al*. 2000). Isolates that cause encephalitis appear to cluster in sequence types 1 and 12 (Stothard *et al*. 1998). As is the case in the genus *Naegleria*, pathogenic strains belong to different species, or sequence types, in the genus *Acanthamoeba*.

Group I introns are also present in the SSU rDNA of *Acanthamoeba* albeit in only two species out of 16 investigated (Gast *et al*. 1994, Schroeder-Diedrich *et al*. 1998) compared to nine out of 26 *Naegleria* spp. investigated (Table 3).

It was suggested that the sequence types of cyst morphology group I in *Acanthamoeba* should probably be reclassified as one or more different genera. The situation resembles, therefore, what we observed in the genus *Vahlkampfia*, where two new genera were erected on the basis of the SSU rDNA sequences. But, as for the species reclassification, no formal proposal for genera names was made for *Acanthamoeba*. There is around 10 to 11% difference between types that could be reclassified as distinct genera in *Acanthamoeba*. Therefore, it could be argued that cluster 6 in *Naegleria*, in which *N. pussardi* and *N. chilensis* differ by about 12% from the other *Naegleria* spp. in their SSU rDNA, could also be reclassified as another genus. This would mean we would be even more like splitters than like lumpers. However, the decision to rename two *Vahlkampfia* spp. into new genera (Brown and De Jonckheere 1999) was based on a SSU rDNA difference of around 40%, and not 10%. Indeed, one has to realise that the genus *Naegleria* branched off much earlier than *Acanthamoeba* and, therefore, species in the genus *Naegleria* have had more time to diverge than species in the genus *Acanthamoeba*. Therefore, 10% evolutionary distance means more in the younger *Acanthamoeba* genus than it does in the ancient *Naegleria* genus. In species that evolved during different timescales the molecular distances can not be compared in the same way for the definition of a species.

The genus *Balamuthia*

Like *Naegleria* and *Acanthamoeba*, *Balamuthia mandrillaris* invades the brain and lungs of experimentally infected mice after IN instillation (Visvesvara *et al*. 1993). Several human brain infections, previously reported to be caused by *Acanthamoeba*, have later been attributed to *B. mandrillaris* (Visvesvara *et al.* 1990). While it was first believed *B. mandrillaris* was a leptomyxid amoeba, a recent phylogenetic analysis of the SSU rDNA of the Leptomyxidae, revealed that *B. mandrillaris* does not belong to the Leptomyxidae, but to the Acanthamoebidae (Zettler *et al*. 2000). Until

now, only one species has been recognized in the genus *Balamuthia*, and no group I introns are reported to be present.

ECOLOGY

Naegleria spp. are found in water and soil but they cannot live in seawater. The pathogenic *N. fowleri* prefers water with high temperatures and, therefore, grows abundantly in thermal waters, be it geothermal or industrial cooling water. Because of special circumstances, South Australia is about the only place where *N. fowleri* is present in public water supplies. In these water supplies a positive association between *N. fowleri* was found with temperature, bacteriological counts and other *Naegleria* spp., while a negative association was evident with free and total chlorine residuals (Esterman *et al*. 1984). In a swimming pool that had been identified as the source of an outbreak of 16 PAM cases, *N. fowleri* and other thermphilic *Naegleria* spp. were found to proliferate in a cavity behind a damaged wall of the pool (Kadlec *et al*. 1980).

Although a lot has been published on the presence of *N. fowleri* in thermal waters, very little research has been done on the accompanying *Naegleria* spp. in these waters. The first other thermophilic *Naegleria* spp. to be recognised was *N. lovaniensis*, which seems to be quite prevalent. The presence of *N. lovaniensis* has been taken as a signal that conditions are right for the growth of *N. fowleri*. It is of significance, therefore, that high numbers of *N. lovaniensis* were found in two hospital hydrotherapy pools (De Jonckheere 1982b). In aquaria *Naegleria* is the predominant amoeba genus and a high percentage of these *Naegleria* isolates is thermophilic (De Jonckheere 1979c).

It was only when allozyme and DNA techniques were used that some information on the ecology of other *Naegleria* spp. became available. To my knowledge, there are presently only three laboratories that use allozyme analyses routinely for identification of *Naegleria* spp. isolated over longer periods of time: my Protozoology Laboratory in Brussels, Belgium, Le Laboratoire de Biologie Cellulaire of Pierre Pernin in Lyon, France, and the Protozoology Section of the Australian Water Quality Centre of Bret Robinson in Adelaide, South Australia. In these three laboratories some interesting results concerning the presence of thermophilic *Naegleria* spp. were obtained.

Although good species identification methods are available now for *Naegleria*, it seems that there is still a problem with isolation methods. The recovery rates of *Naegleria* from water is very low by filtration. Although the recovery by centrifugation is much better, it is still only 22% and 57% for trophozoites and cysts, respectively (Pernin *et al*. 1998). I have used the centrifugation method for concentrating water samples since the seventies (De Jonckheere 1978) as it is more efficient, diminishes competition between different species and is less work to quantitate the amoebae.

The Australian Water Quality Centre has a lot of data and a huge collection of strains (Dobson personal communication) but unfortunately, limited data have been published. It was observed in Australia that the upper temperature limit for growth of each described *Naegleria* sp. varies narrowly (<2°C) over many isolates (Robinson *et al*. 1996a). The temperature optimum for each species is approximately 4°C lower than the upper temperature limit. Natural populations of *Naegleria* spp. display temperature-correlated seasonal successions of species occupying different temperature frames. The Australian Water Quality Centre found, for example, that Hope Valley Reservoir in South Australia supports 12 *Naegleria* spp. at densities up to 320 l^{-1} (Robinson *et al*. 1996a). At this place in South Australia, *N. fultoni* (not thermophilic), *N. australienis* and the WA variant of *N. lovaniensis* were found, but never *N. fowleri* nor *N. lovaniensis*, although these two are closely related to the latter one. The recently described species, *N. carteri*, *N. morganensis*, *N. sturti* and *N. niuginensis*, seem to colonise artificial environments, such as water supplies, rather poorly, in contrast to *N. fowleri* and *N. lovaniensis* (Dobson *et al*. 1997). The colonisation of *N. fowleri* in different water supply pipelines in four Australian states occurs as discrete, infrequent events, rather than as continuous contamination, reflecting increased temperature and poor distribution of chlorine (Robinson *et al*. 1996b).

This discontinuous presence of *N. fowleri* is also observed in the cooling water of industries in Belgium (De Jonckheere and van de Voorde 1977a) and in fish farms using heated waters (De Jonckheere *et al*. 1983b). During isolation campaigns over the last two years I found that none of the described thermophilic *Naegleria* spp. apart from *N. fowleri*, *N. lovaniensis*, *N. jamiesoni*, *N. australiensis* and *N. tihangensis*, are encountered in Belgium (De Jonckheere unpublished). When the water temperature drops below 24°C no thermophilic *Naegleria*

are detected. The latter seems to be in contradiction with previous reports in Belgium (De Jonckheere and van de Voorde 1977a, De Jonckheere *et al.* 1983b), however, during the recent campaigns no sediments were sampled. In lakes in Florida pathogenic *Naegleria* were not detected in water samples when the water temperature dropped below 26.5°C, although the organism remained present in sediment samples (Wellings *et al*. 1977). Also, the cooling waters were disinfected with chlorine and, consequently, the *Naegleria* cannot use spots with higher water temperature within the cooling system to proliferate. In previous investigations the temperature of the water where the amoebae proliferated was probably higher than that of the water sampled.

Although the laboratory of Pernin in France has vast information on the occurrence of the other *Naegleria* spp. typed by allozyme, little of it has been published. In France up to 3,000 *N. fowleri* are detected per liter in cooling water and 80 l^{-1} in the river downstream from the electricity power plant (Pringuez *et al*. 1998). In that study it was not indicated how far downstream sampling was conducted. In Poland, pathogenic *N. fowleri* were detected repeatedly up to 13 km downstream from the warm discharge of an electric power station (Kasprzak *et al*. 1982).

There is only minimal information on the occurrence of *Naegleria* spp. other than *N. fowleri* in continents besides Europe and Australia. A study by John and Howard (1995) indicates that *N. australiensis* seems to be prevalent in the USA. Attempts in my laboratory to isolate thermophilic *Naegleria* spp. from African mud samples yielded many strains that did not correspond to species that were described prior to 1990. While no *N. fowleri* could be isolated from the samples, several strains of *N. australiensis* were identified, as well as *W. magna* (De Jonckheere and Bafort 1990). Also, the Australian Water Quality Centre isolated many *Naegleria* strains from all over the world (Dobson personal communication) and some of the newly-described *Naegleria* spp., such as *N. niuginensis*, *N. chilensis* and *N. indonesiensis*, are actually based on unique strains from other continents (Dobson *et al*. 1997). Also, the descriptions of *N. andersoni* and *N. jamiesoni* were based on strains that I had isolated from waters with fish imported from other continents (De Jonckheere 1988a).

Several descriptions of *Naegleria* sp. are based on only one known strain or on strains from only one place, such as *N. jadini*, *N. pussardi*, *N. niuginensis*, *N. galeacystis*, *N. minor*, *N. robinsoni*, *N. chilensis*, *N. indonesiensis*, *N. italica*, *N. morganensis* and *N. fultoni*. The most notoriously restricted occurrence of a *Naegleria* sp. is *N. italica*, which has only been detected in one place in Italy until recently, although the Australian Water Quality Centre, especially, searched for it all over the world (Dobson and Robinson personal communication). These observations on the restricted occurrence of certain *Naegleria* spp. are in conflict with the idea of ubiquitous dispersal of microbial species (Finlay and Clarke 1999). I believe this conflict is caused by the fact that these authors, who support this ubiquitous dispersal of species, use morphotypes as the basis of species, while morphotypes might actually constitute genetically different species. The different morphotypes these authors refer to are far more different in the SSU rDNA sequences than species of *Naegleria*. In this hypothesis most *Naegleria* spp. would be treated as one morphotype and, therefore, it is actually the genus that is ubiquitous, not the different species.

It is sure many more *Naegleria* spp. are awaiting species description and it will be important to keep the type strains in culture collections. As can be noticed in the species descriptions above, not all type strains have been submitted in the past. This is a pity because strains of *Naegleria* can easily be cryopreserved (Simione and Daggett 1976, Kilvington and White 1991, Brown and Day 1993, Menrath *et al*. 1995). The Australian Water Quality Centre keeps a large collection of their isolates cryopreserved and these collections are a goldmine for people like me who investigate the biodiversity of the genus *Naegleria*.

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High Light Exposure Leads to a Sign Change of Gravitaxis in the Flagellate *Euglena gracilis**

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Summary. The unicellular flagellate *Euglena gracilis* orients itself in the water column by means of pronounced phototaxis and gravitaxis. The antagonism of phototaxis and gravitaxis brings the cells in a position in the water column providing them with optimal light conditions for their photosynthetic apparatus (about 30 Wm^{-2}). Long exposure to solar or artificial radiation induces a loss of the negative gravitactic orientation in *Euglena gracilis* or very often a pronounced, persistent (> 4 h) sign change in gravitaxis. The effects on gravitaxis are exclusively due to UV and intensive blue light as experiments with different light qualities revealed. This phenomenon is not caused by the phototaxis photoreceptor or chloroplast processes, because also the colorless and blind (no photoreceptor) *Euglena gracilis* 1f mutant and *Astasia longa* reverse the sign of gravitaxis upon strong radiation. The sign change is oxygen-dependent, because gravitaxis is not affected in oxygen free medium (in wild type *Euglena gracilis*, as well as in the 1f mutant and *Astasia longa*). This indicates the involvement of oxygen radicals as a trigger of gravitaxis sign reversal. As the destruction of the photoreceptor molecules by light leads to a loss of phototaxis, the switch from negative to positive gravitaxis might be an adaptation mechanism of the cells to escape from deleterious radiation even after loss of the ability to perceive the light direction.

Key words: *Astasia longa*, *Euglena gracilis*, gravitaxis, oxygen, phototaxis, sign change, solar radiation, UV.

INTRODUCTION

The unicellular flagellate *Euglena gracilis* uses external stimuli to control its position in the water column. Negative gravitaxis is a very important mechanism, which enables the cells to orient themselves towards the water surface. In addition, the cells show positive phototaxis at low irradiances (1-10 Wm^{-2}) and increasingly pronounced negative phototaxis at higher irradiances. The interaction of phototaxis and gravitaxis allows the cells to find an optimal position in the water column for growth and reproduction (about 30 Wm^{-2}) (Häder and Griebenow 1988). Phototaxis as well as gravitaxis are most likely active physiological mechanisms. The photoreceptor of *Euglena gracilis* is the paraxonemal body, which is attached to the trailing flagellum. The photoreceptor was found to have a paracrystalline structure and to consist of chromoproteins containing pterins and flavins as chromophoric groups (Sineshchekov *et al.* 1994, Brodhun and Häder

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* Dedicated to Dr. Francesco Lenci on the occasion of his 60th birthday

1995). While flavins are identified as the primary photoreceptors of phototaxis, the pterins seem to act as bulk antenna pigments funneling the adsorbed energy to the flavins. The knowledge on the signal transduction chain from the photoreceptor to the flagellum is still limited. Recently, it was found that the flavoproteins have an adenylate cyclase activity (Iseki *et al.* 2002). The current status of research in phototaxis in *Euglena gracilis* is thoroughly described in a recent review article (Lebert 2001).

The mechanism of gravitaxis in *Euglena gracilis* is not fully elucidated. Formerly, gravitaxis was thought to be a physical phenomenon based on a buoyancy effect due to an unequal mass distribution within the cell body (Brinkmann 1968). But the results of many experiments and movement analyses make a physiological mechanism of gravitaxis very likely (Machemer and Bräucker 1996; Lebert and Häder 1996, 1999a; Häder 1997; Häder *et al.* 1997; Lebert *et al*. 1997, 1999; Porst 1998; Tahedl *et al.* 1998; Kamphius 1999; Richter *et al*. 2001a, b).

According to a recent working model of gravitaxis in *Euglena gracilis* the sedimenting cell body (denser than the surrounding medium) exerts a force on the lower membrane and most likely calcium channels. The resulting change of calcium conductance leads to a change of the membrane potential which in turn triggers reorientational movements of the trailing flagellum. Phototaxis as well as gravitaxis were found to be sensitive to UV radiation (Häder and Liu 1990a, Brodhun and Häder 1995). Gravitaxis was shown to be abolished after about 2 h of exposure to solar radiation (Häder and Liu 1990a). Reanalysis of the experimental data indicate that some of the cells in the irradiated culture show an active positive gravitaxis. Subsequent experiments showed that *Euglena gracilis* often shows a sign change in gravitaxis after exposure to solar radiation. The aim of the current paper is to elucidate these effects in detail.

MATERIALS AND METHODS

Organisms and growth conditions

The flagellate *Euglena gracilis* Z was obtained from the algal culture collection of the University of Göttingen (Schlösser 1994). The cells were grown in a mineral medium as described earlier (Starr 1964, Checcucci *et al.* 1976) in stationary cultures in 100 ml Erlenmeyer flasks at about 20°C under continuous light of about 18 Wm^{-2} from mixed cool white and warm tone fluorescent lamps. The colorless mutant *Euglena gracilis* 1f and *Astasia longa* (obtained from the same source as *Euglena gracilis* Z) were grown in complex medium (Starr 1964) in 100 ml Erlenmeyer flasks at about 20°C in the dark.

Exposure of cells to natural solar radiation

Samples used for one experiment were prepared from the same culture. The cells were transferred into custom-made cuvettes, designed for the Erlanger flagellate test (EFT, Häder *et al.* 1997). Each cuvette consists of a holder with four independent compartments. Each compartment is covered with a different filter: WG 280 (transmits all solar radiation), WG 320 (transmits PAR and UV-A), GG 400 (PAR only) and UG 11 (UV-A and UV-B only). All filters are from Schott and Gen., Mainz, Germany. Water was pumped through the stainless steel body of the cuvette holder in order to warrant a stable temperature of 20°C (thermostatically controlled water bath). Three of these cuvettes were used in parallel for one experiment. Samples were taken at defined time intervals from the cuvettes after gentle mixing with a suction pipette. The samples were transferred into slide cuvettes sealed with silicon (see below), and gravitaxis was measured in a dark room (see below). In addition, the recovery of the cells from illumination was determined in darkness. The irradiances during solar exposure were determined with an ELDONET instrument in the UV-B, UV-A and PAR ranges located on the roof of the Department of Biology at the University of Erlangen (Häder *et al.* 1999).

Exposure to artificial radiation

The light source was a Hönle lamp (Dr. Hönle, Martinsried, Germany), which produces a spectrum similar to the solar spectrum (Klisch *et al.* 2001). The irradiances were: PAR 321 Wm^{-2}, UV-A 67 Wm^{-2} and UV-B 1.9 Wm^{-2} at a distance of 65 cm. About 30 ml of cell suspension were transferred into small black plastic boxes, which were placed in a temperature-controlled water bath (20°C). To determine the effects of different light qualities, different samples were covered with different cut-off filters. The following cut-off filters were used for the experiments: filter foil 295 nm (transmits UV-B, UV-A and PAR, Digefra, Munich, Germany), 320 nm (Montagefolie, Nr. 10155099, Folex, Dreieich, Germany), 395 nm foil (PAR only, Digefra) and the glass cut-off filters (Schott and Gen., Mainz, Germany) UG 11 (UV-B and UV-A only), GG 280 (UV and PAR), GG 400 (PAR only), GG 440, OG 540 and RG 645. Dark controls were covered with aluminum foil. Samples were drawn at predefined time intervals after gentle mixing of the cell suspension. The cells were filled into a cuvette and subsequently analyzed with the image analysis software WinTrack 2000 (see below).

Oxygen was removed by addition of NaS_2O_3 (final concentration 3 µM). Experiments concerning oxygen were performed with the following samples: (1) cells + NaS_2O_3 + light, (2) cells + light (no NaS_2O_3), (3) cells + NaS_2O_3 (no light, covered with aluminum); (4) dark control (no NaS_2O_3 and no light).

Motion analysis

Samples of cells were transferred into a custom-made cuvette (0.1 mm depth and 20 mm diameter) made from stainless steel with glass windows (Daimler-Benz Aerospace, Bremen, Germany). In the case of the solar exposure experiments (see above) some drops of

Euglena cell suspension were sealed between two slides by means of silicon (Bayer Silone, high viscous, Bayer, Leverkusen, Germany). Motion analysis was performed with a recently developed cell tracking system (WinTrack 2000, Lebert and Häder 1999b). The system is based on a video A/D flash converter (Meteor, Matrox, Canada) connected to a PCI slot of an IBM compatible computer which digitizes the analog video images from a CCD camera mounted on a horizontally oriented microscope.

The digitized images are transferred to the computer memory. Objects are detected by brightness differences between cells and background. The movement vectors of all motile cells on the screen are determined by subsequent analysis of five consecutive video frames (movement vectors of the objects from frame 1 to frame 5). In addition to orientation and velocity of the cells, motility, area and cell form are determined as well as several statistical parameters. The *r*-value indicates the precision of (gravitactic) orientation and ranges from 0 (random orientation) to 1 (precise orientation):

$$r = \frac{\sqrt{(\sum \sin\alpha)^2 + (\sum \cos\alpha)^2}}{n}$$

where α is the deviation from the stimulus direction (here acceleration) and n the number of recorded cell tracks. The angle theta indicates the mean movement direction of a cell culture. The increment of the angle is clockwise (see one of the circular histograms in the figures).

Image analysis was performed in darkness to avoid any phototactic or photophobic effect on the orientation of the cells. To exclude the evaluation of immotile cells, which sediment in the vertical cuvette, the software accepted only cells with a speed faster than the sedimentation velocity (about 20 μms^{-1}). In all experiments the movement of the cells was visually monitored by the experimenters on screen in order to avoid any mistakes of data acquisition of the obtained cell tracks by the software.

RESULTS

Effects of artificial radiation on gravitaxis

Green *Euglena gracilis* Z cells generally showed a pronounced sign change (negative to positive) of gravitaxis within 70-195 min of artificial solar radiation (Figs 1, 2). Positive gravitaxis persists for more than 150 min under dim light conditions but after 12 h the cells showed negative gravitaxis again (data not shown). In the colorless *Astasia longa* and *Euglena gracilis* 1f mutant the kinetics of gravitactic sign change was even faster. Most of the samples showed a pronounced positive gravitaxis after 30-45 min of radiation. But not all cells showed a fast reversal of gravitaxis. In some experiments (*Euglena gracilis* Z, mutant 1f, *Astasia longa*) a pronounced sign change in gravitaxis was obtained only after about 240 min of radiation or even longer (data not shown).

Determination of the effectiveness of light quality on gravitaxis

The light regime was the same as in the previous experiment. The samples were covered with different filters as described above. The calculated dose for each filter is shown in Table 1. After about 90 min most of the cells exposed to UV only (UG 11 filter) showed a pronounced positive gravitaxis. After 195 min also the 280 nm samples reversed their gravitactic orientation, while in the 400 and 440 nm samples a loss of orientation of the cell movement was visible (Fig. 3). During recovery in dim light the precision of positive gravitaxis in the samples, which performed a gravitactic sign change decreased within 4 h. After 12 h all samples showed negative gravitaxis again (data not shown).

Determination of the role of oxygen on the gravitactic sign change

The experiments were solely performed with colorless cells (*Astasia longa* and *Euglena gracilis* 1f), to avoid any influence of photosynthetically produced oxygen. In oxygen-free samples (addition of 3 μM Na_2SO_3) a sign change was never detected. The cells showed a pronounced negative gravitaxis during the course of the experiment, while the untreated light controls changed from negative to positive gravitaxis very fast. The gravitaxis of the dark controls (with and without Na_2SO_3) was not affected. Fig. 4 shows a representative result of an experiment with *Euglena gracilis* 1f. The swimming velocity of the cells in oxygen free medium was decreased. The responses of *Astasia longa* and *Euglena gracilis* 1f were very similar. At least four independent measurements were performed for each experiment.

Effects of solar radiation on gravitaxis

The cells which were exposed to solar radiation showed different responses to the radiation depending on the filter (Fig. 5). The perceived dose during the course of the experiment is shown in Table 2. The sign change in the samples exposed to solar radiation in the presented experiment was not as pronounced as in the experiments with artificial radiation. The tendency of positive gravitaxis was seen from the circular histograms (not shown). Although the percentage of upward swimming cells considerably decreased in all samples, a pronounced sign change in gravitaxis was only visible in samples, which were exposed to UV-A and PAR (320 nm cut-off filters). The samples covered with the 395 nm cut-off filters did not show a pronounced sign

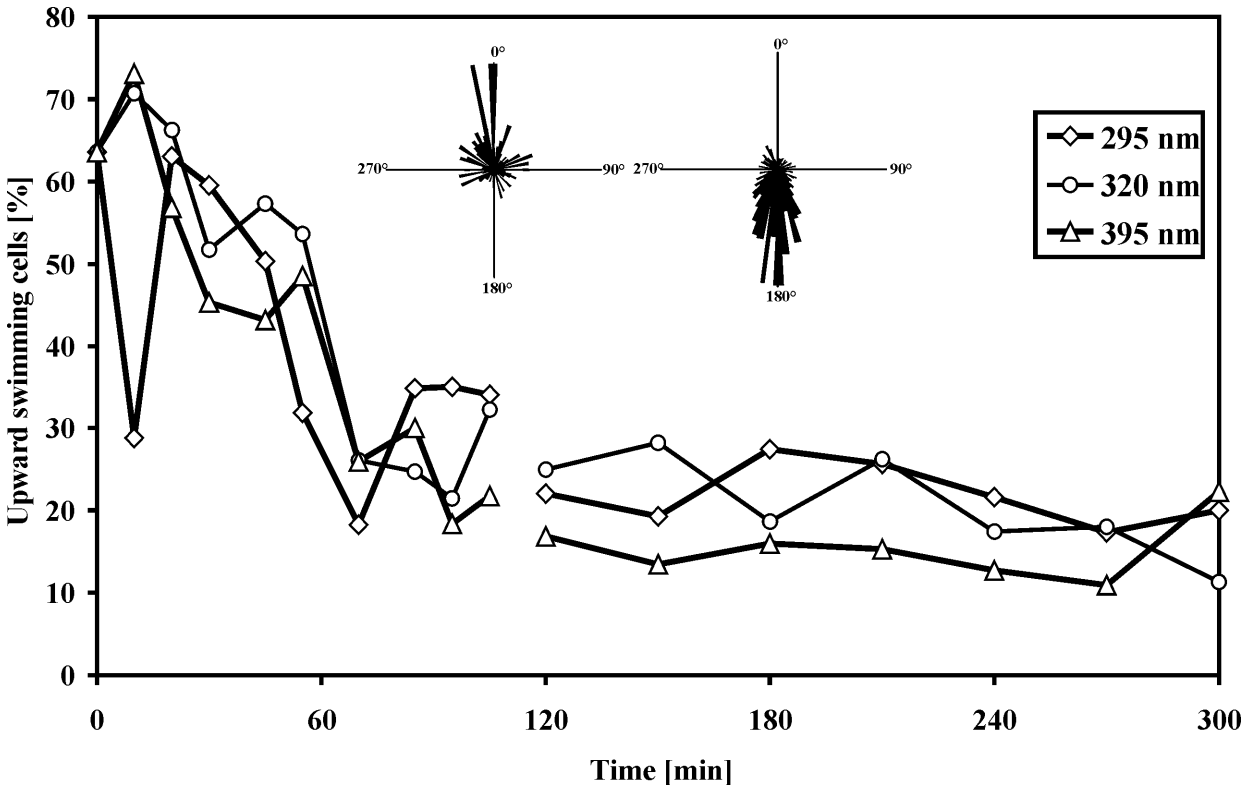

Fig. 1. Effect of artificial simulated solar radiation on gravitactic orientation of *Euglena gracilis*. The diagram shows the percentage of upward swimming cells (120° cone around the vertical) after radiation. The samples were covered with different cut-off filters (295 nm, 320 nm and 395 nm). The decrease in upward swimming cells in combination with the corresponding circular histograms reveal the sign change to positive gravitaxis. Theta indicates the mean movement direction of the cell culture and is indicated by the lines in the histograms

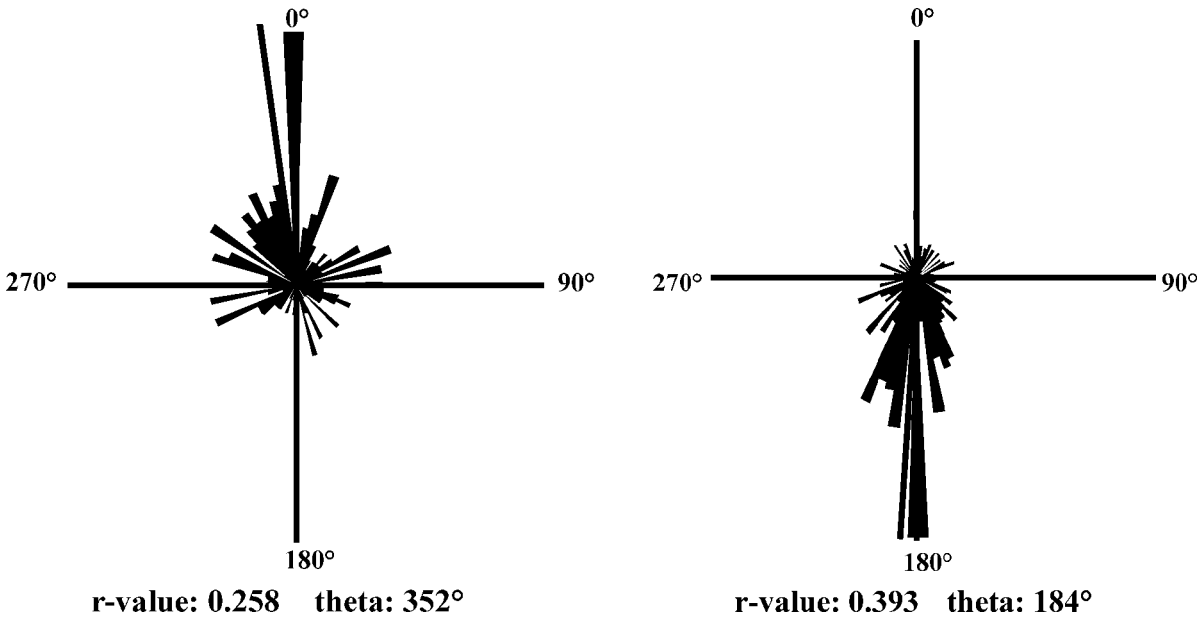

Fig. 2. Typical example of light-induced sign change of *Euglena gracilis* gravitaxis after irradiation shown as circular histograms. Here the effect of 90 min UV-A and PAR irradiation is shown. The length of each single sector indicates the amount of cells swimming in this direction (details see text). The r-value indicates the precision of orientation; theta indicates the mean movement direction of the population (for details see text), and is indicated by the lines in the histograms

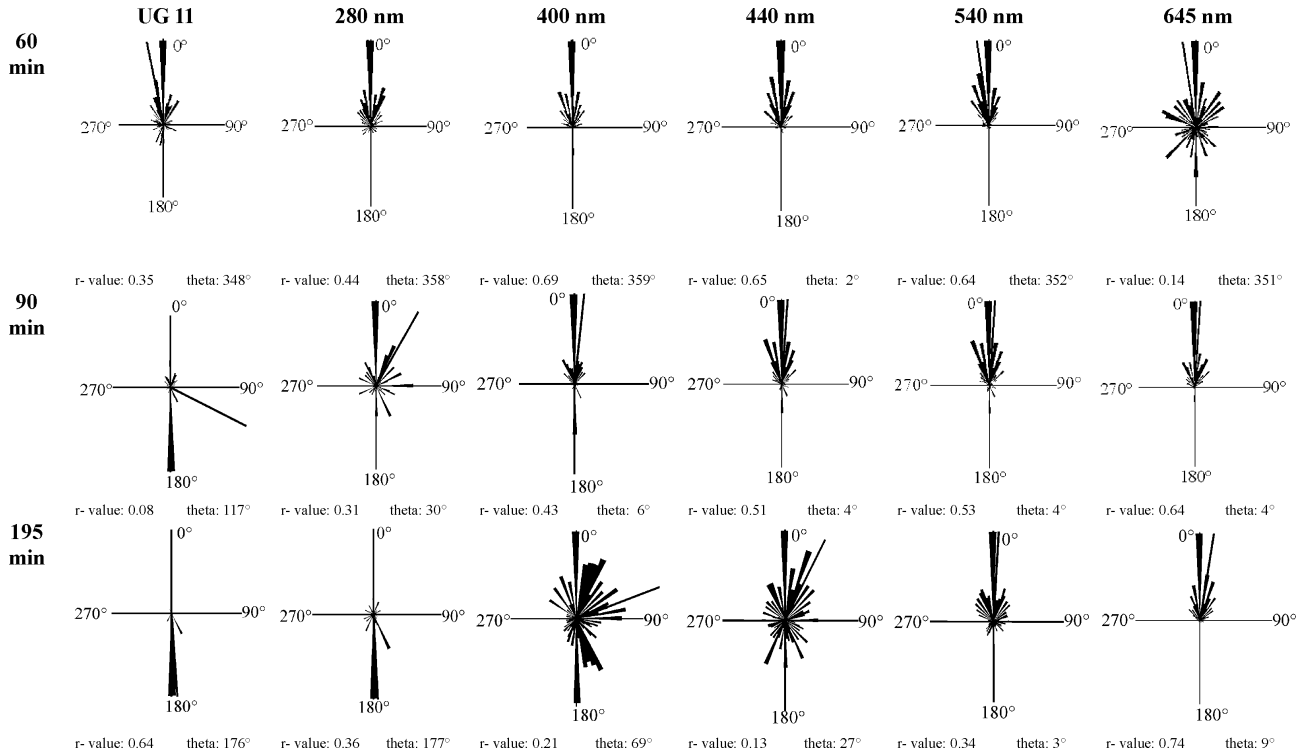

Fig. 3. Effect of light quality on graviorientation in *Euglena gracilis*. A pronounced sign change in gravitaxis (negative to positive) occurred in the samples which were exposed to UV only or to UV plus PAR. The movement behavior is represented as circular histograms. The length of each individual sector indicates the percentage of cells swimming in this direction. The r-value indicates the precision of orientation; (for details see text), theta indicates the mean movement direction of the cell culture and is indicated by the lines in the histograms

Table 1. Calculated radiation doses [J m^{-2}] of artificial radiation under the various filters used in the experiment presented in Fig. 3

Time	UG11	WG 280	WG 400	WG 440	OG 540	RG 645
45 min	74774	879956	670216	511007	319151	77197
90 min	149547	1759913	1340431	1022015	638302	154395
195 min	324019	3813144	2904268	2214366	1382988	334522

Table 2. Calculated radiation doses [J m^{-2}] of solar radiation under the various filters used in the experiments presented in Figs 5 and 6

Time	30 min	120 min	240 min	360 min
Dose PAR [J m^{-2}]	759534	1575479	3206058	4483337
Dose UV-A [J m^{-2}]	173651	362318	735939	1014727
Dose UV-B [J m^{-2}]	5630	12007	24560	32508

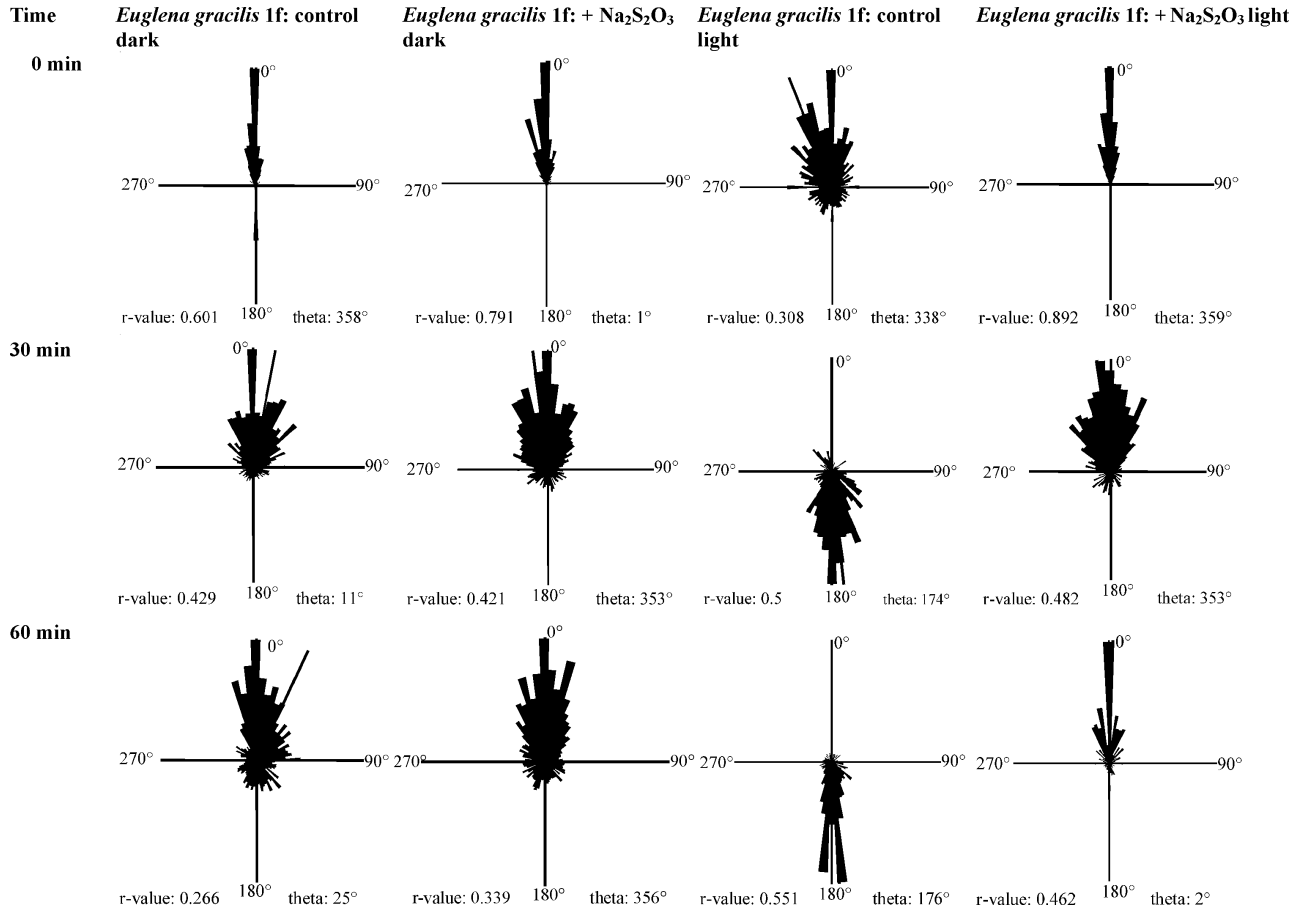

Fig. 4. Influence of oxygen on gravitactic sign change in *Euglena gracilis*. In the absence of oxygen (addition of 3 µM Na_2SO_3) no gravitactic sign change was detected (fourth column), while the control cells (oxygen + light) showed a pronounced reversal of gravitaxis. The dark controls (first and second columns) were not considerably affected. The r-value indicates the precision of orientation; (for details see text), theta indicates the mean movement direction of the cell culture and is indicated by the lines in the histograms

change. The data indicate a loss of gravitactic orientation in the course of radiation. But there is a tendency of positive gravitaxis after longer solar exposure in some of the samples (360 min). The samples which were exposed to UV-B, UV-A and PAR (280 nm cut-off filter) showed positive gravitaxis after 120 min, which disappeared in the subsequent measurements. Cells which perceived UV only (UG 11 filter) lost their orientation after long exposure (240 min) but did not show any indication of a sign change in gravitaxis. In contrast to the other samples, in which the cells were found to accumulate at the bottom of the cuvettes the cells of the UG11 samples were equally distributed in their medium. After 12 h in the darkness all samples had completely recovered (data not shown). The time course of sign change is also culture dependent, because in other experiments a much faster sign change of the cells was detected (data not shown).

DISCUSSION

The results of the experiments show that gravitaxis is influenced by light. UV and blue light lead to a loss of gravitactic orientation and very often also to a pronounced sign change in gravitaxis (from negative to

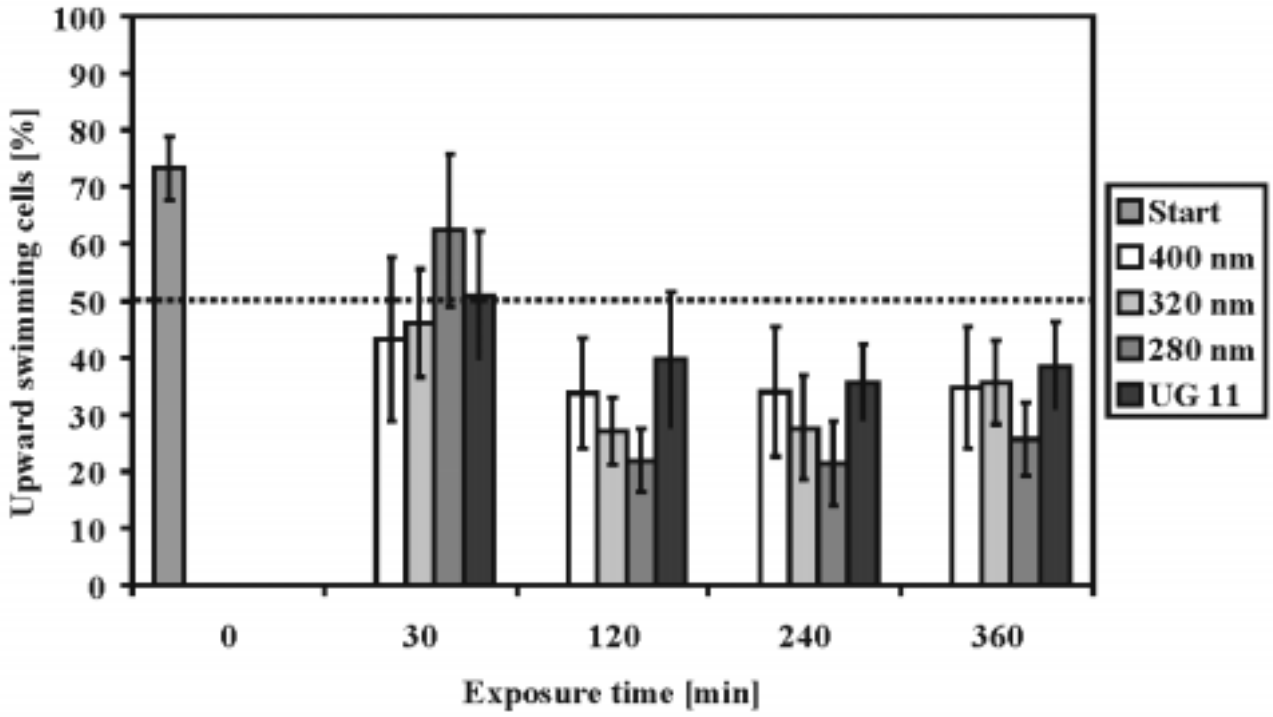

Fig. 5. Effect of solar radiation on the graviorientation of *Euglena gracilis*. The diagram shows the percentage of upward swimming cells (120° cone around the vertical). The samples were covered with different filters (cut-off filters WG 280, WG 320 and WG 400 nm; UG 11 exclusively transmits UV-B and UV-A). Although the percentage of upward swimming cells declined in all samples, only the 320-nm samples switched from negative to a pronounced positive gravitaxis (details see text)

positive), which persists for several hours in darkness or dim light, respectively. The time scale of this phenomenon is very variable, depending on the culture used for the experiments. In some experiments the cells totally reversed gravitaxis within 30 min, other samples needed longer irradiation times to perform a gravitactic sign change (up to 4 h). For this reason it was not possible to determine a dose response curve of gravitactic sign change. A role of the photoreceptor for phototaxis, the paraxonemal body, can be excluded, because also colorless mutants as well as *Astasia longa*, both of which lack a paraxonemal body showed a pronounced sign change. Also photoinduced reactions inside the chloroplasts can be ruled out, because these cells are chloroplast-free. But it is very obvious, that the phenomenon is based on light-induced reactions with oxygen. In the absence of oxygen (performed by addition of NaS_2O_3) no sign change was detected even after long exposure to light. Most likely oxygen radicals or reactive oxygen species trigger the gravitactic sign change. The oxygen receptor in the ciliate *Loxodes* is likely the cytochrome-c-oxidase (see below). The underlying mechanism in *Euglena* is currently intensively investigated by means of metabolic inhibitors.

In addition, it can be stated that only UV and short-wavelength visible light (around 400 nm) can induce a gravitactic sign change. *Euglena* cells are very sensitive to solar UV radiation, and impacts on the photosynthetic apparatus and the photoreceptor pigments of *Euglena gracilis* have been demonstrated (Häder and Brodhun 1991, Brodhun and Häder 1995). Tirlapur *et al.* (1992) revealed an enormous increase in the intracellular calcium concentration, as well as a pronounced decrease in the calmodulin concentration and effects on the nucleus in *Euglena gracilis* upon UV-B radiation. So it is advantageous for the cells to switch to positive gravitaxis after exposure to high solar radiation. An impact on the motility and gravitactic orientation in *Euglena gracilis* was already detected in earlier studies. Gerber *et al.* (1996) measured a polychromatic action spectrum of inhibition in motility of *Euglena*. The experiments revealed a strong effect of UV-B and UV-A, but also the blue light range was effective. This is in good agreement with the results obtained in the present study. The effect

of artificial and solar radiation on gravitaxis has been demonstrated by Häder and Liu (1990a). The strong impact on gravitaxis was interpreted as a possible effect on an active gravireceptor or flagellar protein. The active sign change of gravitaxis after long UV exposure was already mentioned by the authors. It is possible that the reversal of the cells was not completed, because of the limited duration of the experiments (120 - 200 min). The circular histograms as well as the kinetic diagrams in this paper indicate a beginning gravitactic reversal. It is obvious that the positional regulation of *Euglena gracilis* due to the proposed interplay between phototaxis and negative gravitaxis (see introduction) is not the only control mechanism. In the case that the photoreceptors are destroyed by UV the cells are not longer able to regulate their position by means of gravi- and photoreceptors. If the cells were blinded by UV, as demonstrated by Häder and Liu (1990a) with a resulting loss in phototaxis, the negative gravitaxis would guide them to the water surface where they would encounter deleterious radiation. The described phenomenon might be an adaptation mechanism in the case that the cells cannot avoid a high radiation regime (e.g. shallow habitat, as simulated in the described experiments). Positive gravitaxis in *Euglena gracilis* can be found under certain circumstances. In fresh cultures *Euglena* cells very often show positive gravitaxis during the first days of cultivation. Also by manipulation of the properties of the medium gravitaxis can be switched to positive (Richter 2000). Increased salinity (10 g/l NaCl) often leads to positive gravitaxis. Even after transfer of the cells to the standard medium the positive gravitaxis persists for many days (unpublished observations). After recovery from the columella stage (permanent cysts), which can be induced by experimental increase of the osmolarity of the medium (Höfler and Höfler 1952), the cells show a pronounced positive gravitaxis (unpublished results). These observations make it very likely that *Euglena* is able, in analogy to phototaxis, to switch actively between negative and positive gravitaxis. At least under laboratory conditions negative gravitaxis is the favored of the two. Also other gravitactic microorganisms are described to show a sign change in gravitaxis upon radiation. *Prorocentrum micans*, a marine dinoflagellate, normally shows a pronounced negative gravitaxis, which switches to a positive one upon short exposure to solar or artificial UV radiation (Eggersdorfer and Häder 1991, Sebastian *et al.* 1994). Also the dinoflagellate *Gymnodinium* (Y-100), which has been shown to be sensitive to solar UV-radiation reversed actively from negative gravitaxis to positive gravitaxis (Tirlapur *et al.* 1993). The dinoflagellate *Peridinium gatunense,* in contrast, seems to sediment upon excessive exposure to solar radiation in order to avoid cellular light-induced damages (Häder and Liu 1990b). The negative gravitaxis of *Dunaliella bardawil* is not impaired by light; in this case avoidance is only due to negative phototaxis (Jimenez *et al.* 1996). The ciliate *Paramecium* was shown to change between positive gravitaxis upon illumination and negative gravitaxis in the darkness (Fox 1925). Also temperature was described to influence gravitaxis in *Paramecium*. A strong oxygen dependency of gravitaxis was reported in *Paramecium* and *Loxodes*. It was found that low oxygen concentrations of the medium induce a pronounced negative gravitaxis in *Paramecium*, while higher oxygen pressure (>1.2 mg/l) lead to a deterioration of gravitaxis (Hemmersbach-Krause *et al.* 1991). *Loxodes,* a unicellular ciliate normally shows a pronounced positive gravitaxis in the presence of oxygen, while under conditions of anoxia the cells were described to show negative gravitaxis (Fenchel and Finlay 1986). As *Loxodes* only shows a low activity of catalase and superoxide dismutase the cell are very sensitive to high oxygen pressure (Finlay *et al.* 1986). In *Loxodes* probably cytochrome-c-oxidase is the oxygen receptor, because incubation with KCN led to a loss of gravitactic orientation (Finlay *et al.* 1986). In *Anabaena variabilis* singlet oxygen generated in high light was found to be responsible for phototactic reaction by means of an unknown signal processor (Nultsch and Schuchart 1985).

The results obtained in the present study also strongly indicate, that gravitaxis is an active physiological mechanism and not a passive physical effect based, e.g., on buoyancy (Brinkmann 1968, Kessler 1992). Some experiments indicate that the mechanism of gravitactic orientation itself is probably sensitive to excessive UV-B radiation, because also positive gravitaxis disappeared after long exposure to UV radiation. This is another proof for active graviperception in *Euglena gracilis*.

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PROTOZOOLOGICA

The Frequency of Eimeriidae Species in the Domestic Geese in Kars Province of Turkey

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Summary. This study was designed to determine the prevalence of coccidia species in the domestic geese (*Anser anser domesticus*) in Kars province, Turkey. The study involved 400 randomly selected geese of different age and localities. The faecal samples were collected between May and October, 2001 from the cloaca of individual animals. The samples were examined by using saturated salty water flotation technique. OPG (oocysts per gram of faeces) counts in faecal samples containing oocysts were calculated by modified McMaster technique. The sporulation of coccidia oocysts was done in potassium dichromate solution at 27°C. Coccidian oocysts were detected in 80.8% of the samples and seven different species of Eimeriidae were identified. The species identified in this study were *Tyzzeria parvula* (81.7%), *Eimeria anseris* (11.2%), *E. fulva* (16.7%), *E. hermani* (13.3%), *E. nocens* (2.8%), *E. stigmosa* (31.6%), and *E. truncata* (37.8%). The OPG counts in infected geese ranged from 100 to 144 000. The rate of mixed infections was 64.4% and that of pure infection was 35.6%. The rate of mixed infections caused by two different coccidia species was 43%. During this study, necropsies were performed on 32 goslings that died of clinical diarrhea. Endogenous developing forms (meronts, gamonts) of coccidia species were seen in the intestine of 21 (65.6%) geese. Of these, 5 (5/21) had characteristic lesions of intestinal coccidiosis. Examination of kidneys revealed endogenous developing forms of *E. truncata* in the tubulus of 4 goslings (4/21).

Key words: Eimeriidae, geese, Kars, Turkey.

Abbreviations: OPG - oocysts per gram of faeces.

INTRODUCTION

Coccidiosis is an important protozoan disease of domestic animals. The disease is manifested by severe symptoms such as diarrhea, dehydration, and deaths in young animals (Mimioğlu *et al*. 1969, Levine 1985).

Although coccidiosis has frequently been reported in the domestic geese, information on its clinical characteristics and economic importance is limited (Soulsby 1986). Two types of coccidiosis, intestinal and renal coccidiosis, are described in geese. Renal coccidiosis caused by *Eimeria truncata* (Railliet and Lucet, 1891) Wasielewski, 1904 is widespread throughout the world. It is commonly reported in goslings between 3 weeks to 3 months old and causes high morbidity and mortality (Mimioğlu *et al*. 1969, Gajadhar *et al*. 1983a), which reaches up to 100% in goslings in acute episodes (Tolgay 1973, Soulsby 1986).

Eimeria anseris Kotlán, 1933, *E. kotlani* Gräfner and Graubmann, 1964, *E. nocens* Kotlán, 1933, *E. stigmosa* Klimeš, 1963 and *Tyzzeria parvula* Klimeš, 1963 are the most common species involved in intestinal

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coccidiosis of geese. Additionally, experimental infections have been induced by coccidia species of wild geese such as *E. fulva* Farr, 1953, *E. hermani* Farr, 1953, *E. magnalabia* Levine, 1951 and *E. striata* Farr, 1953 (Pellérdy 1974, Gajadhar *et al*. 1983a, Levine 1985).

Merdivenci (1983) reported *Eimeria anseris* and *E. truncata* in the domestic geese in Turkey, but there is no detailed study addressing to the infection rate and epidemiology of coccidiosis. Six percent (107000/1795000) of the total geese population of Turkey is raised in the Kars province (Anonymous 1999). Geese rearing is most often performed as a small family enterprise and constitutes a very important portion of meat production, which plays a role in the economy of the province.

This study was therefore carried out to determine the frequency of Eimeriidae species in the domestic geese in Kars, Turkey.

MATERIALS AND METHODS

The study animals were randomly (simple lottery) selected from clinically healthy geese (*Anser anser domesticus*) from different localities in Kars, Turkey. Visits were made between May and October, 2001 to collect samples from the geese of different age. In total, 400 faecal samples were taken from the cloaca of geese and goslings individually.

Faecal samples were examined for the presence of coccidia oocysts by centrifugal flotation technique using saturated salty water solution. A modified McMaster technique was used to determine OPG (oocysts per gram of faeces) counts in samples positive for coccidia oocysts. The coccidia oocysts were sporulated in 2.5% (w/v) potassium dichromate solution in Petri dishes at 27°C (Mimioğlu *et al*. 1969, MAFF 1986). The identification of coccidia species was made as previously described (Pellérdy 1974, Gajadhar *et al*. 1983a, Hiepe 1983, Levine 1985, Chauve 1988, Rommel *et al*. 1992).

Necropsies were carried out on goslings that died of severe diarrhea of 32 geese. Intestines and kidneys were preserved in 10% formalin solution. Sections were taken from these tissues in 5 µm thickness and stained with hematoxylin and eosin.

Statistical analysis of the data was performed using Epi - info 6.0. A Yates' corrected chi-squared test was used to compare the differences between proportions (Dean *et al*. 1994). A probability of $P<0.05$ was accepted as statistically significant.

RESULTS

The prevalence of Eimeriidae species was found to be 80.8% (323/400) in the domestic geese raised in Kars. The infection rate with coccidia species in different localities and age groups are given in Table 1. There was no statistically significant difference in the total infection rate in different age groups ($P = 0.18$) and localities ($P = 0.4$).

Seven Eimeriidae species were identified in this study. The most prevalent species was *Tyzzeria parvula* (81.7%, 264/323) (Table 2). The distribution of Eimeriidae species in different age groups is shown in Table 2.

OPG counts were determined and results were given in Table 3. OPG numbers were less than 5001 in 65.6% (212/323) of the geese. The comparison between age groups according to OPG counts revealed that number of animals with <1000 OPG was significantly higher in the group aged between 5-6 month than the others ($P<0.001$), while the proportion of animals with 10001-25000 OPG was significantly higher in the 1-2 months group ($P=0.028$). No significant difference was noted in other groups and OPG counts.

Mixed infections were 64.4% (208/323) and infection with single coccidia species was 35.6% (115/323) in this study. The rate of mixed infections caused by two different coccidia species was 43%. The maximum number of Eimeriidae species present in a sample was 7. However, the maximum numbers of oocyst species found was 1 or 2 (Table 4).

Coccidia oocysts identified in this study are shown in Figs 1-7.

The endogenous developing forms (meronts, gamonts) of coccidia species were found in the intestines of 21 of 32 necropsied goslings. Of these, 5 (5/21) had oedematose and hyperemic intestinal mucosae, and the intestinal content was watery and brownish red. Microscopically, hyperemic vessels, epithelial desquamates, and endogenous forms of coccidia in lamina propria (Fig. 8) and epithelial cells of the intestinal villi were seen. Macroscopic lesions were not observed in the kidneys. Histological examination of the kidneys revealed tubular nephrosis and tubular necrosis. Endogenous developing forms of *E. truncata* were also encountered in the epithelium of the renal tubules of 4 (4/21) goslings (Fig. 9). Furthermore, the tubules were dilated up to 3-4 times of the normal size.

DISCUSSION

No detailed study was previously carried out on coccidiosis in the domestic geese in Turkey. In a previous study conducted by Merdivenci (1983) in Turkey the

Table 1. The infection rate (% in brackets) with coccidia species in the domestic geese according to localities and age groups

Locality	n	x	1-2 months n	1-2 months x	3-4 months n	3-4 months x	5-6 months n	5-6 months x
Paşaçayırı	52	38 (73.1)	5	5 (100)	-	-	47	33 (70.2)
Akçakale	35	29 (82.9)	35	29 (82.9)	-	-	-	-
Aynalı	55	45 (81.8)	42	34 (81.0)	13	11 (84.6)	-	-
Doğruyol	32	28 (87.5)	21	19 (90.5)	11	9 (81.8)	-	-
Merkez	30	24 (80.0)	23	18 (78.3)	-	-	7	6 (85.7)
Akçalar	60	51 (85.0)	25	21 (84.0)	35	30 (85.7)	-	-
Arpaçay	60	52 (86.7)	-	-	60	52 (86.7)	-	-
Çalabaş	40	31 (77.5)	-	-	40	31 (77.5)	-	-
Borluk	36	25 (69.4)	36	25 (69.4)	-	-	-	-
Total	400	323 (80.8)	187	151 (80.8)	159	133 (83.7)	54	39 (72.2)

n-number of samples examined; x - number and percentage of samples diagnosed positive

Table 2. The distribution (% in brackets) of Eimeriidae species according to age groups

Age	x	*Tyz. parvula*	*E. anseris*	*E. fulva*	*E. hermani*	*E. nocens*	*E. stigmosa*	*E. truncata*
1-2 months	151	116 (76.8)	9 (6.0)	24 (15.9)	25 (16.6)	4 (2.7)	36 (23.8)	46 (30.5)
3-4 months	133	114 (85.7)	17 (12.8)	22 (16.5)	4 (3.0)	2 (1.5)	40 (30.1)	70 (52.6)
5-6 months	39	34 (87.2)	10 (25.6)	8 (20.5)	14 (35.9)	3 (7.7)	26 (66.7)	6 (15.4)
Total	323	264 (81.7)	36 (11.2)	54 (16.7)	43 (13.3)	9 (2.8)	102 (31.6)	122 (37.8)

E. - Eimeria; *Tyz - Tyzzeria*; x - number of infected animals

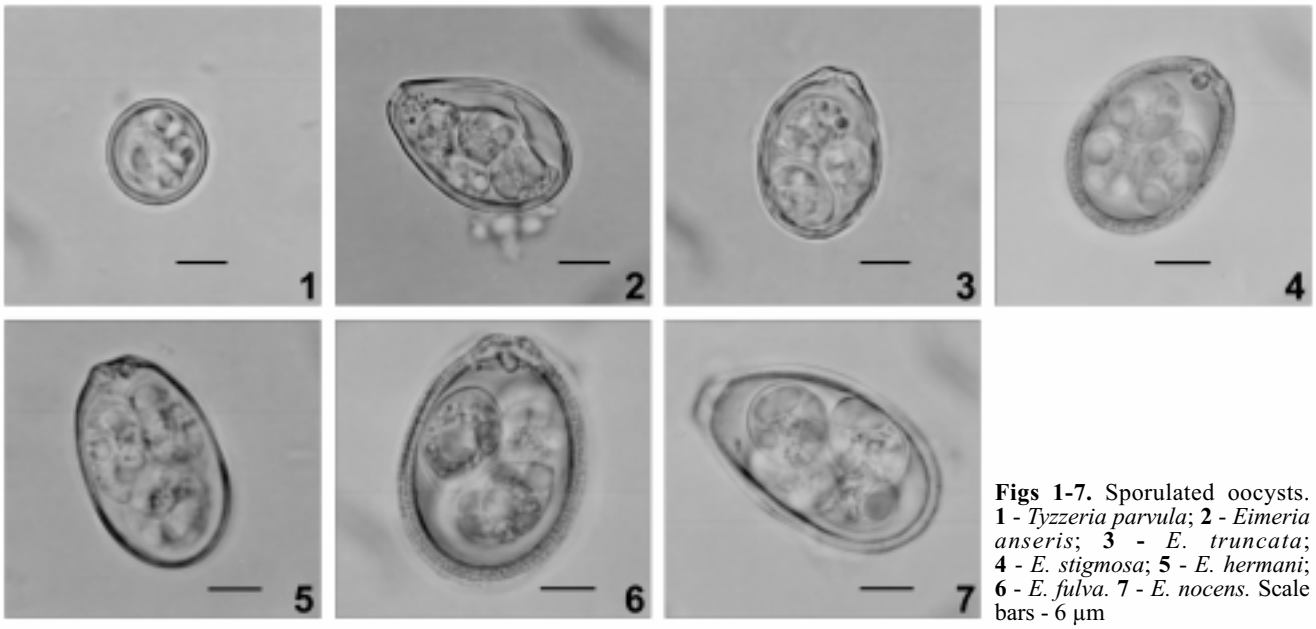
Table 3. The dispersion of the number (% in brackets) of infected animals according to OPG counts and age groups

Age	OPG < 1000	1000-5000	5001-10000	10001-25000	25001-50000	> 50000	Total
1-2 months	31 (20.5)	58 (38.4)	18 (11.9)	27 (17.9)$^{+}$	10 (6.6)	7 (4.6)	151
3-4 months	41 (30.8)	50 (37.6)	17 (12.8)	15 (11.3)	5 (3.8)	5 (3.8)	133
5-6 months	23 (59.0)*	9 (23.1)	3 (7.7)	1 (2.6)	1 (2.6)	2 (5.1)	39
Total	95 (29.4)	117 (36.2)	38 (11.8)	43 (13.3)	16 (5.0)	14 (4.3)	323

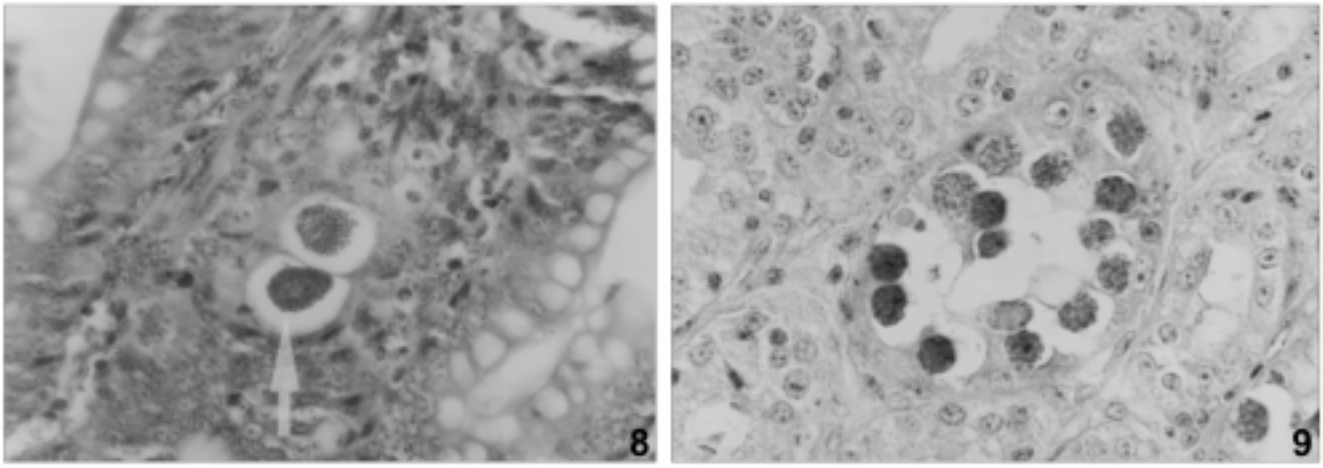
OPG - oocyst per gram of faeces; * $P<0.001$; $^{+}$ $P=0.028$

Table 4. The number of Eimeriidae species prevalent in individual goose faecal samples

No. of Eimeriidae species present in faecal samples	1	2	3	4	5	7
Percentage of 323 faecal samples	35.6	43.0	15.8	4.3	0.9	0.3



Figs 1-7. Sporulated oocysts. **1** - *Tyzzeria parvula*; **2** - *Eimeria anseris*; **3** - *E. truncata*; **4** - *E. stigmosa*; **5** - *E. hermani*; **6** - *E. fulva*. **7** - *E. nocens.* Scale bars - 6 µm



Figs 8, 9. 8 - intestine of goose with meronts in lamina propria; **9** - kidney of goose with gamonts in tubular epithelial cells. H&E, x 400

Eimeriidae species (*Eimeria anseris* and *E. truncata*) were the only species encountered in the domestic geese. The prevalence of coccidia species, the different species of Eimeriidae, and OPG counts in domestic geese in Kars were investigated in this study. Domestic geese population of the Kars province increase from May to November. Goslings are usually fed on pasture and slaughtered in winter months except for breeding geese. This study was carried out between May and October. Because, the disease is very important in young animals in these months.

Tyzzeria parvula, *Eimeria anseris*, *E. fulva*, *E. hermani*, *E. nocens*, *E. stigmosa,* and *E. truncata* were identified in the domestic geese in Kars. These were similar species identified in domestic geese in France (Chauve 1988). *Tyzzeria parvula* was the most prevalent species followed by *E. truncata* and *E. stigmosa*. *Eimeria truncata,* causative agent of renal coccidiosis, was found to be 37.8% in this study. This is similar to that reported by Gajadhar *et al*. (1983b) who found the prevalence of renal coccidiosis as 45.3% in wild geese.

Two types, intestinal and renal coccidiosis, in geese are reported (Tolgay 1973, Gajadhar *et al*. 1983a, Hiepe 1983, Soulsby 1986, Rommel *et al*. 1992). In this study, excretion rate of coccidia oocysts was 80.8% in the geese, but OPG counts were found to be low. The lower OPG counts may indicate a latent infection. It may also be due to the fact that animals were on pasture which might have hindered transmission of coccidia oocysts between the geese. However, caution should be exercised when extrapolating OPG counts in poultry species as they exhibit irregularity in time and number of excreted oocysts.

Meronts and gamonts were seen in 21 of 32 necropsied goslings. However, characteristic macroscopic and microscopic intestinal lesions were encountered in only 5 of the animals which had also typical macroscopic and microscopic lesions as described previously by other researchers (Pellérdy 1974, Gajadhar *et al*. 1983a, Rommel *et al*. 1992). Macroscopically, small whittish nodulus reported previously in the studies carried out in different countries by authors (Pellérdy 1974, Gajadhar *et al*. 1983a, Rommel *et al*. 1992) were not observed in the kidneys of necropsied geese, but meronts and gamonts of *E. truncata* were found in the kidney in 4 goslings.

It is concluded that coccidia species are very common in the domestic geese raised in Kars province, Turkey. This may imply that geese are under potential risk of developing clinical coccidiosis. It is therefore necessary to carry out further detailed studies on epidemiology of coccidiosis and experimental infections in order to understand and control the disease.

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Haemoproteids (Haemosporida: Haemoproteidae) of Wild Birds in Bulgaria

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Summary. 21 species of parasites of the genus *Haemoproteus* were found during the period 1999-2001 in the blood of 773 wild birds of 77 species (mostly passerines). Data on the morphology, size, their hosts, and the prevalence and intensity of invasion of each of these parasite species were gathered. The most commonly found parasite species were: *H. belopolskyi, H. lanii, H. balmorali, and H. payevskyi.* Two parasite species encountered very rarely in the Palearctic Zone: *H. velans* and *H. caprimulgi*, were also found. The prevalence of the invasion was especially high (up to 66.7%, n=30) in the case of *H. lanii* in the host genus *Lanius* (Laniidae). The total prevalence of the invasion of the birds studied was 18.5%. The highest prevalence found in the shrike family (Laniidae): 66.7% (n=30). A high rate was also found in the flycatcher family (Muscicapidae): 27.3% (n=33); the warbler family (Sylviidae): 22.8% (n=351); and the thrush family (Turdidae): 15.0% (n=81). The lowest rate was established in the sparrow family (Ploceidae): 3.45% (n=58). The highest rate of prevalence was found during spring, with a maximum in May (an average of 37.8%). Locally nesting migratory birds were more commonly invaded (a prevalence of 25.3%) than locally resident birds (5.8%). Spring migrants were also more frequently invaded (31.4%) than fall migrants (13.3%). Most invasions were of low intensity (between 1 and 10 parasites per 100 microscope fields at magnification 2000x). In only a few instances intensive invasions were observed; for example, in the host genus *Lanius* (Laniidae), between 200 and 330 parasites per 100 microscope fields were noted.

Key words: Haemosporidians, *Haemoproteus*, morphometry, prevalence, wild birds.

INTRODUCTION

Haemoproteids (family Haemoproteidae Doflein 1916) in wild birds are relatively well investigated, mainly in Western and Northern Europe, North America and South Asia. They are very poorly investigated in South-eastern Europe and neighbouring regions of Asia Minor (Bennet *et al.* 1982, Bishop and Bennet 1992,Valkiunas 1997). From the Balkans, data on these parasites have been published for Macedonia (Wülker 1919), for Greece (Wenyon 1926, Pasiotou *et al*. 1992, Theodoridis *et al*. 1998) and for Bulgaria (Valkiunas *et al*. 1999). In these publications, blood parasites found were identified only to genus level, and rarely to species, and the number of bird species studied was small. For the Balkan region, only three species of bird haemoproteids were identified: *H. columbae* (Pasiotou *et al*. 1992), *H. belopolskyi* (Valkiunas *et al*. 1999), and *H. payevskyi* (Valkiunas *et al*. 1999). This is a very small number of species, since on the Balkan Peninsula there are more than 450 species of birds and there are surely much more parasite species to be found. The purpose of this article is to enlarge the knowledge of the diversity of the blood parasites of wild birds in Bulgaria, of their hosts, and of seasonal variation trends in the prevalence.

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MATERIALS AND METHODS

Blood smears of 773 wild birds of 77 species (31 families and 12 orders) were studied. The birds were caught during the whole year from 1997 to 2001, mainly in three places in Bulgaria: the village Nova Cherna, Silistra District (567 birds caught); and the village Chelopechene (91) and Vrana Campsites (62), Sofia District.

Only 53 birds were caught in other regions of Bulgaria (Rupite, Blagoevgrad District, Sofia, Nissovo, Russe District, Atanassovsko Lake, Burgas District). The birds were caught in vertical mist nets, and blood was taken by cutting the longest claw of each specimen. The families and species of birds studied are shown in Table 1.

From each bird caught, three (rarely 2) blood smears were prepared and then fixed in methanol for 5 min and stained with Giemsa. The smears were studied through a Zeiss microscope (200x, 400x and 2000x). During the work with oil immersion lens (2000x), 600 microscope fields were used. Identification of the haemoproteids found was made through the use of Valkiunas (1997). Bird classification published by Voous (1977) was used. To calculate the intensity of invasion all parasites per 100 microscope fields at magnification 2000x were count.

Measurements (in micrometers) are given only for parasites with more than 20 gametocytes measured. To verify the degree of reliability of the data, Fisher's Criterion is used (Plochinsky 1970). In these comparisons, the degree of probability (p) is stated in the text.

RESULTS

A total of 21 species of *Haemoproteus* were found in the blood of the birds studied (n=773). The species composition and data on their morphology, their hosts and their distribution across Bulgaria are given below. Data on the prevalence are shown in the text for each host species of bird.

Haemoproteus belopolskyi Valkiunas, 1989

Morphology. Gametocyte is usually ameboid. A fully-grown gametocyte can encircle the nucleus of an affected erythrocyte. The nucleus of the parasite is situated closer to one of the poles of the gametocyte, and it is usually attached to the cell membrane of the affected erythrocyte. The granules are medium in size and they are dispersed in the cytoplasm. Their number varies between 4 and 17, rarely rising to 20. The measurements of the parasites and the host erythrocytes are shown in Table 2. Three cases of invasion of 1 erythrocyte by 2 gametocytes were found in the blood of Garden Warbler (*Sylvia borin*).

Hosts in Bulgaria: *Acrocephalus schoenobaenus* (16.1%, n=112), *A. palustris* (32.3%, n=31), *A. scirpaceus* (41.2%, n=17), *A. arundinaceus* (5.3%, n=75), *Sylvia nisoria* (4 cases of 4 examined), *S. borin* (2 cases of 7 examined), *S. atricapilla* (33.3%, n=42), *S. curruca* (6.3%, n=16), *S. communis* (5 cases of 7 examined), *Hippolais icterina* (2 cases of 4 examined), *Phylloscopus trochilus* (1 case of 5 examined), *Ph. sibilatrix* (27.3%, n=11).

Intensity of invasion

Intensity	Number of cases	%
below 1	11	18.0
1-10	36	59.0
10-100	13	21.3
over 100	1	1.6

Maximal intensity registered in blood of Garden Warbler (*Sylvia borin*) was 182 parasites per 100 microscope fields (16.09.2000, Nova Cherna).

Localities: Nova Cherna, Rupite, Vrana and Chelopechene (61 cases). Found in both local breeding birds and spring and fall migrants.

Notes. Widely distributed in the Palaearctic, Ethiopic and Indomalayan zoogeographical zones. In the list of hosts, the species *Acrocephalus scirpaceus* has not been previously included (Valkiunas 1997).

Haemoproteus payevskyi Valkiunas, Iezhova et Chernetsov, 1994

Morphology. Gametocytes are of uniform type, with rounded ends. The nucleus is large and clear, and located in the centre of the gametocyte. The gametocyte does not fill the poles of the affected erythrocyte. The granules are medium in size, rarely large, and they number between 5 and 21. The measurements of the parasites and the affected erythrocytes are shown in Table 2.

Hosts in Bulgaria: *Acrocephalus arundinaceus* (9.3%, n=75) and *A. palustris* (3.2%, n=31).

Intensity of invasion

Intensity	Number of cases
below 1	0
1-10	4
10-100	3
over 100	1

Localities: Nova Cherna (8 cases) and Chelopechene. Found in both spring migrants (6 cases) and local breeding birds (2 cases).

Notes. Marsh Warbler (*A. palustris*) is a new host of this parasite (Bishop and Bennet 1992, Valkiunas 1997).

Haemoproteus coraciae Mello et Afonso, 1935

Morphology. A fully-grown gametocyte does not encircle the nucleus of the affected erythrocyte. The margins of the gametocyte are most often entire, rarely ameboid. Gametocyte usually does not fill the poles of the erythrocyte, but sometimes a fully-grown gametocyte can fill them. The nucleus of the parasite is clear cut and located centrally. The granules are small, rounded, most often between 5 and 10 in number.

Host in Bulgaria: *Coracias garrulus* (2 cases of 2 examined)

Intensity of invasion. Found twice with intensities of 9 and 17 parasites per 100 fields.

Locality: Nova Cherna, in May.

Notes. The species is found in the Indomalayan and Ethiopic zoogeographical zones, and also in the South and Central Palaearctic (Valkiunas 1997).

Haemoproteus fringillae Labbe, 1894

Morphology. A fully-grown gametocyte does not encircle the nucleus of the affected erythrocyte. The gametocyte usually does not adhere to the host cell membrane in the central area. The fully-grown gametocyte fills the poles of the erythrocyte and usually displaces its nucleus. The parasite nucleus is often triangular and displaced slightly towards one of the polar zones. The pigment granules are small, rarely medium-sized, 8-15 in number.

Hosts in Bulgaria: *Fringilla coelebs* (1 case of 3 examined), *Coccothraustes coccothraustes* (2 cases of 2 examined).

Intensity of invasion. Below 1 in 2 cases and 5 in the third case.

Localities: Nova Cherna (1 case) and Vrana (1 case) in the blood of resident birds in breeding season and in autumn.

Notes. Widely distributed species, found in all zoogeographical zones except the Australian. Host species are of 2 families: Fringillidae and Emberizidae (Valkiunas 1997).

Haemoproteus velans Coatney et Roudabush, 1937

Morphometry. A fully-grown macrogametocyte is often circumnuclear. Circumnuclear microgametocytes were not seen. The gametocyte displaces the host cell nucleus and often does not adhere to its membrane. Invaded erythrocytes are longer than the others. The macrogametocyte nucleus has clear margins but the nucleus of the microgametocyte is diffuse. Pigment granules vary in size and are dispersed in all parts of the cytoplasm. Their number is usually between 15 and 26. Volutine granules were not seen. The measurements of the parasites and the host erythrocytes are shown in Table 3.

Host in Bulgaria: *Dendrocopus syriacus* (1 case of 1 examined)

Intensity of invasion. The only invasion found was 81 parasites per 100 fields.

Locality: Rupite, Blagoevgrad district, in a resident breeding bird.

Notes. It is reported for Palaearctic Wrynecks (*Jynx torquilla*) wintering in India (Valkiunas 1997). Our finding proves its distribution in the Palaearctic. The parasites observed by us lack volutine granules and so differ from the description in Valkiunas (1997).

Haemoproteus attenuatus Valkiunas, 1989

Morphology. A large space between the gametocyte and the host cell membrane is usually seen only in the central zone. So the gametocyte is thin in the central zone and broad at the ends. The cytoplasm is divided in areas differing slightly in colour. The parasite nucleus is closer to one of the poles. The pigment granules a re medium or small in size, usually more than 20 in number. If the granules are small, their number is higher.

Hosts in Bulgaria: *Luscinia luscinia* (2 cases of 7 examined), *Erithacus rubecula* (6.3%, n=16).

Intensity of invasion. Three invasions found with 2, 4 and 17 parasites per 100 fields respectively. It is interesting that the highest intensity was found in the blood of a Robin (*Erithacus rubecula*) caught on 10.03.2001 at Chelopechene in winter.

Localities: Nova Cherna and Chelopechene. Found in the blood of spring (2 cases) and fall migrants (1 case) only.

Notes. Reported from Curonian Spit in Baltic Sea up to now; 1 host species Robin (*Erithacus rubecula*) (Valkiunas 1997). Thrush Nightingale (*Luscinia luscinia*) is a new host for the species.

Haemoproteus lanii Mello, 1936

Morphology. Circumnuclear gametocytes may persist, but usually they are very rare. A fully-grown gametocyte has entire margins. The parasite nucleus is centrally located and adheres to the erythrocyte nucleus. Rarely the parasite nucleus is located in one of the poles. The pigment granules are medium sized or large, 5-16 in number. The measurements of the parasites and

Table 1. Number of the examined birds (% shown only in cases with more than 10 examined individuals) and the prevalence of the invasions with different species of haemoproteids. Abbreviations of the haemoproteid names: Hant - *H. anthi,* Hate - *H. attenuatus,* Hbal - *H. balmorali,* Hbel - *H. belopolskyi,* Hcap - *H. caprimulgi,* Hcor - *H. coraciae,* Hdol - *H. dolniki,* Hfri - *H. fringillae,* Hher - *H. herodiadis,* Hhir - *H. hirundinis,* Hlan- *H. lanii,* Hmin - *H. minutus,* Hmot - *H. motacillae,* Hnoc - *H. noctuae,* Hori - *H. orioli,* Hpal - *H. pallidus,* Hpas - *H. passeris,* Hpay - *H. payevskyi,* Hpic - *H. picae,* Hvel - *H. velans,* Hwen - *H. wenyoni*

Families/species	Number of examined birds	Number of infected birds	%	Species of haemoproteids
Pelecanidae	14	0	0	
Pelecanus onocrotalus (Bruch)	14	0	0	
Ardeidae	7	3		Hher
Ixobrychus minutus (L.)*	7	3		Hher
Anatidae	1	0		
Anas platyrhynchos (L.)	1	0		
Accipitridae	1	0		
Accipiter nisus (L.)	1	0		
Scolopacidae	1	0		
Tringa nebularia (Gunn.)	1	0		
Columbidae	1	1		H. sp.
Streptopelia turtur (L.)*	1	1		H. sp.
Cuculidae	3	0		
Cuculus canorus	3	0		
Strigidae	3	1		Hnoc
Asio otus (L.)*	1	1		Hnoc
Otus scops (L.)	1	0		
Athene noctua (Scop.)	1	0		
Caprimulgidae	2	1		Hcap
Caprimulgus europaeus (L.)*	2	1		Hcap
Alcedinidae	21	1	4.8	H. sp.
Alcedo atthis (L.)*	21	1	4.8	H. sp.
Coraciidae	2	2		Hcor
Coracias garrulus (L.)*	2	2		Hcor
Picidae	12	1	8.3	Hvel
Dendrocopus major (L.)	3	0		
D. syriacus (Hemp. et Eher.)*	3	1		Hvel
D. medius (L.)	2	0		
Picus canus (Gm.)	2	0		
P. viridis (L.)	1	0		
Jynx torquilla (L.)	1	0		
Hirundinidae	21	1	4.8	Hhir
Hirundo rustica (L.)	16	0		
Riparia riparia (L.)	4	0		
Delichon urbica (L.)*	1	1		Hhir
Motacillidae	25	2	8.0	Hmot, Hant
Anthus trivialis (L.)*	21	2	9.5	Hmot, H. ant
Motacilla flava (L.)	4	0		
Prunellidae	5	0		
Prunella modularis (L.)	5	0		
Turdidae	81	12	14.8	Hmin, Hbal, Hate
Turdus merula (L.)*	14	3	21.4	Hmin, H. sp.
T. philomelos (Brehm)	6	0		
T. iliacus (L.)	1	0		
Phoenicurus phoenicurus (L.)*	14	1	7.1	Hbal
Erithacus rubecula (L.)*	16	3	18.8	Hbal, Hate
Saxicola rubetra (L.)*	3	1		Hbal
Luscinia luscinia (L.)*	7	4		Hbal, Hate
L. megarhynchos (Brehm)	20	0		

Table 1 (contd.)	Number of examined birds	Number of infected birds	%	Species of haemoproteids
Sylvidae	351	80	22.8	Hbel, Hpay, Hwen
Sylvia nisoria (Bech.)*	4	4		Hbel
S. borin (Bodd.)*	7	2		Hbel
S. atricapilla (L.)*	42	15	35.7	Hbel, Hwen
S. communis (Latham)*	7	5		Hbel
S. curruca (L.)*	16	1	6.3	Hbel
Acrocephalus arundinaceus (L).	75	11	14.7	Hpay, Hbel
A. palustris (Bech.)*	31	11	35.5	Hpay, Hbel
A. scirpaceus (Herm.)*	17	7	41.2	Hbel
A. schoenobaenus (L.)*	112	18	16.1	Hbel
Hippolais icterina (Vieil.)*	4	2		Hbel
Phylloscopus trochilus (L.)*	5	1		Hbel
Ph. sibilatrix (Bech.)*	11	3	27.3	Hbel
Ph. collybita (Vieil.)	4	0		
Locustella fluviatilis (Wolf)	7	0		
L. luscinioides (Savi)	9	0		
Muscicapidae	33	9	27.3	Hbal, Hpal
Ficedula parva (Bech.)	6	0		
F .semitorquata (Hom.)	1	0		
F. albicollis (Temm.)	3	0		
F. hypoleuca (Pall.)	5	0		
M. striata (Pall.)*	18	9	50.0	Hbal, Hpal
Aegithalidae	2	0		
Aegithalos caudatus (L.)	2	0		
Remizidae	4	0		
Remiz pendulinus (L.)	4	0		
Paridae	46	0		
Parus major (L.)	34	0		
P. caeruleus (L.)	11	0		
P. palustris (L.)	2	0		
Sittidae	5	0		
Sitta europaea (L.)	5	0		
Certhiidae	3	0		
Certhia brachydactyla (Brehm)	1	0		
C. familiaris (L.)	2	0		
Troglodytidae	8	0		
T*roglodytes troglodytes*	8	0		
Laniidae	30	20	66.7	Hlan
Lanius collurio (L.)*	27	19	70.4	Hlan
L. minor (Gm.)*	3	1		Hlan
Corvidae	3	1		Hpic
Pica pica (L.)	2	0		
Garrulus glandarius (L.)*	1	1		Hpic
Oriolidae	3	2		Hori
Oroilus oriolus (L.)*	3	2		Hori
Sturnidae	1	0		
Sturnus vulgaris (L.)	1	0		
Ploceidae	58	2	3.5	Hpas
Passer domesticus (L.)	28	0		
P. hispaniolensis (Temm.)*	14	2	14.3	Hpas
P. montanus (L.)	17	0		
Fringillidae	26	3	11.5	Hfri, Hdol
*Fringilla coelebs (L.)**	3	1		Hfri, Hdol
Carduelis chloris (L.)	12	0		
C. carduelis (L.)	9	0		
Emberizidae	3	0		
Emberiza citrinella (L.)	1	0		
E. calandra (L.)	2	0		
TOTAL	773	143	18.5	

Table 2. Measurements of *Haemoproteus belopolskyi* and *H. payevskyi.* Abbreviations: A - average measurement, GR - number of granules, L - length, lim - range, LNE - length of the nucleus of the erythrocyte, LNG - length of the nucleus of the gametocyte, NDR - nucleus displacement ratio, SD - standard deviation, W - width, WNE - width of the nucleus of the erythrocyte, WNG - width of the nucleus of the gametocyte

	H. belopolskyi (n=30)			*H. payevskyi* (n=20)		
Measurement	lim	A	SD	lim	A	SD
Uninfected erythrocytes						
L	9.2-11.3	10.68	0.74	12-14.5	13.25	0.86
W	6.0-7.2	6.56	0.44	6.0-8.0	7	0.75
LNE	4.8-7.0	5.51	0.56	6.0-7.0	6.44	0.5
WNE	2.0-3.0	2.41	0.27	2.0-3.0	2.52	0.34
Erythrocytes infected by macrogametocytes						
L	10.2-13.0	11.58	0.65	13-15.5	14.53	0.69
W	5.8-7.2	6.72	0.42	6.0-7.0	6.7	0.42
LNE	4.8-6.0	5.48	0.44	5.5-7.0	6.47	0.53
WNE	2.0-2.8	2.39	0.26	1.5-2.0	2.27	0.32
Erythrocytes infected by microgametocytes						
L	11.0-12.3	11.67	0.5	14-15	14.7	0.48
W	6.0-8.0	7.02	0.53	6.5-8.0	7.23	0.44
LNE	5.0-6.0	5.4	0.44	6.0-7.0	6.23	0.31
WNE	2.0-2.7	2.32	0.26	2.0-2.5	2.16	0.21
Macrogametocytes						
L	11.0-21.0	14.05	1.71	11.0-15.0	13.6	1.05
W	5.8-7.2	6.72	0.42	2.8-3.0	2.98	0.06
LNG	2.0-3.5	2.6	0.43	2.0-3.0	2.21	0.33
WNG	1.3-2.2	1.75	0.29	1.5-2.0	1.9	0.21
NDR	0.6-1.0	0.88	0.12	0.5-0.85	0.66	0.12
GR	4-15	9.13	3.13	13-20	15	2.45
Microgametocytes						
L	12.5-16	14.2	1.18	11-15	13.2	1.11
W	2.3-3.0	2.81	0.27	3.0 -4.0	3.15	0.34
LNG	4.0-6.0	5.45	0.83	1.8-2.1	1.97	0.09
WNG	1.5-3.0	2.3	0.59	1.2-1.7	1.4	0.17
NDR	0.75-1.0	0.84	0.07	0.67-0.75	0.72	0.04
GR	6-14	8.9	2.47	10-16	13.6	1.84

the host erythrocytes are shown in Table 3. The invasion of 1 erythrocyte by 2 gametocytes was registered on a few occasions.

Hosts in Bulgaria: *Lanius collurio* (70.4%, n=27), *Lanius minor* (1 case of 3 examined)

Intensity of invasion

Intensity	Number of cases	%
below 1	2	10.5
1-10	7	36.8
10-100	8	42.1
over 100	2	10.5

Maximal intensity: 335 parasites per 100 fields in the blood of a Lesser Grey Shrike (*Lanius minor*).

Localities: Nova Cherna, Atanassovsko Ezero (Burgas district), Chelopechene, Nisovo (Russe district) (20 cases). Found in both breeding birds and migrants.

Notes. Distributed in Palaearctic, Ethiopic and Indomalayan zoogeographical zones (Valkiunas 1997).

Haemoproteus orioli Mello, 1935

Morphology. A fully-grown gametocyte fills the poles of the infected erythrocyte, but does not encircle its nucleus. The parasite nucleus is often situated at one of the ends. The parasite displaces the host cell nucleus and the NDR is between 0.5 and 1. The pigment granules vary in size and number between 4 and 18.

Host in Bulgaria: *Oriolus oriolus* (2 cases of 3 examined)

Intensity of invasion. Registered only once with intensity of 4 parasites per 100 fields.

Locality: Nova Cherna. Found in blood of a breeding bird.

Table 3. Measurements of *Haemoproteus velans* and *H. lanii.* Abbreviations: A - average measurement, GR - number of granules, L - length, lim - range, LNE - length of the nucleus of the erythrocyte, LNG - length of the nucleus of the gametocyte, NDR - nucleus displacement ratio, SD - standard deviation, W - width, WNE - width of the nucleus of the erythrocyte, WNG - width of the nucleus of the gametocyte

	H. velans (n=30)			*H. lanii* (n=20)		
Measurement	lim	A	SD	lim	A	SD
Uninfected erythrocytes						
L	11-13	12.3	0.53	10-13	11.61	0.72
W	6.0-8.0	6.84	0.58	6.0-8.0	6.84	0.5
LNE	5.0-7.0	6.14	0.48	5.0-6.5	5.8	0.45
WNE	2.0-3.0	2.39	0.36	2.0-2.7	2.19	0.22
Erythrocytes infected by macrogametocytes						
L	12-15	14.06	0.78	11.2-13.5	12.14	0.59
W	6.0-8.0	7.11	0.48	6.0-9.0	7.1	0.64
LNE	5.0-6.5	5.8	0.37	5.0-6.5	5.68	0.55
WNE	2.0-3.0	2.27	0.35	2.0-3.0	2.24	0.24
Erythrocytes infected by microgametocytes						
L	12.5-15.5	13.88	0.8	12-14	12.65	0.61
W	6.0-8.0	7.00	0.63	6.0-8.0	7.04	0.57
LNE	5.0-7.0	5.79	0.51	4.5-6.5	5.68	0.48
WNE	2.0-3.0	2.31	0.34	2.0-2.5	2.19	0.22
Macrogametocytes						
L	14-26	20.0	3.43	12.5-20.0	16.15	2.0
W	2.5-4.5	3.25	0.45	2.0-3.5	2.77	0.44
LNG	2.0-5.0	3.26	0.61	1.5-3.0	2.18	0.4
WNG	2.0-3.0	2.32	0.38	1.0-2.0	1.32	0.39
NDR	0-1	0.53	0.29	0.57-0.9	1	0.79
0.08						
GR	15-35	23.86	4.91	8-14	10.5	1.93
Microgametocytes						
L	13.5-20.0	16.37	1.61	13-21	14.8	2.07
W	2.5-4.5	3.44	0.5	2.0-3.5	2.74	0.46
LNG	5.0-5.5	3.0-6.0	4.49	0.85		
WNG	3.0	1.0-3.5	2.29	0.62		
NDR	0-0.8	0.4	0.18	0.5-1.0	0.83	0.12
GR	13-28	20.6	3.8	8-15	11.2	2.0

Notes. Distributed in Palaearctic, Ethiopic and Indomalayan zoogeographical zones (Valkiunas 1997).

Haemoproteus herodiadis Mello, 1935

Morphology. A fully-grown gametocyte does not fill the poles of the infected erythrocyte and often has no contact with either its cell membrane or its nucleus. The gametocyte has entire margins. The young gametocyte never adheres to the host cell nucleus. The parasite nucleus is in the central zone and varies in size. The pigment granules are small, 8-16 in number. The measurements of the parasites and the host erythrocytes are shown in Table 4.

Host in Bulgaria: *Ixobrychus minutus* (3 cases of 7 examined).

Intensity of invasion. A very low rate of invasion was registered in 2 cases - below 1. In the third case the intensity was 5 parasites per 100 fields.

Locality: Nova Cherna. Found only in May.

Notes. Reported for Holarctic and Indomalayan zoogeographical zones (Valkiunas 1997).

Haemoproteus balmorali Pierce, 1984

Morphology. A fully-grown gametocyte usually doesn't fill the poles of the infected erythrocyte and doesn't encircle its nucleus. The erythrocyte nucleus is displaced by the parasite. The gametocyte adheres to the host cell membrane at the polar zones, but usually does not do so in the central zone. The parasite nucleus is clear, oval to ellipsoid, situated most often close to the

Table 4. Measurements of *Haemoproteus herodiadis* and *H. balmorali.* Abbreviations: A - average measurement, GR - number of granules, L - length, lim - range, LNE - length of the nucleus of the erythrocyte, LNG - length of the nucleus of the gametocyte, NDR - nucleus displacement ratio, SD - standard deviation, W - width, WNE - width of the nucleus of the erythrocyte, WNG - width of the nucleus of the gametocyte

	H. herodiadis (n=10)			*H. balmorali* (n=20)		
Measurement	lim	A	SD	lim	A	SD
Uninfected erythrocytes						
L	14-16	14.68	0.8	12-13.5	12.44	0.54
W	7.0-8.0	7.13	0.32	6.2-7.5	6.92	0.42
LNE	6.0-8.0	7.16	0.6	5.2-7.0	5.94	0.54
WNE	2.6-3.0	2.85	0.17	2.5-3.0	2.65	0.2
Erythrocytes infected by macrogametocytes						
L	12-18	15.25	1.74	13-14.3	13.38	0.47
W	6.0-8.0	7.33	0.76	6.5-8.0	7.17	0.41
LNE	5.5-8.0	6.7	0.71	6.0-6.5	6.1	0.21
WNE	2.0-3.0	2.5	0.34	2.2-3.0	2.67	0.3
Erythrocytes infected by microgametocytes						
L	14.5-17	15.75	0.95	13-14	13.53	0.5
W	6.5-8.0	7.22	0.6	6.5-8.0	7.05	0.37
LNE	5.8-8.0	6.83	0.65	5.8-6.5	6.1	0.2
WNE	2.0-3.0	2.22	0.32	2.2-3.0	2.7	0.31
Macrogametocytes						
L	11-13.5	12.45	0.76	16-19	17.55	1.04
W	2.0-3.0	2.76	0.39	2.0-3.0	2.17	0.33
LNG	2.0-4.5	3.32	0.71	2.0-3.0	2.77	0.39
WNG	1.0-3.0	1.95	0.54	1.2-2.0	1.82	0.3
NDR	0.67-0.8	0.72	0.04	0.85-1.25	0.98	0.11
GR	9-15	11.9	2.88	?	?	?
Microgametocytes						
L	11-14.5	13.45	1.23	15-19.5	17.25	1.38
W	2.5-3.0	2.9	0.21	1.5-3.2	2.3	0.48
LNG (n=3)	4.2-5.0	7.5-9.0	8.25	0.49		
WNG (n=3)	1.5-2.0	1.5-3.2	2.3	0.48		
NDR	0.44-0.8	0.72	0.12	0.64-1	0.9	0.11
GR	11-20	13.5	2.76	?	?	?

poles and adhering to the host cell membrane. Volutine granules are present, so counting the pigment granules is difficult. The microgametocyte cytoplasm is darker coloured at the ends and all the granules are gathered there. The measurements of the parasites and the host erythrocytes are shown in Table 4.

Hosts in Bulgaria: *Muscicapa striata* (50 %, n=18), *Luscinia luscinia* (2 cases of 7 examined), *Saxicola rubetra* (1 case of 3 examined), *Erithacus rubecula* (12.5 %, n=16), *Phoenicurus phoenicurus* (7.1 %, n=14).

Intensity of invasion

	Number of cases	%
below 1	2	13.3
1-10	6	40.0
10-100	6	40.0
over 100	1	6.6

Maximal intensity registered: 290 parasites per 100 fields in blood of a Robin (*Erithacus rubecula*), Nova Cherna, 14.09.2001.

Localities: Nova Cherna and Chelopechene. Found on 15 occasions in the blood of migrants.

Notes. Reported for a great number of hosts of the families Turdidae and Muscicapidae. Found in the Palaearctic, Ethiopic and Indomalayan zoogeographical zones (Valkiunas 1997).

Haemoproteus passeris Kruse, 1890

Morphology. A young gametocyte adheres to only the cell membrane of the infected erythrocyte. The fully grown gametocyte occasionally can fill the poles of the infected erythrocyte and does not encircle its nucleus. The gametocyte has entire or ameboid margins. The parasite nucleus is clear cut, situated closer to one of the ends. The pigment granules are medium-sized, rarely

Table 5. Measurements of *Haemoproteus passeris* and *H. pallidus.* Abbreviations: A - average measurement, GR - number of granules, L - length, lim - range, LNE - length of the nucleus of the erythrocyte, LNG - length of the nucleus of the gametocyte, NDR - nucleus displacement ratio, SD - standard deviation, W - width, WNE - width of the nucleus of the erythrocyte, WNG - width of the nucleus of the gametocyte

	H. passeris (n=10)			*H. pallidus* (n=10)		
Measurement	lim	A	SD	lim	A	SD
uninfected erythrocytes						
L	11-12	11.48	0.48	11.5-13.5	12.45	0.63
W	6.0-7.0	6.25	0.42	6.0-7.0	6.4	0.46
LNE	5.0-6.0	5.62	0.39	5.6-6.5	6.06	0.24
WNE	2.0-3.0	2.51	0.35	2.0-2.5	2.25	0.2
Erythrocytes infected by macrogametocytes						
L	12-13	12.47	0.42	12-15	13.17	0.82
W	6.0-7.0	6.38	0.49	6.0-7.0	6.2	0.35
LNE	5.2-6.0	5.77	0.39	5.7-7.0	6.06	0.35
WNE	2.0-3.0	2.53	0.25	2.0-2.2	2.02	0.06
Erythrocytes infected by microgametocytes						
L	11.5-13	12.3	0.67	13-14.8	13.28	0.62
W	6.0-7.0	6.33	0.38	6.0-7.0	6.25	0.33
LNE	5.0-6.2	5.73	0.37	5.5-6.4	6.04	0.24
WNE	2.0-3.0	2.47	0.3	2.0	2.0	0
Macrogametocytes						
L	11-16	13.55	1.57	13-15.5	13.85	0.78
W	2.0-2.4	2.04	0.13	2.0-2.5	2.07	0.16
LNG	2.0-3.5	2.39	0.51	1.5-2.0	1.9	0.22
WNG	2.0-2.5	2.05	0.16	0.7-1.5	1.08	0.29
NDR	0.71-1	0.92	0.1	0.75-1.1	0.96	0.1
GR	8-18	11.9	3.31	10-15	12.8	1.75
Microgametocytes						
L	12.5-19.5	15.3	1.89	13.5-16	14.65	1.13
W	2.0-3.0	2.22	0.36	2.0-3.0	2.27	0.34
LNG	?					
WNG	?					
NDR	0.5-1	0.81	0.15	0.8-1.0	0.95	0.07
GR	9-15	12	2	10-15	12.5	1.7

small, oval to ellipsoid, 8-15 in number. In the microgametocyte the granules are gathered in the poles. The measurements of the parasites and the host erythrocytes are shown in Table 5.

Hosts in Bulgaria: *Passer hispaniolensis* (14.3%, n=14)

Intensity of invasion. Found twice with intensities of 12 and 16 parasites per 100 fields, respectively.

Locality: Nova Cherna. Found in local resident birds in breeding season.

Notes. Found in all zoogeographical zones (Valkiunas 1997)

Haemoproteus pallidus Valkiunas et Iezhova, 1991

Morphology. In comparison with other haemoproteids the gametocyte of *H. pallidus* is obviously paler. The fully-grown gametocyte does not fill the poles of the infected erythrocyte. The gametocyte usually adheres to the host cell nucleus but does not adhere to its cell membrane. The parasite nucleus is situated closer to one of the ends. The microgametocyte nucleus is diffuse and very difficult to measure. The pigment granules are small to medium, most often between 10 and 15 in number. Erythrocytes infected by 2 gametocytes are not rare. Measurements of the parasites and the host erythrocytes are shown in Table 5.

Host in Bulgaria: *Muscicapa striata* (11.1%, n=18)

Intensity of invasion. Found twice in mixed invasions with *H. balmorali*. Intensities registered were 12 and16 parasites per 100 fields respectively.

Localities: found at Nova Cherna in a fall migrant and at Chelopechene in a spring migrant.

Notes. Known for the Palaearctic and Ethiopic zoogeographical zones (Valkiunas 1997).

Table 6. Measurements of *Haemoproteus noctuae.* Abbreviations: A - average measurement, GR - number of granules, L - length, lim - range, LNE - length of the nucleus of the erythrocyte, LNG - length of the nucleus of the gametocyte, NDR - nucleus displacement ratio, SD - standard deviation, W - width, WNE - width of the nucleus of the erythrocyte, WNG - width of the nucleus of the gametocyte

Measurement (n=10)	lim	A	SD
Uninfected erythrocytes			
L	14-16	14.82	0.68
W	7.0-8.0	7.65	0.53
LNE	5.8-8.0	6.68	0.7
WNE	2.5-3.5	2.9	0.29
Erythrocytes infected by macrogametocytes			
L	14-16.5	15.32	0.81
W	7.5-9.0	8.25	0.54
LNE	5.0-7.0	6.19	0.69
WNE	2.0-3.0	2.62	0.42
Erythrocytes infected by microgametocytes			
L	13.5-16.0	15.25	0.75
W	7.5-9.2	8.27	0.52
LNE	6.0-7.3	6.51	0.46
WNE	2.3-3.0	2.69	0.26
Macrogametocytes			
L	18.5-26.5	22.6	2.67
W	2.0-3.0	2.8	0.35
LNG	2.5-4.0	3.35	0.58
WNG	1.5-3.0	2.12	0.30
NDR	0.8-1.0	0.91	0.09
GR	17-25	20.7	2.58
Microgametocytes			
L	20-24	21.6	1.15
W	2.0-3.0	2.43	0.5
LNG (n=3)	8.0-8.5		
WNG (n=3)	2.0		
NDR	0.8-1.0	0.92	0.08
GR	14-22	17.7	2.16

Table 7. Total prevalence of invasions with haemoproteids of wild birds in different parts of Eurasia

Place (country)	Total prevalence %	Source
England	3.8-8.6	Pierce and Mead (1976, 1977, 1978)
Poland	6.4-15.6	Dymowska and Żukowski (1965), Ramisz (1965), Dymowska and Żukowski (1968)
Chechoslovakia (former)	11.8	Kučera (1981)
Kazakchstan	13.7	Jakunin (1972)
Greece	19.6	Theodoridis *et al*. (1998)
Curonian Spit in the Baltic Sea (Kursiu peninsula)	34.8	Valkiunas (1985)
Bulgaria	18.5	present study

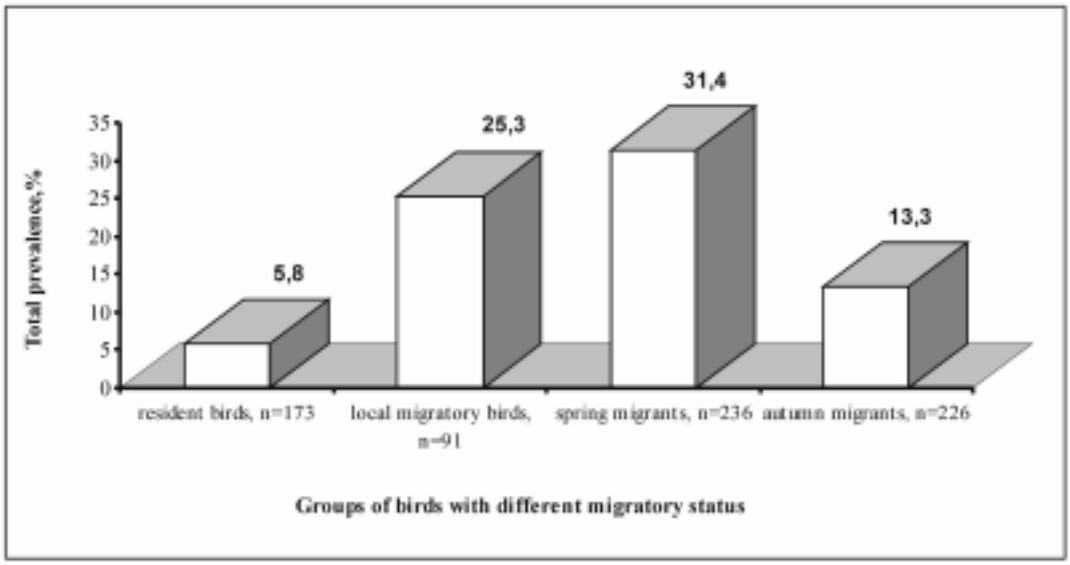

Fig. 1. Total prevalence of haemoproteid invasions of birds with different migratory status in Bulgaria

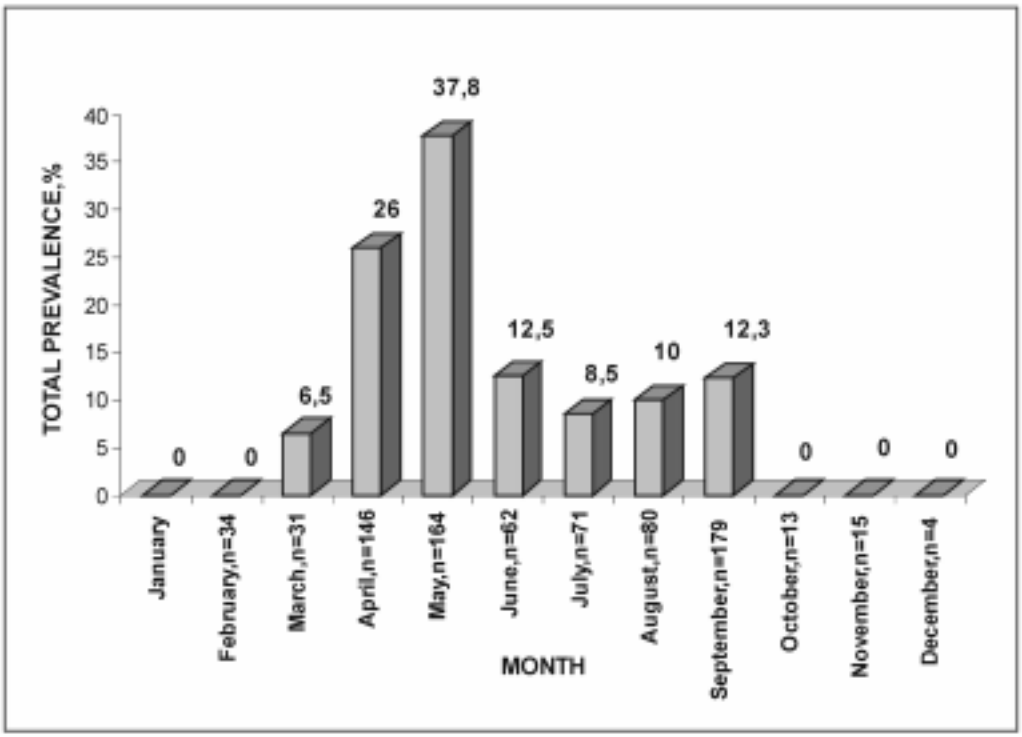

Fig. 2. Total prevalence of haemoproteid invasions by months

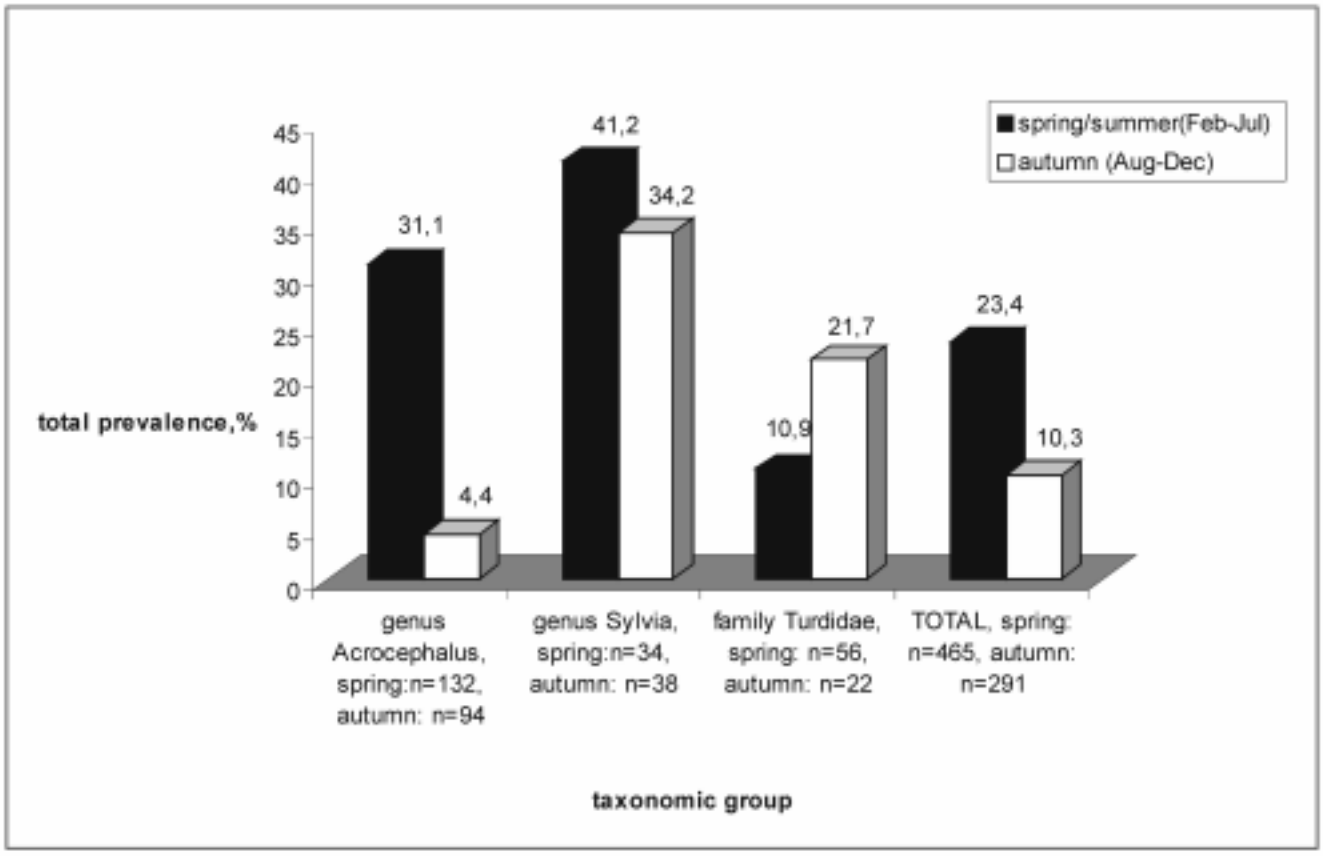

Fig. 3. Seasonal differences in the total prevalence of the haemoproteid invasions: a comparison among different taxonomic groups of birds

***Haemoproteus picae* Coatney et Roudabush, 1937**

Morphology. A fully-grown gametocyte fills the poles of the infected erythrocyte and has a tendency to encircle its nucleus. Nevertheless a case with a fully encircled erythrocyte nucleus was not observed. The gametocyte has entire margins. The parasite nucleus is ellipsoid, situated closer to one of the ends and to the host cell membrane. The pigment granules are medium-sized to large, 10-18 in number. The largest granules are ellipsoid.

Host in Bulgaria: *Garrulus glandarius* (1 case of 1 examined)

Intensity of invasion. Found only once with a very low rate of infection (below 1).

Locality: Rupite, Blagoevgrad district. Found in the blood of a local breeding bird.

Notes. Distributed in Holarctic (Valkiunas 1997).

***Haemoproteus anthi* Mello, 1935**

Morphology. A fully-grown gametocyte fills the poles of the infected erythrocyte and strongly displaces its nucleus. The gametocyte has a tendency to encircle the erythrocyte nucleus, but a case with fully encircled erythrocyte nucleus was not observed. The parasite nucleus is situated closer to one of the ends. The pigment granules medium in size, 8-14 in number.

Host in Bulgaria: *Anthus trivialis* (4.8 %, n=21)

Intensity of invasion. Found only once with an infection rate of 2.

Locality: Nova Cherna. Found in the blood of a fall migrant.

Notes. Distributed in the Palaearctic, Ethiopic and Indomalayan zoogeographical zones (Valkiunas 1997).

***Haemoproteus motacillae* Bennet et Pierce, 1990**

Morphology. A gametocyte adheres to the host cell membrane in the polar zones, but often does not do so in the central zone. The gametocyte has entire or ameboid margins. The fully-grown gametocyte fills the poles of the infected erythrocyte and does not encircle its nucleus. The parasite nucleus is ellipsoid, situated close to the cell membrane of the infected

erythrocyte. The pigment granules are ellipsoid, large in size, number between 5 and 14.

Host in Bulgaria: *Anthus trivialis* (4.8%, n=21)

Intensity of invasion. Found only once with an infection rate of 9.

Locality: Nova Cherna. Found in the blood of a fall migrant.

Notes. Distributed in the Palaearctic, Ethiopic and Indomalayan zoogeographical zones (Valkiunas 1997).

Haemoproteus noctuae Celli et Sanfelice, 1891

Morphology. Circumnuclear parasite**.** A fully-grown gametocyte does not adhere to the host cell nucleus. The gametocyte has entire or ameboid margins. The parasite nucleus is large, centrally located, rarely displaced towards one of the ends. The pigment granules are small to large, between 15 and 29 in number. In the microgametocyte granules are often gathered at the poles. The measurements of the parasites and the host erythrocytes are shown in Table 6.

Host in Bulgaria: *Asio otus* (1 case of 1 examined)

Intensity of invasion. Found only once with an infection rate of 18.

Locality: Nova Cherna. Found in the blood of a breeding bird.

Notes. Distributed in all zoogeographical zones (Valkiunas 1997).

Haemoproteus hirundinis Sergent et Sergent, 1905

Morphology. A fully-grown gametocyte fills the poles of the infected erythrocyte. The gametocyte has entire margins. The pigment granules vary from small to large, around 10 in number.

Host in Bulgaria: *Delichon urbica* (1 case of 1 examined)

Intensity of invasion. Found only once with a very low rate of infection (below 1).

Locality: Vrana. Found in spring.

Notes. Distributed in the Holarctic, Ethiopic and Indomalayan zoogeographical zones (Valkiunas 1997).

Haemoproteus minutus Valkiunas et Iezhova, 1992

Morphology. Ever a fully-grown gametocyte is rarely longer than the host cell nucleus and never fills its poles. The gametocyte has entire margins. The parasite nucleus is situated closer to one of the poles, sometimes has a terminal position. The pigment granules are small, 4-10 in number.

Host in Bulgaria: *Turdus merula* (7.1%, n=14)

Intensity of invasion. Found only once with an infection rate of 10.

Locality: Nova Cherna. Found in a resident breeding bird.

Notes. Distributed in the Palaearctic. Described from Curonian Spit in the Baltic Sea in the blood of a Blackbird (*Turdus merula*). Other hosts are not known (Valkiunas 1997).

Haemoproteus caprimulgi Williams, Bennet et Mahrt, 1975

Morphology. A circumnuclear parasite**.** The young gametocytes and some of the grown gametocytes have no contact with either the host cell membrane or the host cell nucleus. The parasite nucleus has a variable location. The parasite margins are entire or ameboid. The pigment granules are 16-29 in number.

Host in Bulgaria: *Caprimulgus europaeus* (1 case of 2 examined)

Intensity of invasion. Found only once with an infection rate of 1.

Locality: Nova Cherna. Found in a spring migrant.

Notes. Distributed in the Holarctic (mainly Nearctic) and Indomalayan zoogeographical zones, rarely in the Palaearctic (Valkiunas 1997). In the description of the species (Valkiunas 1997) 2 types of growth are reported: the first with a strong displacement of the host cell nucleus, and the second (more rare) with circumnuclear gametocytes. In our samples, only circumnuclear parasites were observed (second type of growth).

Haemoproteus wenyoni Mello, Sa, Sousa, Dias et Noronha, 1916

Morphology. A gametocyte fills the poles of the infected erythrocyte, but never encircles its nucleus. The parasite margins are entire or ameboid. The gametocyte adheres to the host cell membrane at the polar zones, but often does not do so in the central zone. The parasite nucleus is clear cut, ellipsoid and situated closer to one of the poles. The pigment granules are small, usually between 18 and 20 in number.

Host in Bulgaria: *Sylvia atricapilla* (2.4%, n=42)

Intensity of invasion. Found only once with an infection rate of 9.

Locality: Rupite, Blagoevgrad district. Found in a local breeding bird on 30.04.2001.

Notes. Widely distributed species, found in all zoogeographical zones except the Australian. Rare in the Palaearctic. In the original description of the species (Valkiunas 1997) ameboid gametocytes were not observed, but volutine granules were found. So there is a difference between the original and our description.

***Haemoproteus dolniki* Valkiunas et Iezhova, 1992**

Morphology. A gametocyte fills the poles of the infected erythrocyte, but never encircles its nucleus. The parasite margins are entire. The gametocyte adheres to the host cell membrane at the polar zones, but often does not do so in the central zone. The erythrocyte nucleus is not displaced by the gametocyte. The pigment granules are medium in size, most often between 12 and 15 in number.

Host in Bulgaria: *Fringilla coelebs* (1 case of 3 examined)

Intensity of invasion. Found only once with an infection rate of 12.

Locality: Vrana. Found in a local breeding bird.

Notes. Distributed in the Palaearctic zoogeographical zone. The only known host species is Chaffinch (*Fringilla coelebs*) (Valkiunas 1997).

Prevalence and intensity of the invasion

The total prevalence of invasion of the birds studied is 18.5%. The prevalence of invasion varies considerably among different families of birds (Table 1). The highest prevalence is found in Laniidae: 66.7% (n=30). A high rate was also found in Muscicapidae: 27.3% (n=33); Sylviidae: 22.8% (n=351); and Turdidae: 15.0% (n=81). A low rate was found in Ploceidae: 3.45% (n=58). Of 46 birds studied from Paridae, none was invaded.

Data on the invasion of the best-investigated bird family - Sylvidae show differences in prevalence between some species and genera (Table 1). Prevalence in the genus *Sylvia* is 37.3% (n=76), which is nearly double the rate of the genus *Acrocephalus* (20.0%, n=235), (Fisher's exact test, p>0.99, n=311). The comparison between the prevalence of invasion of the genera *Sylvia* and *Acrocephalus* is not affected by other factors than the host species (genus). We chose these two genera because they are suitable for such a comparison. First they include closely related species of birds, long-distance migrants, infected with the same species of haemoproteids, caught in the same time and in 75% of the cases at one site (Nova Cherna). Additionally the samples are enough by number. The age and the sex structure of the birds caught were of the same order. Of 16 birds of the genus *Locustella* studied, no invasion was found.

According to their migration status, the birds studied could be divided into 4 groups: local resident birds; local migratory birds (caught in the period 20.05 - 31.07 or individuals proved to be local breeders out of this period); spring migrants (caught in the period 1.02 - 20.05); and fall migrants (caught in the period 1.08 - 31.10). Birds with clear appearance of breeding, captured before 20.05, were considered as local breeders and birds from the species, which do not breed in Bulgaria, caught after 20.05 were considered as spring migrants. Local migratory birds are more often invaded than local resident birds (Fig. 1): 25.3% (n=91) and 5.8% (n=173), respectively (Fisher's exact test, p>0.999, n=264). Data on winter and late autumn periods were not included in the graph.

In Fig. 1 it is shown that spring migrants are more often invaded than fall migrants: 31.4% (n=236) and 13.3% (n=226), respectively (p>0.999, Fisher's exact test, p>0.999, n=462). This difference could be explained, on the one hand, by spring relapses of most haemosporidioses, and, on the other hand, by invasion of the birds during migration and wintering (Valkiunas 1997).

When studying the prevalence of invasions of birds by haemoproteids by months, the maximum was found in May (Fig. 2). The total prevalence in spring and summer is 23.4% (n=465), which is considerably higher than the autumn prevalence of 10.3% (n=291) (Fisher's exact test, p>0.999, n=756). This disparity varies among different taxonomical groups of birds and for some families, such as Turdidae; the rate is the opposite of that for birds as a whole (Fig. 3). In such cases, birds migrating from the north are probably from populations more heavily invaded than Bulgarian populations of the same species. Supporting this presumption is the fact that the invasion prevalence of local breeding birds is 12.5% (n=265), just over half the rate of transitory migrants (22.5%) (n=462), (Fisher's exact test p>0.999, n=727).

The intensity of haemoproteids invasions varies greatly. Most invasions are of low intensity, between 1 and 10 parasites per 100 microscope fields. This fact supports Valkiunas's (2000) assertion that heavily invaded birds are difficult to capture by the use of nets because they are more stationary. Very high rates of invasion have

been found in both spring and autumn. The highest intensity registered is 335 parasites per 100 microscope fields in the blood of a Lesser Grey Shrike (*Lanius minor*) (25.05.1999, Nova Cherna).

Three cases of mixed invasions of haemoproteids were observed in this study. Two Spotted Flycatcher (*Muscicapa striata*), were invaded with *H. balmorali* and *H. pallidus*, and a Marsh Warbler (*Acrocephalus palustris*), with *H. payevskyi* and *H. belopolskyi*. Similar cases have been reported frequently by other authors (Valkiunas 1997). Mixed invasions by haemoproteids and other groups of blood parasites (*Plasmodium, Leucocytozoon*) were observed in this study on 10 occasions. The prevalence of invasion by haemoproteids of birds infected with other genera of haemosporidians is 18.5 percent (n=54), a rate that is the same as that of all birds studied.

DISCUSSION

Of the 21 species of Haemoproteids found, 19 are new for the Balkan Peninsula. Finding *H. velans* in the Palaearctic is of considerable interest. To date, the only evidence of the distribution of this species in the Palaearctic has been finding in the blood of Palaearctic Wryneck (*Jynx torquilla*) wintering in the Indomalayan zoogeographical zone (Valkiunas 1997). The species *H. caprimulgi*, found in the blood of *Caprimulgus europaeus* in Bulgaria, is also very rare in the Palearctic (Valkiunas 1997). *H. attenuatus* was found in Bulgaria for the first time outside its typical locality. Several species of haemoproteids were found in new host species of birds: in Syrian Woodpecker (*Dendrocopus syriacus*) - *H. velans*; in Marsh Warbler (*Acrocephalus palustris*) - *H. payevskyi*; in Thrush Nightingale (*Luscinia luscinia*) - *H. attenuatus*; and in Reed Warbler (*Acrocephalus scirpaceus*) - *H. belopolskyi*.

In birds of local breeding populations, 14 species of parasites were found. Another 3 species were found in the blood of spring and fall migrants (*H. attenuatus, H. balmorali, H. pallidus*), 2 only in fall migrants (*H. anthi* and *H. motacillae*), and 1 only in spring migrants (*H. caprimulgi*).

The total prevalence of invasion of the birds studied is 18.5%. For a comparison with some other areas of Eurasia, information is provided in Table 7. These studies are comparable because the species composition, the number of birds studied, and the periods during which the birds were captured are similar. From these data, it is clear that the percentage of invaded birds in Bulgaria is higher than most other regions, including England, Poland, former Czechoslovakia and Kazakhstan. The prevalence of haemoproteids invasion in Bulgaria is lower than that of Curonian Spit in the Baltic Sea.

Variations in prevalence among the bird families have been reported by other authors (Jakunin 1972, Kučera 1981, Valkiunas 1985). Kučera (1981) identified the following families of passerines as the most heavily invaded in Central Europe: Hirundinidae, Emberizidae, Fringillidae, Paridae, Muscicapidae and Laniidae. All of these except the last 2 are among the least invaded in Bulgaria. At the same time, families such as Sylvidae, Turdidae and Motacillidae are considerably more often invaded in Bulgaria, as compared with Central Europe. It is possible that these differences depend not only on geographic location, but also on the year in which the birds are gathered.

In our study local migratory birds are more often invaded than local resident birds. Similar results were reported by Kucera (1981) for Central Europe. Valkiunas (1997) noted that in different studies there are contradictory data on this topic. The contradictions are mainly due to the differences in the composition of the species of the birds studied and the periods of capture (Valkiunas 1997).

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Redescription of the Rare Heterotrophic Flagellate (Protista) - *Phyllomitus undulans* Stein, 1878, and Erection of a New Genus - *Pseudophyllomitus* gen. n.

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Summary. *Phyllomitus undulans* Stein 1878 is redescribed by light-microscopy from live material found in marine sediments of Botany Bay, New South Wales, Australia. This species is 11-21 μm long, is somewhat flexible, has no gullet or pocket and has two flagella that adhere to each other and which arise at the anterior end of the cell. To accommodate the taxa without adhering flagella, which had previously been assigned to this genus, a new genus (*Pseudophyllomitus* gen. n.) is erected and contains 4 new combinations; *Pseudophyllomitus apiculatus* comb. n., *Pseudophyllomitus granulatus* comb. n., *Pseudophyllomitus salinus* comb. n., *Pseudophyllomitus vesiculosus* comb. n. One more new combination *Hemistasia amylophagus* comb. n. is introduced for one other species, *Phyllomitus amylophagus*. The evolutionary affinities of these genera (*Phyllomitus* and *Pseudophyllomitus*) cannot be established on the basis of present information, and they are placed among the protists as *incertae sedis*.

Key words: flagellate, kathablepharids, *Phyllomitus*, protist, Protista *incertae sedis, Pseudophyllomitus.*

INTRODUCTION

Heterotrophic flagellates are important numerically and ecologically in aquatic ecosystems both in the water column and in the benthos (e.g., Azam *et al*. 1983, Lee and Patterson 2002). Despite their importance, their taxonomy has until recently been little studied. Recently, the marine species have been the subject of a number of taxonomic studies (e.g., Vørs 1992a, b; Ekebom *et al*. 1996; Larsen and Patterson 1990; Thomsen *et al.* 1991, 1997; Patterson and Simpson 1996; Tong 1997a, b, c; Tong *et al*. 1997, 1998; Al-Qassab *et al*. 2002; Bernard *et al*. 2000; Lee and Patterson 2000; Lee 2002).

Many species of heterotrophic flagellates have been badly described and some species were introduced without descriptions or drawings (e.g., Lackey 1961, Lackey and Lackey 1970). Early descriptions may lack reference to characteristics, which have since proved necessary to distinguish species. Many species of heterotrophic flagellates are rare (Lee 2001) and have not been reported since their original descriptions. *Phyllomitus* is one such taxon. It was erected by Stein (1878) to accommodate *Phyllomitus undulans* from a freshwater site in Germany. This species was described as 21-

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30 µm long, is flexible and has two flagella that adhere to each other and emerge from a small depression (Figs 1a-f). Since then, 6 species have been included in *Phyllomitus* by Klebs (1893), Ruinen (1938), Lackey (1940), Skuja (1948) and Larsen and Patterson (1990). Later species do not have the adhering flagella mentioned by Stein (1878). This may be a useful diagnostic character for the genus (Larsen and Patterson 1990, Patterson and Zölffel 1991) and the assignment of these 6 species is therefore questionable.

Phyllomitus undulans is redescribed here to provide this species with a clear morphological identity, and a new genus, *Pseudophyllomitus,* is proposed to accommodate the taxa without adhering flagella which had previously been assigned to this genus.

MATERIALS AND METHODS

Phyllomitus undulans was encountered three times from September to November 1999, during a survey of the diversity of heterotrophic flagellates from marine sediments at Botany Bay in New South Wales, Australia (151°7' E; 33°59' S). The surface water temperature was 15-19.5°C, pH was 7.55-7.73 and salinity was 33-35 ‰. Sediments were taken from intertidal sandy sediments to a depth of about 1 cm from 1 m^2 quadrat using a flat spoon. The sediments were placed in plastic trays in 1 cm deep layers. Coverslips (No.1 22 x 22 mm) were placed on lens tissue laid on the sediments. After 12-24 h, flagellates were observed using a Zeiss Axiophot microscope equipped with photographic and video facilities (Lee and Patterson 2000).

RESULTS

Phyllomitus undulans Stein, 1878 (Figs 2a-e)

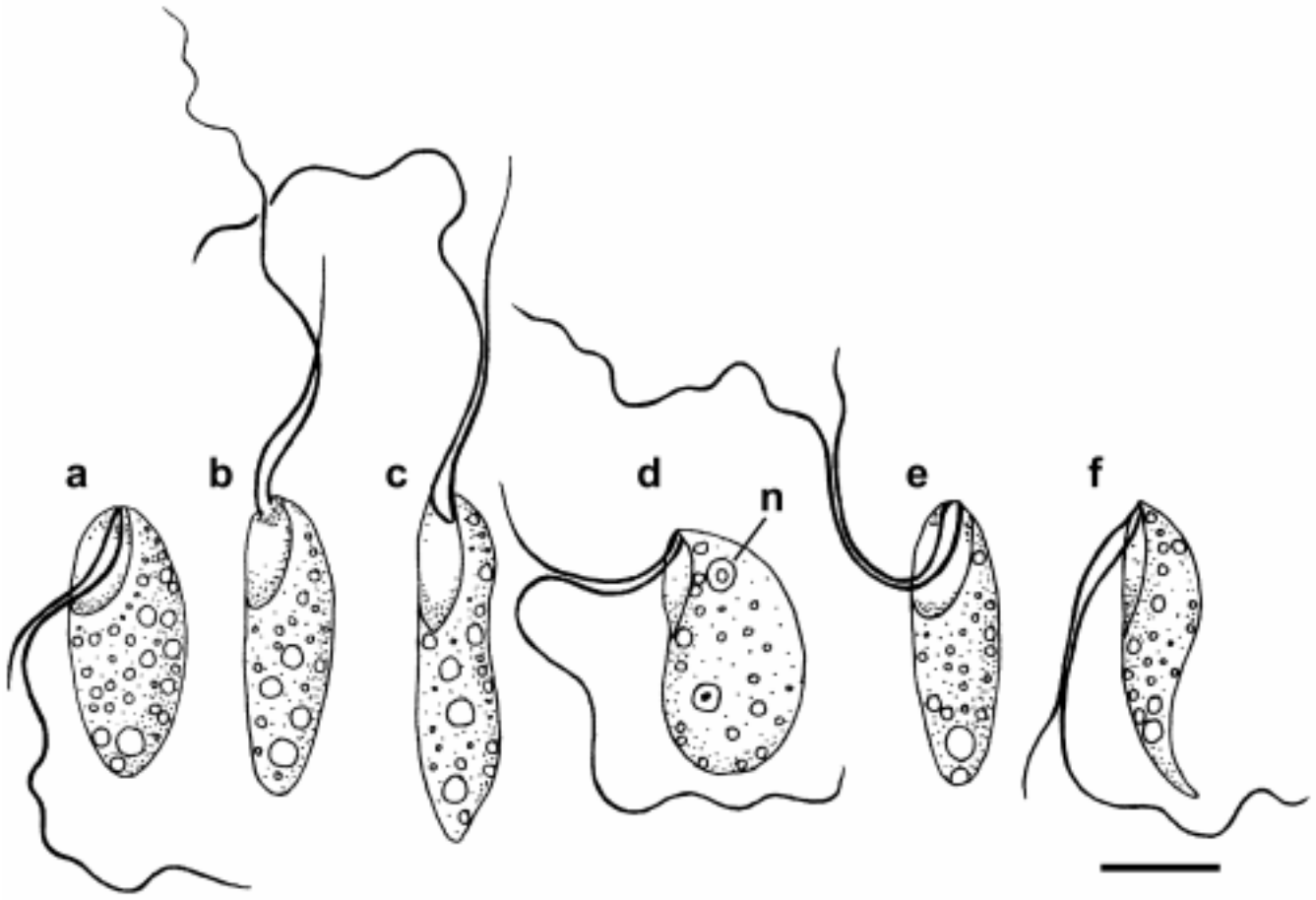
Cells are 11-21 µm long, slightly flexible with two flagella that adhere to each other. The cells are elongate and are pointed at both ends. The cells appear to be convex dorsally and concave ventrally. The flagella arise at the anterior end of the cell, appear to be similar in length about 3 times longer than the cell, and have an undulating beat. No anterior depression or pocket was observed. The cell surface is smooth and the nucleus is located on the mid-anterior part of the cell. Cytoplasmic strands may arise from the posterior end of the cell. The cells contained some food materials. The cells may attach to the substrate by the posterior end of the cell. Three cells were observed.

This species was also occasionally found at Moreton Bay Prawn Farm (Queensland, Australia) with cell lengths from 13 to 21 µm and reported without a name (Blackmore 1997). Only one record (Rico 1985) of the type species has been made. This was without a description or photos and was from soil samples in the San Juan Tezompa (Mexico).

DISCUSSION

Phyllomitus undulans is a distinctive species of flagellate because it has two flagella, which adhere to each other. The cells described by Stein (1878) and observed here have two adhering flagella and somewhat a pointed posterior end. The cell length ranges overlap, although I note the magnification given by Stein (1878) appears to be inaccurate, and common organisms reported by him are 1.5-2 times bigger than reported by others. This species was originally reported with an anterior depression but this could be an inaccurate description of the concave ventral side. Consequently, the cells described here are regarded as *P. undulans.*

The following species have been included in *Phyllomitus* (Fig. 3): *Phyllomitus amylophagus,* Klebs, 1893, *P. apiculatus* Skuja, 1948, *P. granulatus,* Larsen et Patterson, 1990, *P. salinus* Lackey, 1940, *P. vesiculosus* Larsen et Patterson, 1990, *P. yorkeensis,* Ruinen, 1938. None of these species have two adhering flagella. *Phyllomitus yorkeensis* was transferred to *Palustrimonas* by Patterson and Simpson (1996) because the body of *P. yorkeensis* is not very plastic and has two opposed flagella, which insert subapically and close together in separate grooves. *Phyllomitus amylophagus* (Fig. 3a) is transferred to *Hemistasia* (*H. amylophagus* comb. n.). It does not have two adhering flagella and it shares cell shape, cell length, flexibility and having a spiral groove with *Hemistasia*. The spiral groove in *H. amylophagus* was not shown in Klebs (1893)'s drawings (Fig. 3a), but he noted it in the text. Additionally, Elbrächter *et al*. (1996) noted that *P. amylophagus* needed re-investigation and may be transferred to *Hemistasia,* and Myl'nikov (personal communication) is also of the view that *Phyllomitus amylophagus* belongs to *Hemistasia* on the basis of ultrastructural evidence (Myl'nikov *et al*. 1998). *Hemistasia amylophagus* most closely resembles *Hemistasia phaeocysticola* (Scherffel, 1900) Elbrächter *et al.* 1996 and these species are difficult to distinguish by light-microscopy. They can be distinguished by electron-microscopy (Myl'nikov *et al*. 1998) because *H. phaeocysticola* has no microtubular prism



Figs 1 a-f. *Phyllomitus undulans* (after Stein 1878), nucleus (n). Scale bar -10 μm

(nemadesm), it has distinct glycocalyx-like coat on the plasmalemma and has more than 11 extrusomes. *Hemistasia amylophagus* has no cysts or cyst-like bodies, no division cyst (Myl'nikov *et al*. 1998), and the cytoplasm of *H. phaeocysticola* has swollen peripheral lacunae (see Elbrächter *et al*. 1996, p127, Fig. 5).

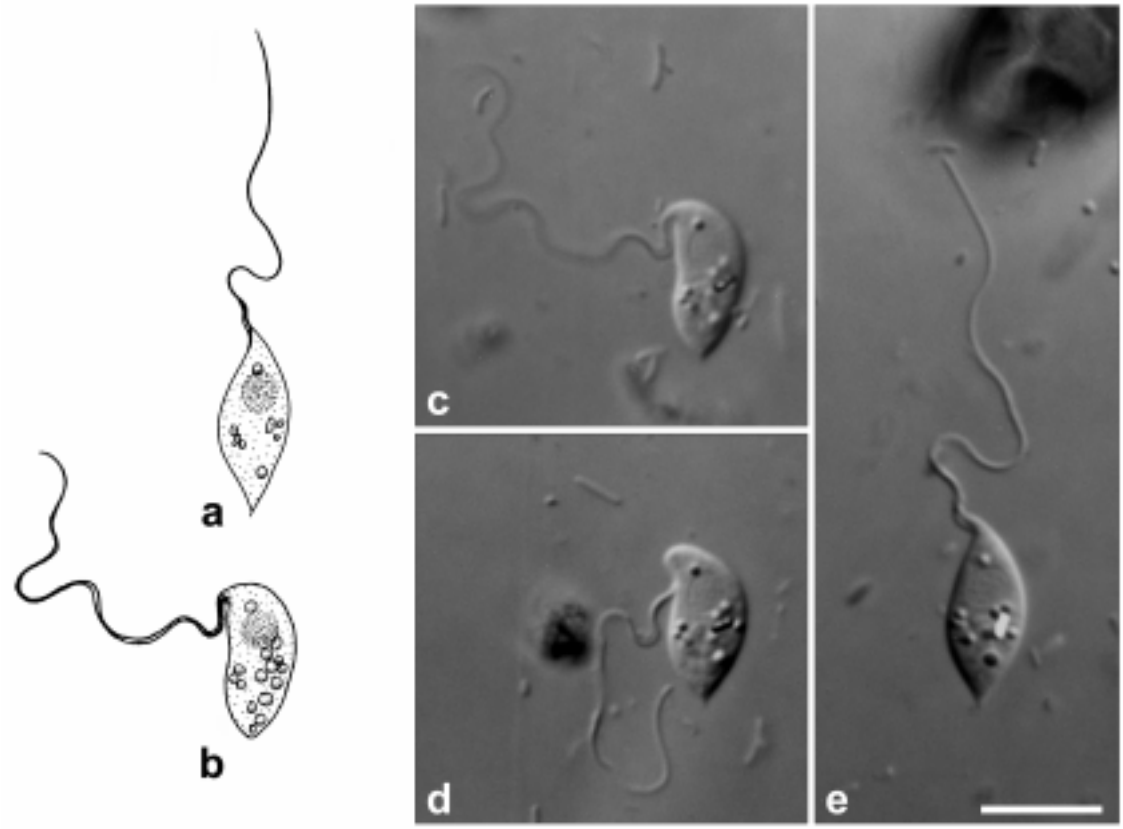
Larsen and Patterson (1990) and Patterson and Zölffel (1991) noted that the adhesion of the two flagella mentioned by Stein (1878) might be a good character to distinguish the genus *Phyllomitus*. On this basis, the remaining species, other than *P. undulans* currently placed in the genus would be best located in a different genus. I adopt their view and erect the new genus *Pseudophyllomitus* to accommodate the taxa without adhering flagella. This genus may contain unrelated taxon (see *P. apiculatus*), but I believe that it is appropriate to retain it in a single genus until more information is available.

***Pseudophyllomitus* gen. n.**

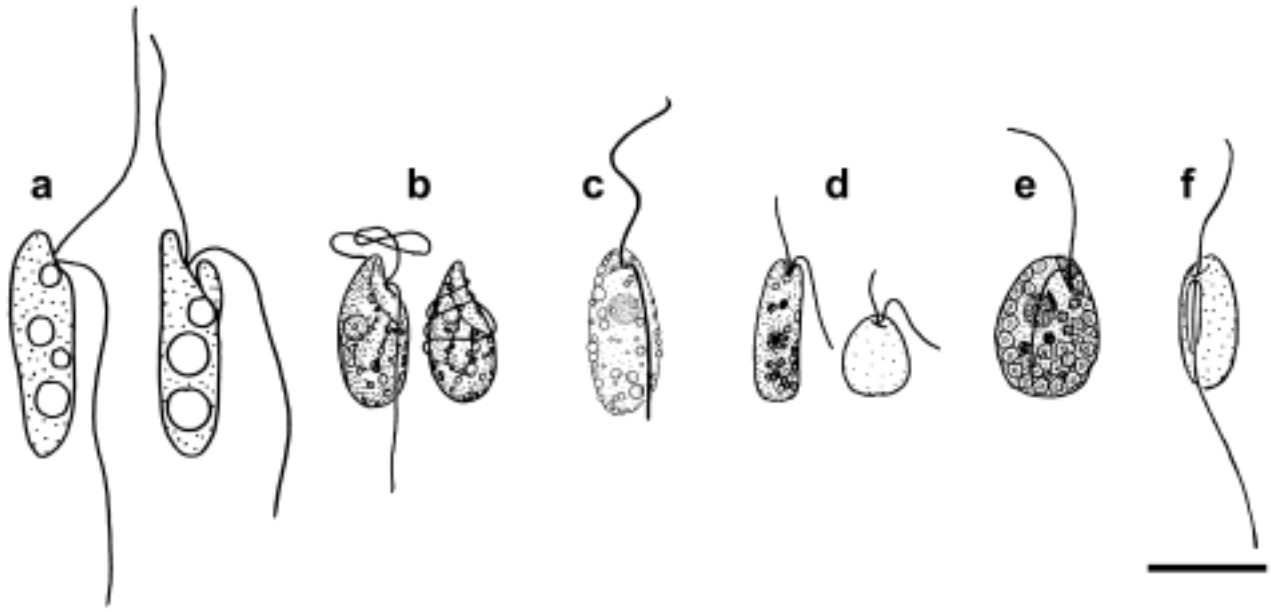
Diagnosis: free-living heterotrophic, flexible, sac-shaped protists with two flagella, which do not adhere to each other and which insert subapically into a gullet or pocket. One flagellum is directed anteriorly and the other one trails posteriorly. Cells swim and may glide. The genus contains 4 nominal species.

Type species: *Pseudophyllomitus granulatus* (Larsen et Patterson, 1990) comb. n.

Discussion of similar taxa: *Pseudophyllomitus* differs from *Phyllomitus* in that its flagella emerge subapically from a gullet or pocket and because one flagellum is directed anteriorly and the other one trails posteriorly. This genus resembles *Colponema* Stein, 1878 in being highly flexible and in having two flagella emerging from a deep gullet of pocket, but is distinguished by the lack of a groove extending the length of



Figs 2 a-e. *Phyllomitus undulans*; **a-b** - drawings of the species; **c-e** - cells from Botany Bay, note the two adhering flagella, DIC images. Scale bar - 10 μm



Figs 3 a-f. **a** - *Hemistasia amylophagus* comb. n. (after Klebs 1893 under the name *Phyllomitus amylophagus*); **b** - *Pseudophyllomitus apiculatus* comb. n. (after Skuja 1948); **c** - *Pseudophyllomitus granulatus* comb. n. (after Lee and Patterson 2000); **d** - *Pseudophyllomitus salinus* comb. n. (after Lackey 1940); **e** - *Pseudophyllomitus vesiculosus* comb. n. (after Larsen and Patterson 1990); **f** - *Palustrimonas yorkeensis* (after Patterson and Simpson 1966 under the name *Phyllomitus yorkeensis*). Scale bar - 10 μm

the cell (Larsen and Patterson 1990). *Pseudophyllomitus* is similar to *Heterochromonas* Pascher, 1912 in being flexible and in cell shape and length, but can be distinguished because the flagella are directed anteriorly in *Heterochromonas*. This genus is also similar to *Palustrimonas* Patterson et Simpson, 1996 which contains only one species (*P. yorkeensis*), but can be distinguished because *Palustrimonas* is not highly flexible or metabolic and the two flagella insert separate prominent grooves.

***Pseudophyllomitus apiculatus* (Skuja, 1948) comb. n. (Fig. 3b)**

Basionym: *Phyllomitus apiculatus* Skuja, 1948

Cells are 11-15 μm long and 4-5.5 μm wide, flexible, somewhat flattened laterally, and narrowed anteriorly with a pocket. Two flagella emerge from the pocket. The anterior flagellum is about the cell length and winds around the anterior end of the cell, and the posterior flagellum is 1.5-2 times the cell length and extends posteriorly. In the resting stage, the two flagella wind spirally around the body. The contractile vacuole is located in the anterior end of the cell. The cells feed on detritus and small algae through the pocket. The cells move by swimming with rotating movements.

This species has been reported from freshwater sites in Germany and Sweden (Skuja 1948, 1956; Steinberg *et al*. 1983). The drawings of Steinberg *et al*. (1983, p308, Abb.1) appear to be different to the original drawing of Skuja (1948) and appear to be a species of *Rhynchobodo* (maybe *R. agilis*). *Phyllomitus apiculatus* mostly resembles *Rhynchobodo conoidea* (Skuja, 1956) Bernard *et al*. 2000 in general appearance, cell length (*R. conoidea* is 11-14 μm long), and in being flexible and in having a ventral pocket and a rotating movement. Both species were recorded in Skuja (1956), but found in different locations. There is one ultrastructural study on organisms with this name (Myl'nikov 1986), but according to Patterson and Zölffel (1991) the cells studied are *Rhynchobodo armata* of Brugerolle (1985). This species may belong to *Rhynchobodo*, sharing with that genus a lateral deep pocket, rotating movements, and its two flagella, which wrap around the body of resting cells. Elbrächter *et al*. (1996) noted that this species needs reinvestigation and may be transferred to *Hemistasia*. I retain this species as a member of the genus *Pseudophyllomitus* until further ultrastructural or molecular information becomes available.

***Pseudophyllomitus granulatus* (Larsen et Patterson, 1990) comb. n. (Fig. 3c)**

Basionym: *Phyllomitus granulatus* Larsen et Patterson, 1990

Cell outline is sac-shaped. Cells are flexible, 7-21 μm long and slightly flattened being elliptical in cross section. Two flagella emerge subapically. The anterior flagellum beats with a sine-wave, is 1.0-1.5 times the cell length and is directed to the front and slightly to the right during swimming. The posterior flagellum inserts to the left of the anterior flagellum, varies in length from 0.5 to 2.5 times the cell length and trails behind the cell when swimming. Cytoplasm is drawn out at the posterior end. Refractile granules underlie the cell surface. The nucleus is located below the anterior pocket, near the centre of the cell and is roundish. The cells contain ingested eukaryotic algae. When eating, food materials are driven into the pocket, and the cell becomes very plastic (almost amoeboid) extending as a very thin layer around the food. Common at times.

This species has been reported from marine sites in subtropical and tropical Australia, Brazil, Denmark, Hawaii and Korea (Larsen and Patterson 1990, Vørs 1992b, Lee and Patterson 2000, Lee 2002). This species is similar to *P. salinus* in general appearance and cell length, but *P. salinus* has shorter flagella. Further studies are required to distinguish these two species.

***Pseudophyllomitus salinus* (Lackey, 1940) comb. n. (Fig. 3d)**

Basionym: *Phyllomitus salinus* Lackey, 1940

Cells are about 12 μm long and 5 μm wide. The cells are cylindrical, typically elongate, and metabolic with two flagella emerging from a subapical depression. The posterior flagellum is about two-thirds the cell length, the anterior one is about 0.5 times the cell length. The nucleus is located in the centre of the cell. Cytoplasm clear, granular, a few small spheres sometimes present. Nutrition and reproduction not ascertained. Very common at times.

This species was found in USA (Lackey 1940).

***Pseudophyllomitus vesiculosus* (Larsen et Patterson, 1990) comb. n. (Fig. 3e)**

Basionym: *Phyllomitus vesiculosus* Larsen et Patterson, 1990

Cells have a sac-shaped body, are 11-15 μm long, and are slightly dorso-ventrally flattened. The cells have two

flagella arising from large anterior depression; the anterior flagellum extends in front of the cell with a long sweeping curve and the posterior flagellum trailing behind. The nucleus is located near the centre of the cell, adjacent to the base of the flagella; a rod or bar lies against one anterior side of the nucleus. The outer region of the cytoplasm is highly vesiculate. The cells move by swimming, frequently in the immediate vicinity of the substratum, with the posterior flagellum dragging against the substratum.

This species has been found from tropical Australia (Larsen and Patterson 1990). It is easily distinguished from other species of *Pseudophyllomitus* because the cell surface is underlain with a layer of vesicles.

Taxonomic position of these genera

Many heterotrophic flagellates have no evident affinities with other types of protists (Patterson 1999). The affinities of most genera studied by light-microscopy only cannot be established without further ultrastructural or molecular studies (Patterson 1999). This situation applies to *Phyllomitus* and *Pseudophyllomitus*.

Early attempts were made to locate *Phyllomitus* on the basis of light microscopy alone, but they have not stood the test of time. Lemmermann (1914) placed *Phyllomitus* (*P. undulans* and *P. amylophagus*) in the family Bodonaceae, and Pringsheim (1944) placed *Phyllomitus* in the family Kathablepharidaceae Skuja 1939 (ICBN) = Kathablepharidae Vørs 1992 (ICZN) at that time erroneously treating this family as a component of the Cryptophyceae. None of the four genera included in this family (*Kathablepharis* Skuja, 1939, *Leucocryptos* Butcher, 1967, *Phyllomitus* Stein, 1878 and *Platychilomonas* Larsen et Patterson, 1990) have anything more than a superficial similarity with the true cryptomonads, leading to the conclusion that these taxa do not belong to the Cryptophyceae (Lee and Kugrens 1991; Lee *et al*. 1991a, b; Vørs 1992a, c; Clay and Kugrens 1999a). *Phyllomitus* should be removed from the kathablepharids and cryptomonads because it lacks the characteristics of both groups such as the rigid body and the ejectisomes (Clay and Kugrens 1999b). Although *Pseudophyllomitus apiculatus* may belong to Kinetoplastida (see above) and *P. granulatus* may belong to stramenopiles due to the beating pattern of the anterior flagellum (see Larsen and Patterson 1990, Lee and Patterson 2000, Lee 2002), *Phyllomitus* and *Pseudophyllomitus* cannot be confidently assigned to any major group of flagellates, and are regarded as Protista *incertae sedis* until ultrastructural or molecular information becomes available.

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Development of *Schroedera plumatellae* gen. n., sp. n. (Microsporidia) in *Plumatella fungosa* (Bryozoa: Phylactolaemata)

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Summary. A new microsporidian, *Schroedera plumatellae*, is described infecting a freshwater bryozoan. The development of the infection is described both at the light and ultrastructural level. The infection induces the formation of hyperplastic testes within the bryozoan host from which cord-like xenomas form. Development of diplokaryotic sporonts is preceded by haplophasic schizogony. Sporogony forms diplokaryotic spores. The spores formed within the xenomas are oval, tapering to one end and were 7.2 ± 0.3 μm by 5.0 ± 0.3 μm with 22-23 turns of the polar filament. Sporogony occurred within the hyaloplasm with the exospore developing into two distinct layers. Phylogenetic analysis showed that the parasite was most closely related to a *Bacillidium* sp. and *Janacekia debaisieuxi*.

Key words: *Bacillidium,* Bryozoa, Microsporidia, *Plumatella fungosa*, *Schroedera plumatellae* gen. n., sp. n.

INTRODUCTION

Phylactolaemate bryozoans are colonial, filter-feeding, hermaphrodites. The testes of these bryozoans develop on the funiculus, a cord of peritoneal cells and muscle fibres that connect the stomach caecum with the body wall. In 1892, Korotneff described a parasite developing within the spermatogonia of *Plumatella fungosa* (Pallas). This parasite was initially named *Myxosporidium bryozoides* within the group "Myxosporidien" but was then transferred into the microsporidian genus *Glugea* (Thélohan 1895). Labbé subsequently transferred the parasite to the genus *Nosema* in 1899.

Since its initial description, *Nosema bryozoides* has been reported in several species of freshwater Bryozoa from around the world. However, these descriptions vary both for the form of the spore and the development of the parasite within the bryozoan hosts. Differences for the site of infection have also been noted between authors. For example, Korotneff (1892) described the development of the parasite as occurring exclusively within the spermatogonia of the bryozoan while Marcus (1941), describing the parasite within *Stolella evelinae* (Marcus), observed parasite development not only within the spermatogonia but also affecting the epithelial cells of the body wall. Marcus (1941) further described the parasite as forming elongated cords from the funiculus that filled the coelom of the bryozoan. These cords are

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contrary to the lobules figured by Korotneff (1892) and Braem (1911). Schröder (1914) comparing the reports of Korotneff (1892) and Braem (1911), highlighted differences between them regarding the development of the parasite, although he attributed this to different interpretations made on the same parasite species. However, Schröder in his paper also included his own observations on *N. bryozoides* and reported on the development of ellipsoidal spores of two different sizes that slightly tapered to one end. The measurements given for the spores also differ between authors. These differences, particularly between the papers of Schröder (1914) and Marcus (1941), suggest that the reports of *N. bryozoides* in the literature are referring to different microsporidian species in bryozoa rather than observations on the same parasite species. Furthermore, the discovery that bryozoans can act as hosts for several species of Myxozoa has led to the suggestion that the parasite described by Korotneff may have been a myxozoan. As such, the name *N. bryozoides* would be synonymous with a myxozoan and therefore could not be used for a microsporidian (Canning *et al.* 1997).

Recently a new species of microsporidian *Nosema cristatellae* has been reported from the bryozoan *Cristatella mucedo* (Canning *et al*. 1997). This parasite was differentiated from previous descriptions of *N. bryozoides* by both its host and tissue specificity. Here we describe a microsporidian infecting the spermatogonia of *P. fungosa*. Development of this parasite is described both at the light and ultrastructual level. Phylogenetic analysis of the parasites' small subunit (SSU) rDNA sequence and aspects of its development suggest that it is a new species that we propose to call *Schroedera plumatellae* gen. n. *et* sp. n. The terms used for the description of the development of this parasite are derived from the glossary of Sprague and Becnel (1999).

MATERIALS AND METHODS

Collection and identification of colony

A bryozoan colony was obtained, attached to a submerged branch collected from the southern end of Airthrey Loch, University of Stirling, Stirling, Scotland in July 2001. Preliminary examination of the colony using a x10 hand lens suggested that it was a single entity with no evidence of fragmentation. It was removed along with the adherent bark, from the branch using a penknife. Macro-invertebrates were carefully removed under an inverted microscope, using forceps and a pipette. The bark pieces were then glued onto the centre of a 9 cm plastic Petri dish using a cyanacrylate adhesive.

Culture and light microscopy of colony

The colony was cultured following the protocol of Morris *et al.* (2002). Briefly, the Petri dishes were suspended vertically in a 7 l plastic aquarium, containing vigorously aerated, artificial freshwater maintained at 20°C. The colony was fed using cultured *Cryptomonas ovata* (Pringsheim), *Synococcus leopoliensis* (Komárek), *Chilomonas paramecium* (Ehrenberg) and *Colpidium striatum* (Stokes). The colony was removed from the aquarium and examined daily at x40 under an Olympus inverted microscope for any parasite development. As bryozoans grown in the laboratory are transparent it is relatively straightforward to identify parasites within them.

At the termination of the study, statoblast morphology was used to verify the infected bryozoan species. The statoblasts were dissected from the colonies and examined with scanning electron microscopy. Observations made on the colony form of the bryozoan, in conjunction with the SEM examination, were used to identify the bryozoan using the published keys of Mundy and Thorpe (1980) and Ricciardi and Reiswig (1994).

Transmission electron microscopy

Portions of colony, were fixed in Karnovsky's fixative for 4 h and rinsed in cacodylate buffer (pH 7.2) overnight. They were then post-fixed in 1% osmium tetroxide for 1 h, dehydrated through an acetone to alcohol series and embedded in Spurr's resin. Ultrathin gold/silver sections were mounted on formvar coated grids and stained with lead citrate and uranyl acetate. The sections were viewed at 80kV using a Phillips 201 electron microscope. Semi-thin sections (1 μm) were also taken from the blocks, stained with methylene blue-fuchsin for examination of infected tissue using light microscopy.

Molecular characterisation

A portion of colony, consisting of 3 zooids that possessed hyperplastic testes was removed and the DNA extracted using an ABGene Magnetic separation kit following the manufacturers protocol. The small subunit (SSU) rDNA gene from this DNA was amplified using primers V1f and 1942r in a PCR reaction as described by Nilsen (2000). When visualised on a gel, this gave a product of the expected size of ~1300bp. This product was excised from the gel and the DNA purified using a GFX column (Pharmacia). The product was sequenced directly with the original primers and a further two sequencing primers '5-TACCAGGGCCGAATGTTTTA-3' and '5-TCACTACCTCTCCCCATAGGGA-3' using the DYEnamic sequencing kit (Amersham Pharmacia). The sequencing was performed on an ABI Prism 377 sequencer (Perkin Elmer). The initial PCR reaction was repeated and the product sequenced as before to confirm the final sequence.

Phylogenetic analysis

The obtained SSU sequence was aligned to other microsporidial sequences obtained on the GenBank database using ClustalX. These sequences were as follows: *Amblyospora californica* (U68473); *Amblyospora* sp. (U68474); *Amblyospora connecticus* (AF25685); *Amblyospora stimuli* (AF027685); *Bacillidium* sp. (AF104087); *Culicosporella lunata* (AF027683); *Edhazardia aedis* (AF027684);

Encephalitozoon cuniculi (L39107); *Encephalitozoon hellem* (L39108); *Endoreticulatus schubergi* (L07123); *Glugea anomala* (AF104084); *Glugea atherinae* (U15987); *Ichthyosporidium* sp. (L39110); *Janacekia debaisieuxi* (AJ252950); *Kabatana takedai* (AF356222); *Loma* sp. (AF104081); *Microgemma* sp. (AJ252952); *Microsporidium prosopium* (AF151529); *Nosema algerae* (AF069063); *Nosema bombi* (U26158); *Nosema bombycis* (D85504); *Nosema carpocapsae* (AF426104); *Spraguea lophii* (AF0104086); *Thelohania solenopsae* (AF134205); *Trachipleistophora hominis* (AJ002605); *Vairimorpha necatrix* (Y00266); *Vavraia oncoperae* (X74112); *Visvesvaria acridophagus* (AF024658); *Visvesvaria algerae* (AF024656) and *Vittaforma corneae* (L39112). Regions of ambiguous alignment were visually identified and removed. Phylogenetic analyses were conducted on the aligned sequences using MEGA version 2.1 (Kumar *et al.* 2001). The data were analysed using maximum parsimony analysis and neighbour-joining for distance analysis with the Kimura-2 algorithm. Maximum likelihood analysis was also performed on the aligned sequences using fastDNAml (Felsenstein 1981, Olsen *et al*. 1994).

RESULTS

Bryozoa colony

After one week the bryozoan colony, growing on the surface of the Petri dish, appeared parasite free. After 27 days in culture, a portion of the colony appeared to be parasitised. The infection eventually spread throughout the whole colony.

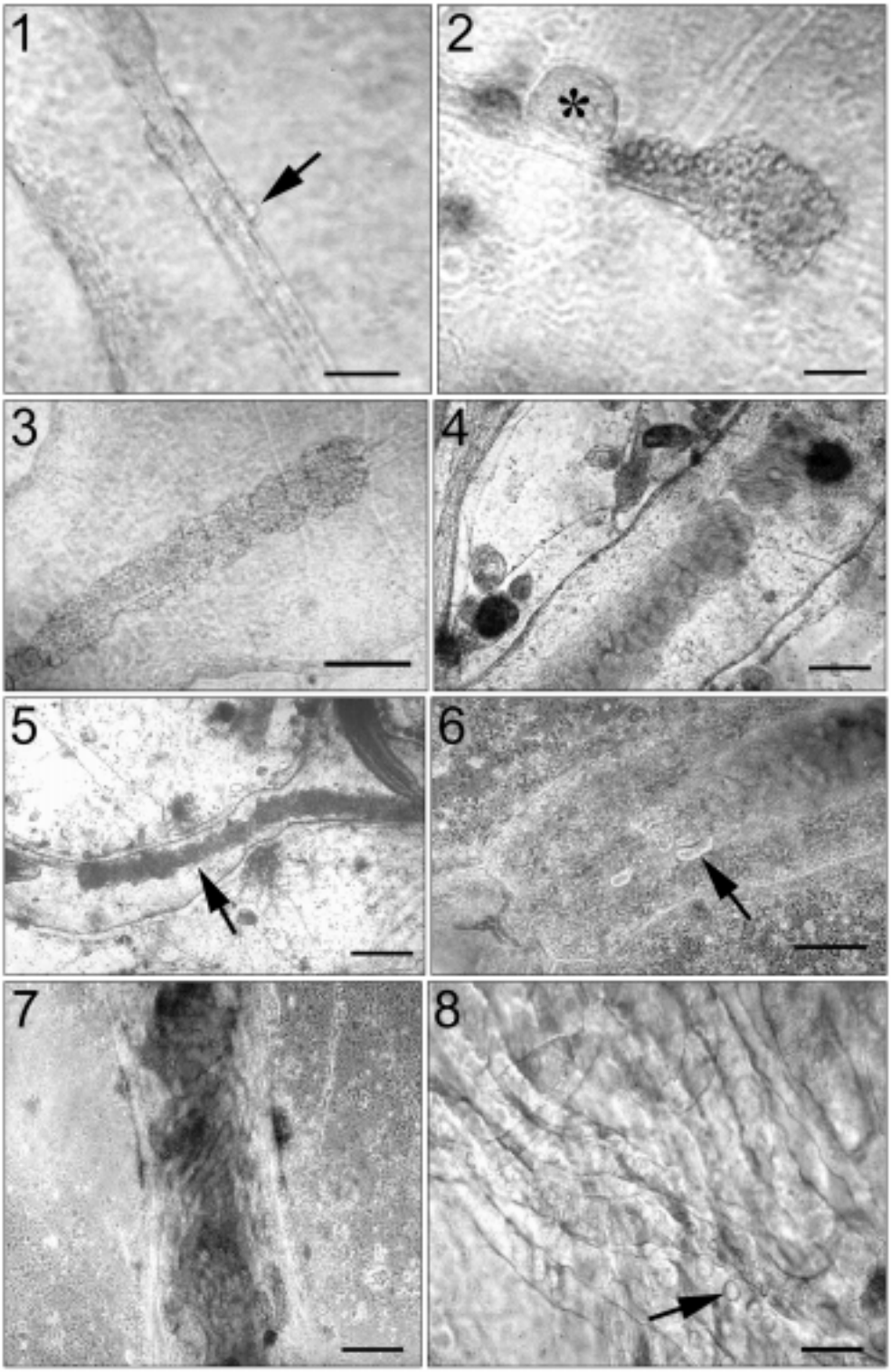
Infected portions of the colony did not produce mature statoblasts, however it was possible to extract mature statoblasts from those branches of the colony that had grown onto the Petri dish before the infection became apparent. Examination of these statoblasts using SEM determined that the bryozoan was *Plumatella fungosa*.

Examination of Microsporidia development by examination of live colonies

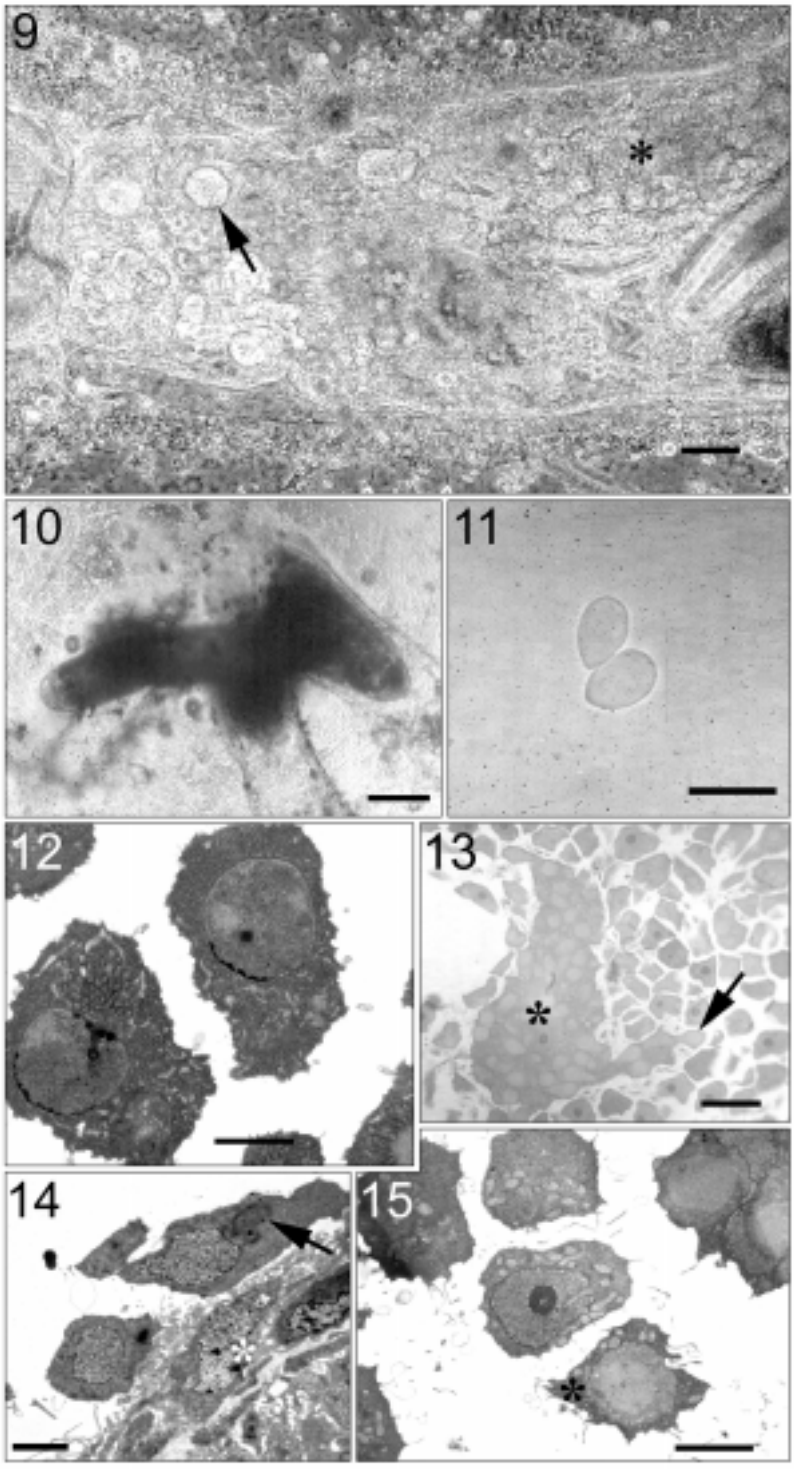
The initial observation of parasitised zooids consisted of single cells appearing on the peritoneum of the funiculus of a zooid (Fig. 1). These cells appeared to replicate, forming bundles of cells along the funiculus (Figs 2, 3). As the infection progressed, the cell bundles produced flagella that hung in the coelom of the bryozoan, wafting in currents of coelomic fluid (Fig. 4). The development of the tissue at this point was indistinguishable from that for the spermatogonia of normal phylactolaemate bryozoan testes. However, unlike normal testes, more of the funiculus became replaced with spermatogonia until it appeared to be totally comprised of these cells. The length of the tissue continued to grow until it was up to three times the length of a normal funiculus (Fig. 5). Seven days after the initial spermatogonic cells were noted, the proliferation of cells appeared to halt and a second developmental phase started (Fig. 6). This took the form of the spermatogonic cells becoming hypertrophied, developing into elongate cord-like xenomas, that hung freely in the coelom of the bryozoan. Flagella were not observed on these cords. As more of the infected cells developed into cords, they totally filled the coloemic space entwining together, forming a tangled mass that surrounded the zooid and the coelom immediately posterior to it (Fig. 7). These masses gave the infected zooids an opaque, white appearance when viewed by the naked eye. Within many of the cords microsporidian spores and developmental stages could be observed (Fig. 8). During this time constrictions were noted along the length of some of the cords, eventually leading to bundles of spores being pinched off (Fig. 9). The bundles of spores being delimited by the plasma membrane of the host cell. The cytoplasm surrounding the spore bundles reduced until it appeared as a thin walled sac. When the coelom had become filled with the parasitised cells the zooid possessing the infected funiculus began to atrophy and degenerate. The degeneration of the zooid sealed the coelom containing the spore masses from the rest of the bryozoa colony, forming a sealed tube full of spore bundles. This portion of the colony containing the spore bundles would gradually shrink until only an opaque mass of spores contained within the chitinous remains of the bryozoan remained (Fig. 10).

Development of hyperplastic testes occurred on all of the zooids in the infected colony. Although the parasite apparently induced testes development and spermatogenesis, mature spermatozoa were never observed, suggesting that the bryozoan was effectively castrated by the infection. Development of statoblasts although noted to begin during the early stages of infection (Fig. 2) was suppressed on parasitised zooids and presumably the developing statoblasts were absorbed as the parasite infection progressed along the funiculus. Parasite development was only ever observed associated with the funiculus of the bryozoan. No other tissues apart from those on the funiculus were noted to be infected with the microsporidian.

Some spore bundles that had become detached from the main mass of spores were observed free in the coelom of the bryozoan host moving from the diseased zooid into 'healthy' areas of the colony. These bundles were ejected from the bryozoan by the lophophore of



Figs 1 - 8. *Schroedera plumatellae* sp. n. **1** - first sign of infection on the funiculus in a living colony of Bryozoa. Arrow indicates cell developing from the peritoneum of funiculus; **2** - development of clump of cells on funiculus. An immature statoblast is also developing upon the funiculus (*); **3** - continued development of cells along the funiculus; **4** - development of spermatids on funiculus, the infected tissue resembling testis with the tails of the spermatids visible hanging in the coelom of the bryozoan; **5** - hyperplasia of spermatogonic tissue. Arrow indicates the expected length of a non-infected funiculus; **6** - start of development of cord-like xenomas from the infected spermatogonia (arrow); **7** - coelom of bryozoan completely filled with cord-like xenomas derived from the spermatogonia; **8** - detail of cord-like xenomas within bryozoan coelom. Within the xenomas developing microsporidian spores can be observed (arrow). Scale bars: 1, 2 - 20 μm; 3 - 100 μm; 4, 6, 7 - 150 μm; 5 - 350 μm; 8 - 30 μm



Figs 9-15. *Schroedera plumatellae* sp. n. **9** - breakdown of xenomas into spore bundles. Arrows indicate discrete bundles of spores that have separated from the xenomas. As more spores develop within the cord-like xenomas the cytoplasm reduces giving the cords a knotted appearance (*). Phase contrast microscopy; **10** - dead part of bryozoan colony filled with spore bundles, giving it an opaque appearance; **11** - fresh spores of *S. plumatellae*; **12** - spermatids associated with normal testis development observed in infected bryozoan colony. Heterochromatin is clearly deposited near the nuclear membrane, with mitochondria clustering around the forming midpiece; **13** - spermatogonic cell (*) infected with haplophasic schizonts. This cell is surrounded by numerous associated spermatids. Note the lobed appearance of parts of the spermatogonic cell (arrow); **14** - spermatids, associated with infected spermatogonic cell, adjacent to the peritoneum of the bryozoan coelom (*). Note irregular shaped nuclei of spermatids and presence of an amorphous structure in one of the spermatids (arrow); **15** - spermatids associated with infected spermatogonic cell (top right hand corner). A spermatid (*) is infected with a schizont. Scale bars: 9 - 75 μm; 10 - 350 μm; 11 - 10 μm; 12 - 2 μm; 13 - 20 μm; 14, 15 - 3 μm

both the infected and neighbouring zooids. When this happened the lophophore would retract, and the spore bundle would be released from between the tentacles. The exact route of exit could not be determined due to compression of the tissue during the retraction of the lophophore. Ejected spore bundles were collected by pipette, transferred to a glass microscope slide and placed under a coverslip. Pressure applied on the coverslip allowed the bundle to rupture and for the number of spores within the bundles to be counted under x200 magnification. Using this method, the contents of 6 spore bundles were examined and found to contain variable numbers of spores from 122 to 312. Some spores at this time were noted to partially extrude an isofilar polar filament of over 40 μm in length. Complete extrusion however, was not noted. The spores were ovoid, tapering to one end and measured 5.0 ± 0.3 μm by 7.2 ± 0.3 μm (n=22) (Fig. 11). Very occasionally, larger spores 10.0 μm by 6.0 μm of an identical shape to the smaller spores were observed.

The infection did not affect all of the zooids in a single branch at the same time, although all branches of the colony were finally infected. The development of the parasites appeared to depend upon the age of the zooid. Young and developing zooids at the end of a branch did not appear to be infected. However, the further away from the growing terminal of the branch the zooid was, the more pronounced the infection. This resulted in the colony quickly becoming fragmented for as fast as zooids were produced by the growing end of a branch of the colony, they were dying at the other end of the colony to be replaced by bundles of spores.

Spore sacs naturally extruded from the bryozoans were occasionally observed to be ingested by neighbouring zooids. Although, it could not be determined whether these spores were capable of infecting other zooids or if the subsequent infection of zooids occurred by sharing a common coelom with the infected colony.

Examination of microsporidian development using fixed tissue

Samples for TEM were taken during the infection within the funiculus tissue, during the production of elongate cord-like xenomas and during the degeneration of a zooid.

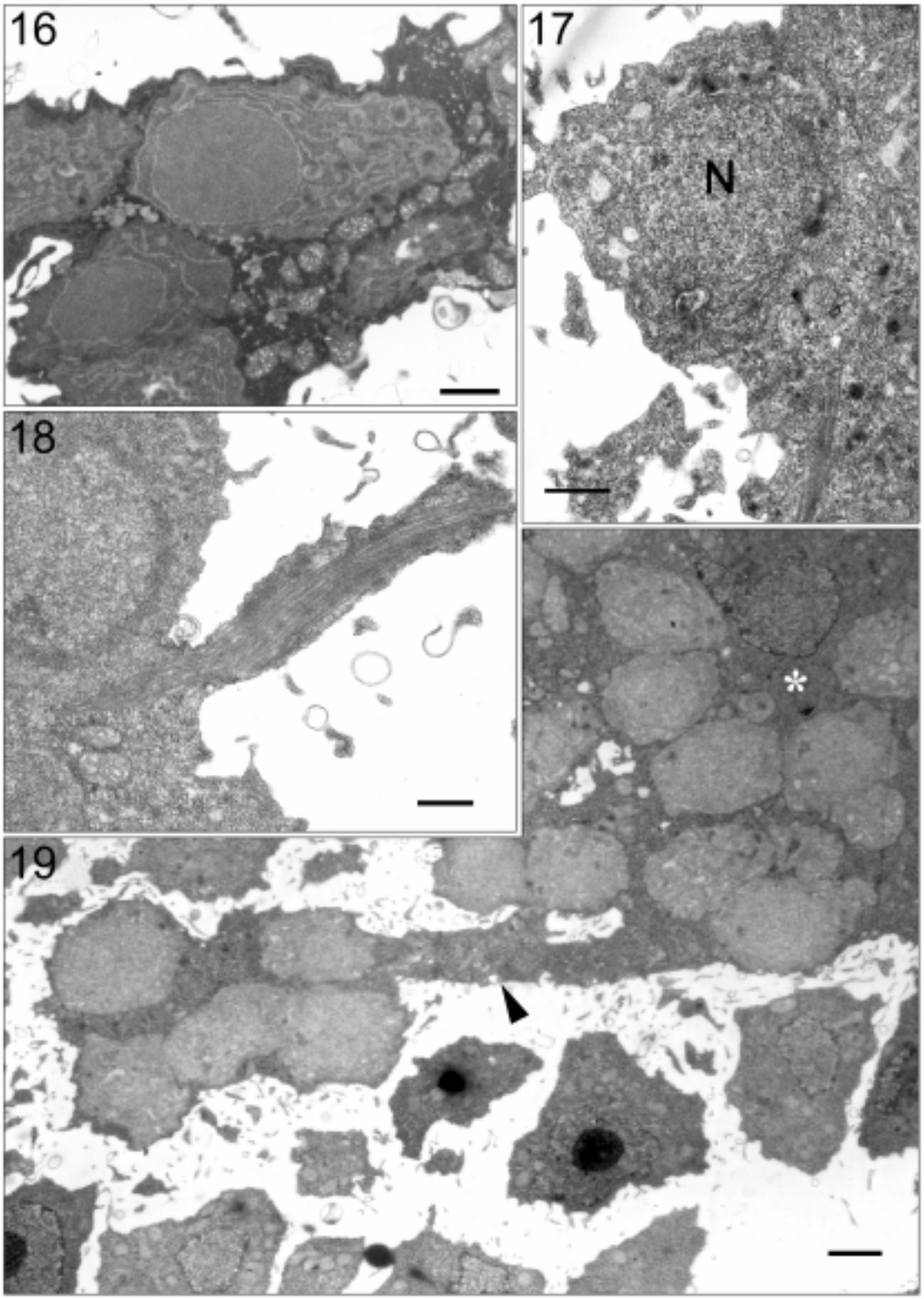
Infection of the funiculus

Sections through the bryozoan demonstrated areas of normal testis development and other, neighbouring areas that comprised of microsporidian infected spermatogonic cells and affected spermatids. The interface between the infected and non-infected parts of the testis, however, could not be determined.

Normal spermatid development was determined by comparison of these areas of the tissue with a previous ultrastructural description of spermatogenesis for *P. fungosa* (Franzén 1982). The observed spermatids were connected to the funiculus and each other by very fine cytoplasmic filaments. They were characterised by the formation of heterochromatin on the nuclear envelope, insertion of a centriole in the nucleus and the formation of midpiece and tail regions (Fig. 12). Full development of spermatozoa possessing developed acrosomes however was not observed.

In contrast, to the non-infected areas of spermatogonic tissue, the infected areas consisted of hypertrophied spermatogonic cells associated with large numbers of surrounded spermatids. The infected spematogonic cell was irregular in shape with short lobed branches of cytoplasm extending from it (Fig. 13). The spermatids, although connected to each other and the spermatogonic cell with fine cytoplasmic threads appeared to be affected by their association with the infected spermatogonic cell. They possessed irregular shaped nuclei with dispersed heterochromatin, often with a pronounced nucleolus. Centriole and associated midpiece/tail development was not noted although they did contain numerous mitochondria. Some affected spermatids appeared possess areas of amorphous material, possibly resembling degenerating midpieces. A few affected spermatids were observed in direct contact with the peritoneum of the bryozoans' coelom (Fig. 14).

Haplophasic schizonts were noted within the hypertrophic spermatogonic cells of the funiculus, and sometimes in the associated spermatids (Fig. 15). The schizonts possessed a round nucleus that occupied approximately half of the parasites' volume, endoplasmic reticulum and dense bodies (Fig. 16). The schizonts resided in direct contact with the host cells' cytoplasm and appeared to replicate by binary division as no chain or rosette formation was observed. Actual division was not noted. The staining intensity of both the host cells and the schizonts varied, even within the same section, with some schizonts easily distinguished from the host cell while others were not (Fig. 17). This may have been an artefact caused by the staining procedure or variations in section thickness, however as the variations appeared consistent in serial sections and within the same section it may represent developmental differences in the formation of xenomas.



Figs 16-19. *Schroedera plumatellae* sp. n. **19** - haplophasic schizont present in spermatogonic cell. Details of schizont structure clearly visible; **17** - schizont present in spermatogonic cell, poorly differentiated from the host cell cytoplasm. Nucleus of schizont indicated with an N.; **18** - microtubules extending from infected spermatogonic cell; **19** - connection of spermatogonia (*) to schizont infected spermatid *via* cytoplasmic extension of spermatogonia (arrowhead). Scale bars: 16 - 1 μm; 17, 18 - 500 nm; 19 - 2.5 μm

In addition to the spermatogonic cells becoming hypertrophied, bundles of microfilaments were observed extending from the infected spermatogonic cells connecting them to spermatids (Fig. 18). The spermatids connected in this way were always noted to contain one or more schizonts (Fig. 19). Often the microtubules would just be surrounded by a thin layer of cytoplasm, but more substantial connections between spermatids and the spermatogonia existed with mitochondria and other organelles occurring along the length of the connecting microtubules. It could not be ascertained whether the microtubules originated from reorganisation of the spermatogonic cell or the connected spermatid.

Development of xenomas into cords

In section, the cord-like xenomas appeared as discrete hypertrophied cells filling the coelom. Unlike the previous phase of development there were no cells, spermatids or otherwise, associated with the xenomas. The surface of the xenomas possessed many fine cytoplasmic filaments of host cell origin. Microtubular extensions were not noted. The cytoplasm of the xenoma was granular with mitochondria dispersed throughout. The xenomas were multi-nucleated possessing enlarged nuclei, with reduced chromatin. The xenomas possessed many diplokaryotic sporonts within them (Fig. 20). Occasional parasite cells were also observed that appeared haplophasic although this may have been due to the plane of section through a sporont. The nuclei of the sporonts appeared to be of similar size, while the cytoplasm contained rough endoplasmic reticulum. Karyogamy by the haplophasic schizonts to form sporonts, or division of the sporonts however, was not observed. Sporonts were observed developing into sporoblasts. At this stage of development the cytoplasm of the sporont would become indistinct making any nuclear or cellular division difficult to assess (Fig. 21). The membrane of the parasite would become locally electron dense caused by the parasite extending fine membranous processes over its surface. Beneath these processes, material would form that separated the parasite from the host cell (Fig. 22). The membranes and the material between them appeared to condense to form the electron dense surface of the developing sporoblast. Complete separation of the parasite and the cytoplasm of the host cell by the formation of an interfacial envelope was not observed.

Early development of sporoblasts was observed within the cords. These stages were oval, and had an electron dense thickening of their plasmalemma, the cytoplasm appearing to be relatively electron lucent compared to that of sporonts and schizonts (Fig. 23). Fixation of the developing sporoblasts was poor and so the processes of sporogony could not be determined.

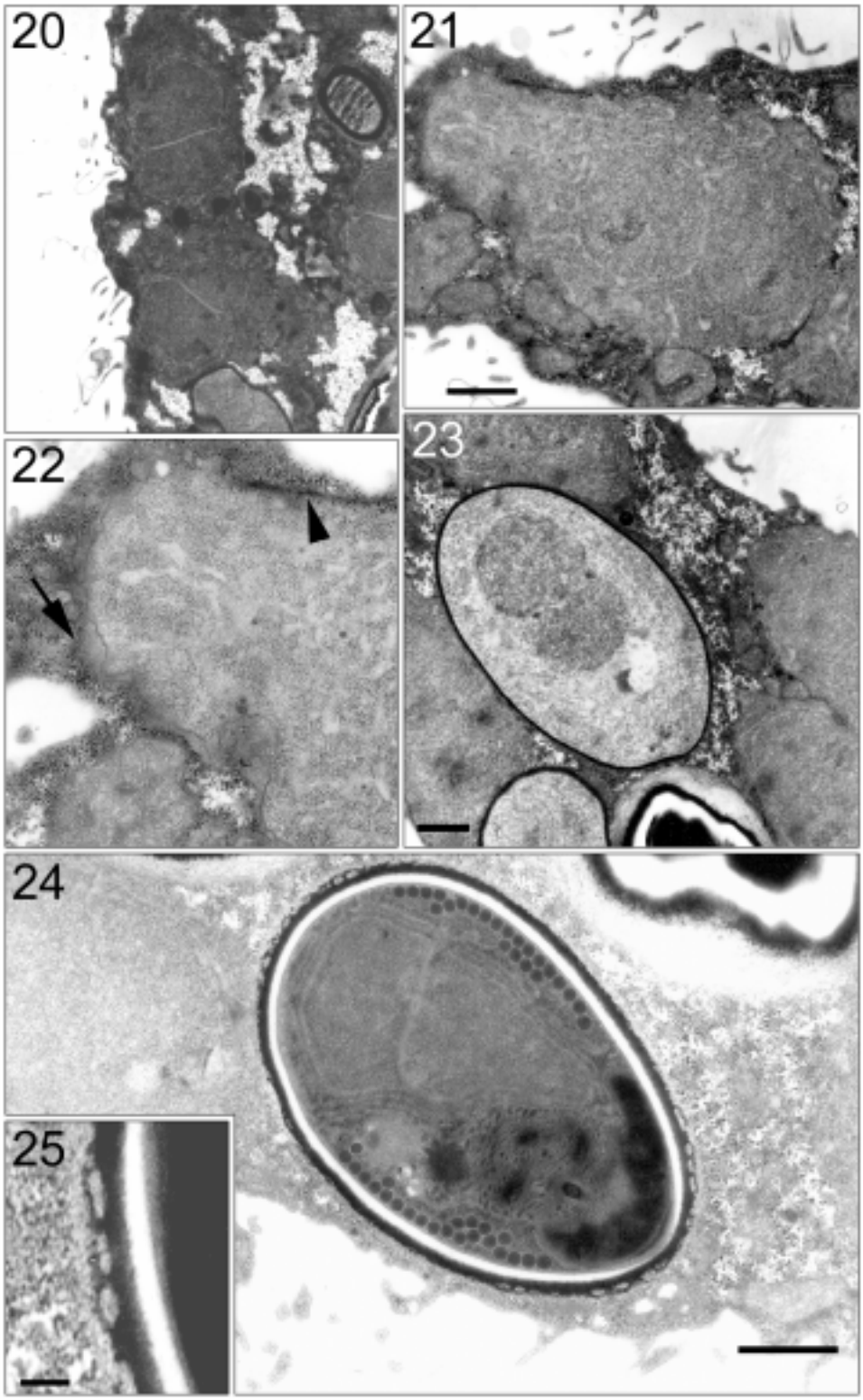
Spore morphology

Examination of the spore mass collected from a degenerating zooid determined that the host cell cytoplasm was very reduced, composed of occasional mitochondria in an electron lucent granular material with spores, sporoblasts and occasional sporonts. Haplophasic schizonts were not present at this stage of the infection.

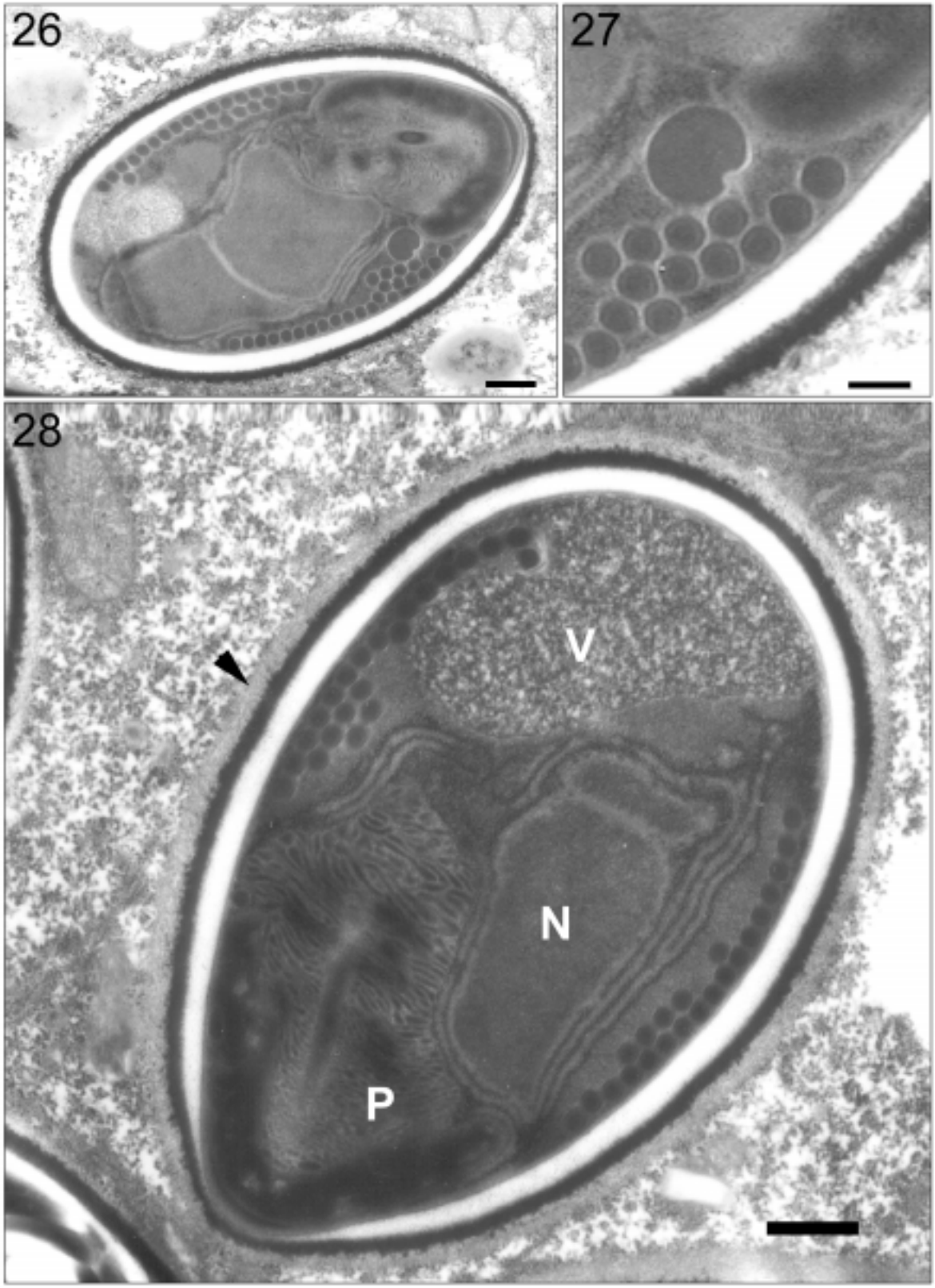
Fixation of the majority of spores was poor. Mature spores were observed to lie directly within the host cell cytoplasm. The fixed spores appeared to be oval, tapering to one end. The spores observed all appeared to be of one type, larger spores as noted under light microscopy were not observed in the sections examined. The spore wall consisted of an electron lucent endospore surrounded by an exospore of two distinct layers. An electron dense layer, surrounded by a glycocalyx-like, granular layer, that could be easily distinguished from the cytoplasm of the host cell (Fig. 24). The thickness of the two outer layers was roughly equal, measuring between 200-250 nm thick except anteriorly, where it was down to 150 nm thick. The electron-dense exospore layer of some spores was observed to possess fine ornamentation towards the anterior and posterior poles, reminiscent of small bubbles (Fig. 25). The spores were diplokaryotic, with nuclei of roughly equal size arranged in tandem. Encircling the nuclei were well-developed cisternae of rough endoplasmic reticulum. The polar filament appeared to be isofilar and was coiled 22-23 times in one to three rows around the spore (Fig. 26). An electron dense body of unknown function was observed at the base of the polaroplast in one of the spores. The polar filament was composed of three layers, alternating between electron lucent and electron dense, with an electron dense core (Fig. 27). The polaroplast consisted of closely packed anterior lamellae with wider less organised posterior lamellae, with a surrounding electron-dense, bell shaped polar sac. The posterior vacuole of the spore contained granular material (Fig. 28).

rDNA analysis

A segment of the SSU rDNA of the parasite was successfully amplified by PCR and sequenced. This segment was 1348 bp in length with a GC content of 42.7%. The Genbank/NCBI accession number is AY135024. When using the BLAST facility, the se-



Figs 20-25. *Schroedera plumatellae* sp. n. **20** - diplokaryotic sporonts. **21** - transformation of sporont into sporoblast. Cytoplasm of the cell becomes increasingly lucent making differentiation of the nuclei difficult; **22** - detail of Fig. 21 demonstrating formation of electron-dense cell membrane of sporoblast. Arrow indicates membranous extensions derived from sporont. Arrow-head indicates condensing of material to form electron-dense membrane; **23** - Sporoblast with electron-dense cell membrane; **24** - spore, demonstrating bubble-like formations on the exospore; **25** - detail of Fig. 24 showing bubble-like extensions to the electron-dense layer of the exospore. Scale bars: 20 - 2 μm; 21 - 1 μm; 22 - 500 nm; 23 - 1 μm; 24 - 1 μm; 25 - 200 nm



Figs 26-28. 26 - *Schroedera plumatellae* sp. n. spore with nuclei of similar sizes and location of unidentified body at the base of the polaroplast; **27** - detail of Fig. 26 highlighting the unidentified body and the structure of the polar filament; **28** - spore of demonstrating granular posterior vacuole (V), lamellae nature of the polaroplast (P), diplokaryon (N) and two distinct layers of material forming the exospore (arrowhead). Scale bars: 26 - 500 nm; 27 - 200 nm; 28 - 500 nm

quence showed highest homology to the sequence in Genbank deposited for *Bacillidium* sp. (AF104087) and *Janacekia debaisieuxi* (AJ252950). Using parsimony, distance analysis and maximum likelihood analysis, trees with very similar overall topology were obtained. All of the analyses consistently placed *S. plumatellae* in a group branching with *Bacillidium* sp. and *J. debaisieuxi* (Fig. 29). The bootstrap support for this group was 100%, independent of the method used. This group appeared within a larger clade that included *Nosema algerae, Thelohania solenopsae, Visvesvaria algerae* and *V. acridophagus*. The grouping of *N. algerae* with *V. algerae* is in accordance to the previous phylogenetic studies on this species that distanced it from other *Nosema* spp. (Müller *et al*. 2000).

DISCUSSION

Only two species of Microsporidia have been identified as parasites of phylactolaemate bryozoans, *Nosema cristatellae* and *N. bryozoides*. The parasite described in this paper is different to the description of *N. cristatellae* in that the host species, host cell type and morphology of the spore are different and therefore we consider that it is not the same species as the parasite described here.

The descriptions of *N. bryozoides* by Korotneff (1892) and Braem (1911) and that of *S. plumatellae* in this study are similar, in that the parasites affect the spermatogonia, are released into the coelom and finally kill the host zooid resulting in a spore filled tube. However, significant differences also exist, *N. bryozoides* is figured to form lobules that develop on the funiculus before detaching to float in the coelom, gradually filling it. The cord-like xenomas observed for *S. plumatellae* were not reported. Marcus (1941) described cord-like xenomas in his description of *N. bryozoides*, however, this author also observed somatic cells becoming infected. These cells were never noted to become infected either in the study presented here or in the previous reports for *N. bryozoides*. As such, it is likely that the parasites described by previous authors are not *S. plumatellae* but other, perhaps related, microsporidian species.

Nilsen (1999) examined the molecular phylogeny of a *Bacillidium* sp. and could not relate it to any other microsporian species included in his analysis. The analysis also demonstrated the presence of four lineages within the Microsporea. The phylogenetic analysis in the present study again suggested four distinct lineages. The *Bacillidium* sp. grouping with *S. plumatellae* and *Janacekia debaisieuxi* which branch from a sister group composed of *Visvesvaria acridophagus, Thelohania solenopsae and Visvesvaria algerae.* The spores of both *Bacillidium* sp. and *J. debaisieuxi* are distinctly different to that of *S. plumatellae*. Members of the genus *Bacillidium* are characterised by rod-shaped spores with a manubroid polar filament, while *J. debaisieuxi* forms oval, uninucleate spores and produces sporogonial plasmodia. Due to these differences it is surprising to find that *J. debaisieuxi* and the *Bacillidium* sp. cluster together in the analyses. The relationship between these species should be examined in more detail as it is possible that they have a life-cycle that involves different forms of spore pertaining to different hosts. Although it has to be noted that the taxonomy of *J. debaisieuxi* is in doubt as it has been suggested that it comprises an assemblage of different species (Sprague *et al.* 1992).

Although *S. plumatellae* does not appear to share many features with *J. debaisieuxi* it does have some common features with the genus *Bacillidium* and other members of the family Mrazekiidae. Notably that sporulation occurs within the host cell hyaloplasm, it is disporous with diplokaryotic nuclei lying in tandem within the spore and it forms a distinctive xenoma. The structure of this xenoma includes fine cytoplasmic extensions on the xenomas' surface and multiple nuclei correlating to the those formed by *Bacillidium criodrili* and *Hrabyeia xerkophora* (Larsson 1986, Lom and Dyková 1990). Furthermore, the differentiation of the exospore into different layers is in keeping with other members the family Mrazekiidae. Usually members of the family Mrazekiidae have rod-like spores, however the genus *Hrabyeia*, is the notable exception to this, forming a tail from the electron-dense exospore material of an ovoid spore. This exospore material being surrounded by a glycocalyx-like layer (Lom and Dyková 1990). We consider that the developmental and phylogenetic data collected during the study of *S. plumatellae* suggest that it is a member of the Mrazekiidae. Although similar to *H. xerkophora* regarding aspects of the spore wall composition and xenoma formation, the absence of a exospore tail and difference in polaroplast construction, suggest significant differences between these species.

From the results of the light and electron microscopy a developmental pathway for the xenoma can be suggested. The parasite infects spermatogonia inducing spermatogenesis and causing this tissue to become

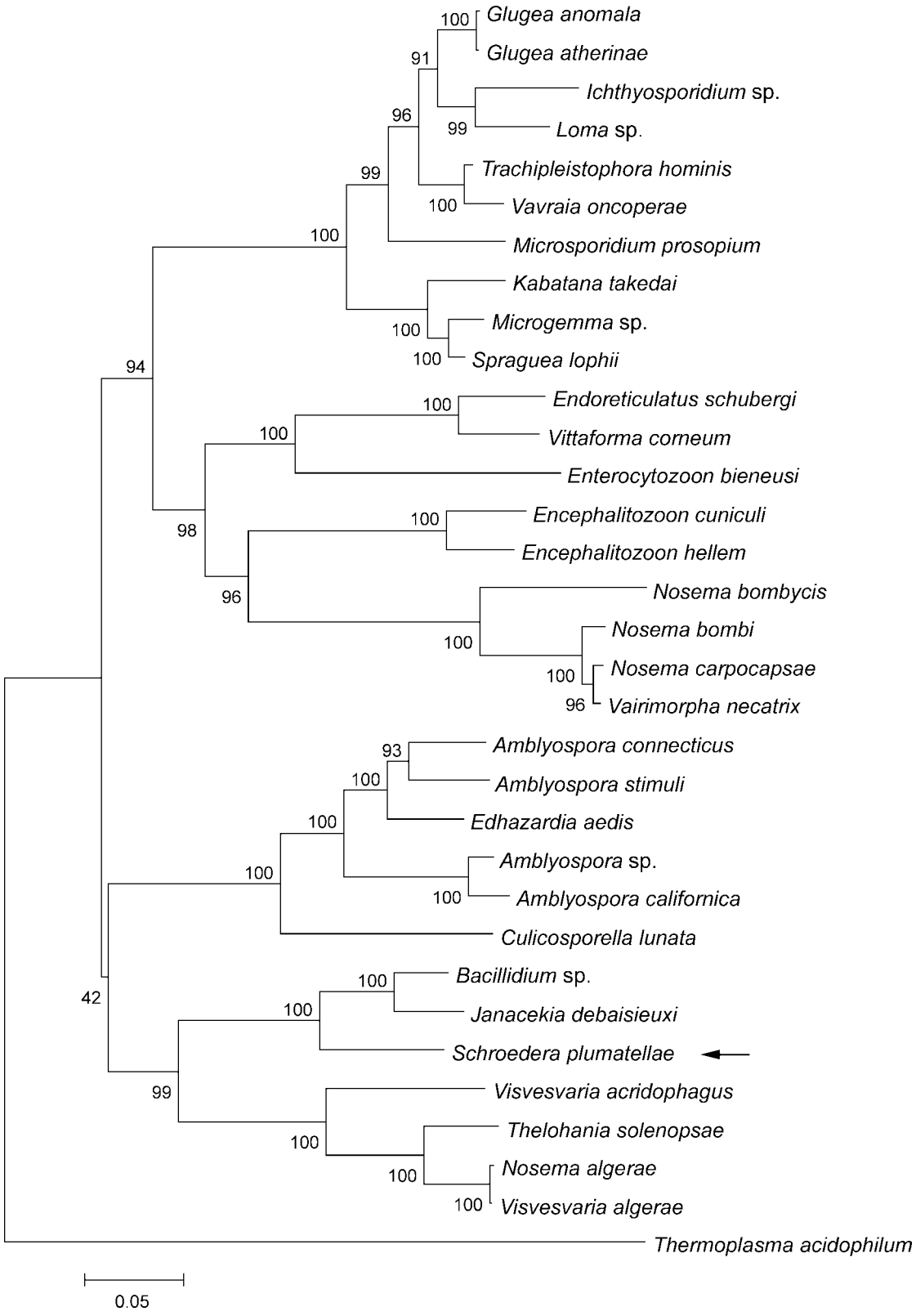


Fig. 29. Tree obtained by 500 bootstrap replicates using Kimura 2-parameter and neighbour joining. The numbers on the branches are a percentage support from the 500 bootstrap resamples. Tree rooted using *Thermoplasma acidophilum* as the outgroup. Branch lengths are proportional to unambiguous changes

hyperplastic. The development of the spermatids associated with the infected spermatogonia into spermatozoa is halted and their development modified, presumably to supply energy and nutrients to the parasitised cell. Either the infected spermatogonia or the connected spermatid develop microtubules that passes to the other cell. This connection allows the cytoplasm of the spermatids and the spermatogonia to fuse, thus forming a multi-nucleated syncitium. Evidence of the fusion of cells is suggested by the lobed appearance of the infected spermatogonia (Figs 13, 19). The microtubules presumably becoming reabsorbed as the two cells merge. As more spermatids are incorporated into the syncitial xenoma so it elongates forming a cord-like structure, with the eventual incorporation of all of the spermatids.

The description of the development of *S. plumatellae* within the host is incomplete. The route of entry into the host is undetermined as are those stages proceeding development on the funiculus. Although it is clear that autoinfection was occurring within the host autoinfective spores were not identified. It is possible that the life cycle of this parasite requires more than one host, and that the microsporidian within this host has already been described. However, until molecular or life-cycle work can elucidate the identity of another host (if there is one) we propose to name the parasite *Schroedera plumatellae* gen. n. *et* sp. n.

TAXONOMY

Schroedera gen. n.

Diagnosis: sporogony diplokaryotic, preceded by haplophasic schizogony. Presumably disporoblastic. Spores are oval, tapering to one end, have an isofilar polar filament arranged in 1-3 rows. The exospore is divided into two distinct layers, an electron dense layer surrounded by a granular layer. Sporophorous vesicles are not produced. The parasite induces xenoma formation that develops in the coelom of the host. Xenomas are multinucleate, derived from interconnected host cells.

Etymology: named in recognition of Dr. Olaw Schröder, who conducted several early studies into bryozoan parasites.

Schroedera plumatellae sp. n.

Schizogony: uninucleate schizonts, divide apparently by binary fission. Number of cycles of schizogony unknown.

Sporogony: presumably disporoblastic. Sporonts diplokaryotic, and develop electron dense surface as they form sporoblasts. Later stages of sporogony did not fix well. Sporophorous vesicle absent.

Spores: ovoid, tapering to anterior end. Dimensions of fresh spores 7.2 x 5.0 μm (±0.3 μm, n=22). A few macrospores observed 10.0 x 6.0 μm. Spore wall as for genus, the two layers forming an exospore 200-250 μm thick. Polar filament, isofilar, composed of three layers of material enclosing an electron dense core, with 22-23 coils arranged in 1-3 rows. The filament is arranged in a single row at the posterior of the spore with 2-3 rows appearing near the anterior of the spore. Thickness of filament 140 nm. Polaroplast with compact membrane vesicles toward anterior and lamellae posteriorly with a surrounding bell shaped, electron dense, polar sac. Polaroplast extends mid-way into the spore. Posterior vacuole contains granular material.

Type host: *Plumatella fungosa* Pallas (Bryozoa, Phylactolaemata)

Host tissue involved: infects spermatogonia to form conspicuous acapsulate xenoma. Host spermatids associated with early xenoma formation.

Type locality: southern end of Airthrey Loch, University of Stirling campus, Stirling, Scotland. The national grid reference being NS 804963.

Type specimens: hapantotype material has been deposited in the collection of the Natural History Museum, London. The material comprises of slides and EM grids of the early stages of parasite development demonstrating schizonts (registration numbers 2002:6:26:1 and 2002:6:26:3) and late stages of development demonstrating sporonts and spore stages (registration numbers 2002:6:26:2 and 2002:6:26:4).

Etymology: name alludes to the host genus.

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ACTA
PROTOZOOLOGICA

Studies on the Morphology and Morphogenesis of *Allotricha curdsi* sp. n. (Ciliophora: Hypotrichida)

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Summary. The morphology and morphogenesis of the freshwater hypotrichous ciliate *Allotricha curdsi* sp. n. was investigated using protargol silver impregnation. The new species is characterized thus: medium-sized, freshwater *Allotricha* 150-210x70-90 µm *in vivo*, ellipsoid with tapered posterior end; contractile vacuole in left half of midbody region; 2 macronuclei; one left and three right marginal cirral rows; 53-61 adoral membranelles; 9-10 frontal and 6-7 ventral cirri; usually with 4 complete dorsal kineties and 2 dorsomarginal kineties; cortical granules absent. The morphogenetic events reveal the following characteristics: (1) AZM in the opisthe arises from the new oral primordium while the proter retains the parental structure in its entirety; (2) 9-10 frontal, 6-7 ventral, and 5 transverse cirri are derived from 5 frontoventral transverse cirral anlagen, together with the undulating membrane anlage; (3) three new right marginal cirral rows derive from 3 separate anlagen, which originate in or near to the rightmost parental marginal row; all other parental cirri are resorbed just before cytokinesis; (4) the generation of the dorsal kineties is of "two-group-mode", 3 left primary dorsal and 2 dorsomarginal anlagen appear as two groups in both dividers and form all dorsal kineties (the 4th kinety is formed by the fragmentation of the 3rd anlage); (5) one caudal cirrus is formed at the posterior ends of the 1st, 2nd and 4th dorsal anlagen respectively.

Key words: *Allotricha curdsi* sp. n., freshwater ciliate, Hypotrichida, morphology and morphogenesis.

INTRODUCTION

The genus *Allotricha* was established by Sterki (1878) for *A. mollis*, a flexible hypotrich with a typical *Oxytricha* cirral pattern but with more than 2 marginal cirral rows. Over the next 120 years the classification of this genus underwent a variety of changes including being reclassified as a subgenus of *Pleurotricha* (Bütschli, 1889) and being declared by Petz and Foissner (1996) as *genus indeterminata* (for detailed discussion on the taxonomy of *Allotricha*, see Berger 1999). The genus was reactivated by Berger (1999) who defined it thus: adoral zone of membranelles formed like a question mark; undulating membranes in *Oxytricha* pattern, or rather straight and arranged almost side by side; frontoventral cirri in V-shaped pattern; post-oral ventral cirri in dense cluster behind buccal vertex; two pre-transverse ventral, and 5 transverse, cirri; two or more right, and one or more left, rows of marginal cirri; caudal cirri present; primordia V and VI of the proter originate from cirrus V/4 and V/3 respectively; no parental marginal rows retained after division; dorsal morphogenesis in *Oxytricha*-pattern.

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Song (2001) subsequently refined the diagnosis for *Allotricha* thus: flexible Oxytrichidae with frontoventral cirri in V-shaped pattern; 3 post-oral ventral cirri in dense cluster underneath (i.e. posterior to) buccal vertex, 2 pre-transverse ventral and 5 transverse cirri; 1 left and 2 or more right rows of marginal cirri; caudal cirri present; no parental marginal rows retained after division.

In August 1996, a freshwater *Allotricha*, collected from a eutrophic pond in northeast China, was brought into laboratory culture. A study of its morphology and morphogenetic processes during binary fission was carried out which revealed it to be a previously unknown taxon. We here provide detailed descriptions based primarily on protargol-impregnated specimens.

MATERIALS AND METHODS

Samples were collected on 23 August 1996 from a small eutrophic pond in Mohe county in the northmost area of P. R. China (52° 55'N, 122° 12'E). The ciliates were cultured in Pringsheim solution, to which some rice grains were added as food source for bacteria. Ciliates were examined *in vivo* using bright field microscopy. The protargol sliver staining method according to Wilbert (1975) was used in order to reveal the infraciliature.

Measurements were performed at a magnification of x1500. Drawings were made with help of a camera lucida. To illustrate the changes during morphogenetic processes, parental cirri are depicted by contour whereas new ones are shaded black.

Terminology is mainly according to Corliss (1979), Hemberger (1982), Berger (1999) and Song and Warren (1999). Definitions of expressions, such as the primordium/a, anlage(n) and undulating membrane anlage are according to Song and Hu (1999).

RESULTS

Allotricha curdsi sp. n. (Figs 1a-c, 4, Table 1)

Diagnosis: freshwater *Allotricha* with broadly ellipsoid body shape, length:width ratio 2:1, tapering posteriorly. *In vivo* 150-210 x 70-90 µm. Dorsoventrally flattened, ratio of width to depth about 2:1. 9-10 frontal, 6-7 ventral, 5 transverse and 3 caudal cirri. 53-61 membranelles in adoral zone. One left and 3 right marginal cirral rows. Four dorsal and 2 dorsomarginal kineties. Two macro- and 2 micronuclei. Cortical granules absent.

Type locality: a small pond near a marsh in Mohe County (52° 55'N, 122° 12'E), Heilongjiang Province, P. R. China.

Type specimens: 1 holotype and 1 paratype slide of protargol impregnated specimens have been deposited in the Laboratory of Protozoology in Harbin Normal University, P.R. China. A second paratype slide is deposited in the Laboratory of Protozoology, College of Fisheries, Ocean University of Qingdao, P. R. China. A third paratype slide is deposited at the Natural History Museum, London, with registration number 2001:10:4:2.

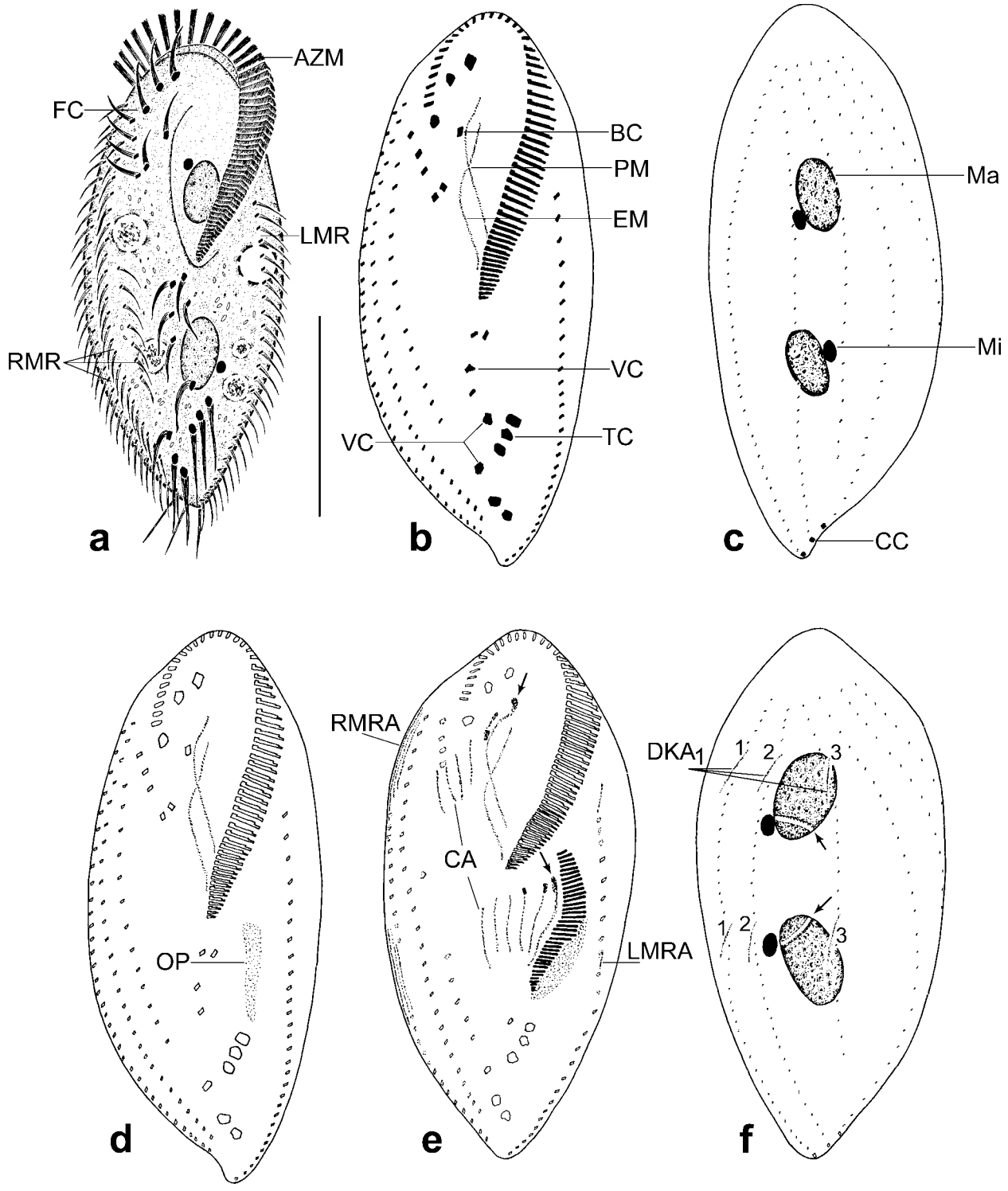
Dedication: named in honour of Professor Colin R. Curds who made many valuable contributions to the taxonomy of ciliates in general and of hypotrichs in particular.

Morphology of *Allotricha curdsi* sp. n. (Figs 1a-c, 4, Table 1)

Body flexible, broadly ellipsoid in shape, tapering posteriorly (Fig. 1a), size *in vivo* 150-210 x 70-90 µm (150-200 x 50-70 µm after protargol impregnation), length to width ratio about 2:1; anterior end rounded, posterior more or less pointed; dorso-ventrally flattened, width to depth ratio about 2:1. Buccal field broadened due to extensive buccal lip. No cortical granules were observed. Cytoplasm colourless with numerous densely packed shining granules and food vacuoles containing flagellates and bacteria. Single contractile vacuole (CV) 12-15 µm in diameter, located near left margin at about mid-body of cell (Fig. 1a). Two widely separated macronuclei lying slightly left of main body axis, each 20-23 µm in diameter *in vivo* and with small spherical nucleoli (Figs 1a, c, 10). Two micronuclei about 5 µm in diameter, always adjacent to macronuclei. Locomotion typically like other oxytrichids.

Proximal end of adoral zone of membranelles (AZM) extends to about mid-body region (i.e. 50% body length), distal region markedly curved. AZM with on average 56 adoral membranelles, bases of largest membranelles about 11-12 µm wide. Most membranelles composed of four rows of kinetosomes (Figs 3b, 4); one short row (*ca* 1.2 µm long) of 3 kinetosomes; one intermediate row (*ca* 8.5 µm long) of 18-20 kinetosomes; two long rows of equal length (12-13 µm), each with 28-30 kinetosomes.

Cilia of frontal cirri about 20-22 µm long *in vivo*, although cilia of three anteriormost frontal cirri and buccal cirrus are noticeably longer. Buccal cirrus (II/2) lies close to right buccal wall at about level of anterior end of endoral membrane (Figs 1b, 4). Cirrus III/2 lies to left and slightly anterior to cirrus IV/3. Ventral cirri in three groups, 2 postoral, 2 pretransverse and 2 or 3 in region of posterior macronucleus (Figs 1b, 4). Five



Figs 1a-f. Non-dividing cell (a-c), early and early-mid stages of morphogenesis (d-f) in *Allotricha curdsi* sp. n.; **a** - ventral view *in vivo*; **b, c** - ventral (b) and dorsal (c) views showing infraciliature (after silver impregnation); **d-e** - ventral views showing early stages in morphogenesis (after protargol impregnation), arrows showing the anterior frontal cirrus forming at anterior end of each undulating membrane anlage; **f** - 1st group of dorsal view, showing development of dorsal kinety anlagen (1-3) and relative position of micronuclei to macronuclei during middle stage of morphogenesis; arrows mark replication bands. AZM - adoral zone of membranelles; BC - buccal cirrus; CA - cirral anlagen; CC - caudal cirri; DKA_1 - 1st group of dorsal kinety anlagen; EM - endoral membrane; FC - frontal cirri; LMR - left marginal row; LMRA - left marginal row anlagen; Ma - macronucleus; Mi - micronucleus; OP - oral primordium; PM - paroral membrane; RMR - right marginal row; RMRA - right marginal row anlagen; TC - transverse cirri; VC - ventral cirri. Scale bar - 80 µm

Table 1. Morphometric data for *Allotricha curdsi* sp. n. All data are based on protargol impregnated specimens. Measurements in µm. Abbreviations: CV - coefficient of variation in %; Max - maximum; Min - minimum; n - number of cells measured; SD - standard deviation; SE - standard error of mean

Character	Min	Max	Mean	SD	CV	n
Length of body	150	200	172.9	16.5	9.6	20
Width of body	70	90	78.1	5.9	7.5	20
Length of adoral zone of membranelles*	60	100	74.8	10.8	14.4	20
No. of adoral membranelles	53	61	56	1.9	3.3	20
No. of frontal cirri**	9	10	9.2	0.4	4	20
No. of buccal cirri	1	1	1	0	0	20
No. of ventral cirri	6	7	6.3	0.57	9.1	20
No. of transverse cirri	5	5	5	0	0	20
No. of caudal cirri	3	3	3	0	0	20
No. of left marginal cirri	19	28	25.9	2.5	9.6	20
No. of cirri in right marginal row 1	26	36	31.9	2.7	8.5	20
No. of cirri in right marginal row 2	23	27	24	1.2	4.9	20
No. of cirri in right marginal row 3	3	15	7	2.8	40.7	20
No. of dorsal kineties***	6	6	6	0	0	20
No. of macronuclei	2	2	2	0	0	20
No. of micronuclei	2	2	2	0	0	20

* total length from proximal to distal end; **Including buccal cirrus (II/2);***Including dorsomarginal kineties [not depicted in table]

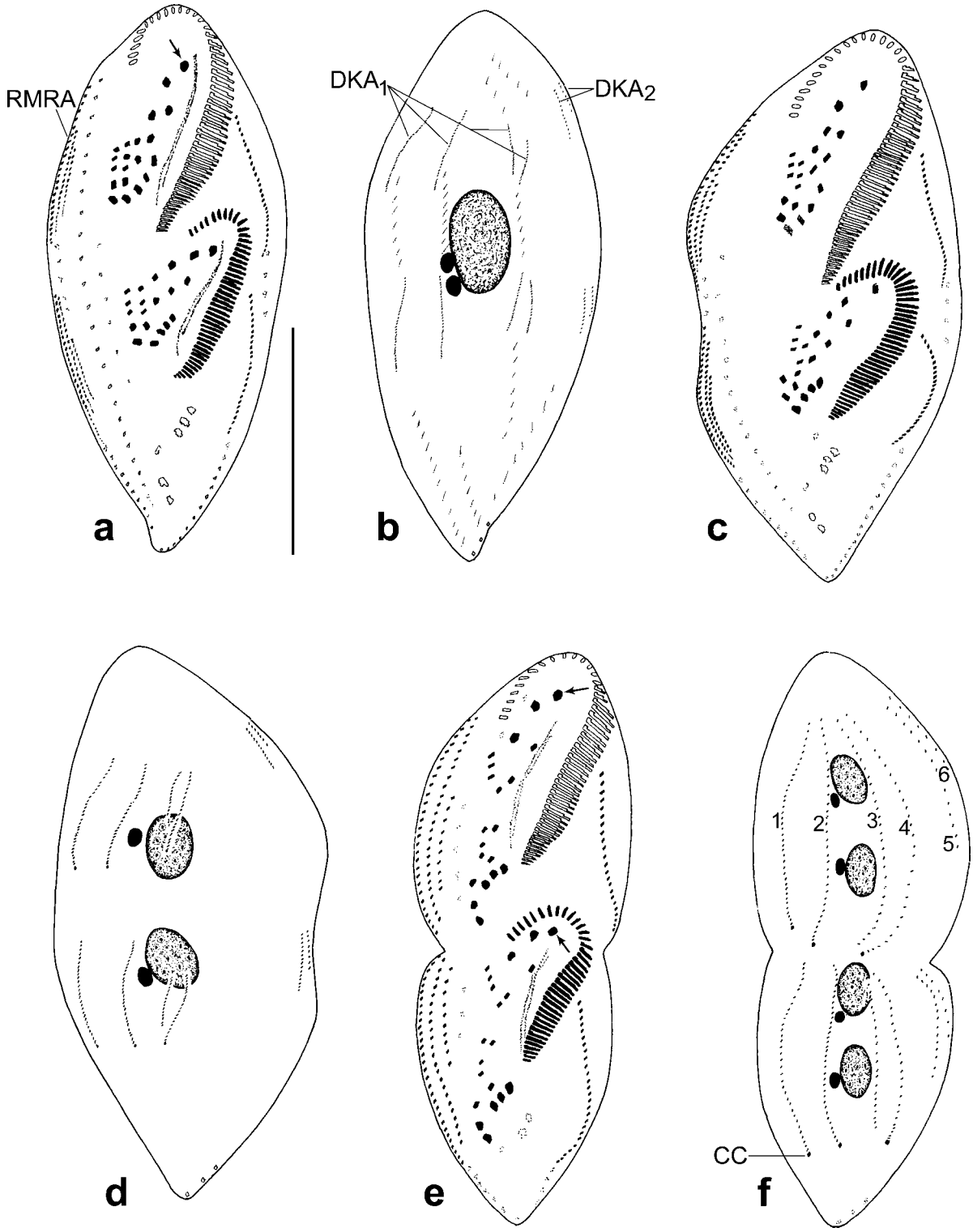
transverse cirri separated into two groups of two and three (Figs 1a-b, 4). Cilia of transverse cirri 42-45 µm long. Marginal cirri arranged in three right and one left marginal rows, those on the right being more conspicuously curved than that on the left. Rightmost marginal row beginning dorsally at anterior end of cell and terminating close to the two rightmost transverse cirri (Figs 1a, b). Left marginal row extending to posterior end of cell and not distinctly curved (Figs 1b, 4). Cilia of marginal cirri 20-23 µm long. Three well-developed caudal cirri (CC) located at ends of dorsal kineties 1, 2 and 4 (DK_1, DK_2 and DK_4 respectively) on right side of posterior end of cell (Figs 1a, c). Cilia of caudal cirri about 32 µm long. Dorsal cilia 2-3 µm long, not easily observed *in vivo* and arranged in 4 dorsal and 2 dorsomarginal rows.

Comparison with the related species

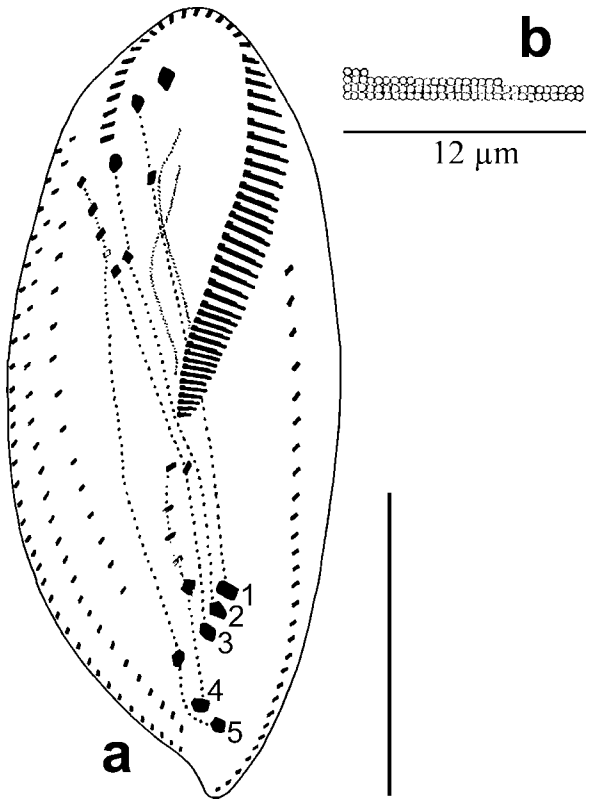
According to Berger (1999) there are two known species of *Allotricha*; *A. mollis* Sterki, 1878 and *A. antarctica* Berger, 1999. *A. curdsi* can be readily differentiated from *A. antarctica* by its body size (150-210 µm *vs*. 90-125 µm long for species of *A. antarctica* from wild), number of adoral membranelles (53-61 *vs.* 35), arrangement of transverse cirri (in two groups of 2 and 3 *vs.* in a hook-shaped row), arrangement of marginal cirral rows (3 right, 1 left *vs*. 2 right, 2 left), arrangement of undulating membranes (curved and intersecting *vs*. straight and parallel), cortical granules (absent *vs*. present), and habitat (freshwater *vs*. soil) (Petz and Foissner 1996, Berger 1999).

The new species bears a much stronger resemblance to *A. mollis* although it can be distinguished from the latter by the body shape (broad ellipsoid, length:width ratio 2:1, tapering posteriorly *vs*. elongate ellipsoid, length:width ratio *ca* 3:1, with rounded ends), length of AZM relative to the body length (about 50% *vs*. 35%), arrangement of transverse cirri (in two groups of 2 and 3 *vs*. in a single oblique row), and cortical granules (absent *vs*. present) (Berger 1999).

One other taxon with which *A. curdsi* could be confused is *Pleurotricha variabilis* Reuter, 1961, which Petz and Foissner (1996) considered a synonym of *Onychodromopsis flexilis*, but which Berger (1999) considers probably to be a member of the genus *Allotricha*. Both these taxa lack cortical granules, or at least they were not observed by Reuter (1961) in *P. variabilis*, and have similar patterns of infraciliature. However, *A. curdsi* differs from the latter species in terms of its body size and shape (150-210 µm long; broadly ellipsoidal with tapering posterior; length:width ratio 2:1 *vs*. 200-220 µm long; elongate ellipsoidal with rounded ends; length:width ratio 3:1); number of frontal cirri (9-10 *vs*. 8) and the length of AZM relative to the



Figs 2a-f. Middle and late stages of morphogenesis in *Allotricha curdsi*. **a, b** - ventral and dorsal views of the same individual. a - ventral view showing the cirral pattern during middle stage. Note the three new right marginal cirri anlagen which develop within or near the rightmost marginal cirral row. Arrow marks the first frontal cirrus which develops from the undulating membrane anlagen; b - dorsal view showing the single fused macronucleus: **c,d** - slightly later stage, ventral and dorsal views of same cell; c - ventral view, showing the frontal cirri starting to migrate and the well developed adoral zone of membranelles of the opisthe; d - dorsal view. Note the newly divided macronuclei and the two groups of dorsal kinety anlagen; **e, f** - late stage, ventral and dorsal views of the same cell; e - ventral view, showing the well-developed cirri and the new cirral patterns of the proter and the opisthe; f - dorsal view showing the six dorsal kinety anlagen in each divider. CC - caudal cirrus; DKA_1 - 1st group of dorsal kinety anlagen; DKA_2 - 2nd group of dorsal kinety anlagen. RMRA - right marginal row anlagen. Scale bar - 80 µm



Figs 3a,b. a - diagrammatic representation of *Allotricha curdsi*, ventral view, showing the origin of the transverse cirri; **b** - the standard structure of a membranelle with four rows of kinetosomes; one short row of 3 kinetosomes; an intermediate row of 18-20 kinetosomes; two long rows of 28-30 kintosomes. Scale bars: a - 80 μm; b - 12μm

body length (50% *vs*. 30-35%) (Reuter 1961, Berger 1999).

Considering the general appearance and size, especially the pattern of ciliature, comparison should be made with the well-known *Pleurotricha lanceolata*, which differs from the current species by lacking caudal cirri (*vs*. present) and in having a rigid (*vs*. flexible) cortex and a more slender body shape (Jeffries and Mellott 1968; Dragesco 1970, 1972; Martin-González *et al.* 1984; Berger 1999).

Morphogenesis (Figs 1d-f, 2a-f, 5-14)

Morphogenesis during binary fission has been described for both previously described species of *Allotricha*, *A. mollis* and *A. antarctica* (Kramer 1988, Petz and Foissner 1996, Berger 1999). The morphogenetic processes in *A. curdsi* do not differ fundamentally from either of these.

The main events in the process of nuclear division in *A. curdsi* are similar to those of other oxytrichids, i.e. the macronuclei fuse into a single mass which splits twice, the last division being completed in the postdividers (Figs 1f, 2b, d, f, 6, 7, 10, 12, 14). Hence this process needs no further comment other than to note that the replication bands were observed at an earlier stage of morphogenesis than is normal for other oxytrichids (Fig.1f, arrows).

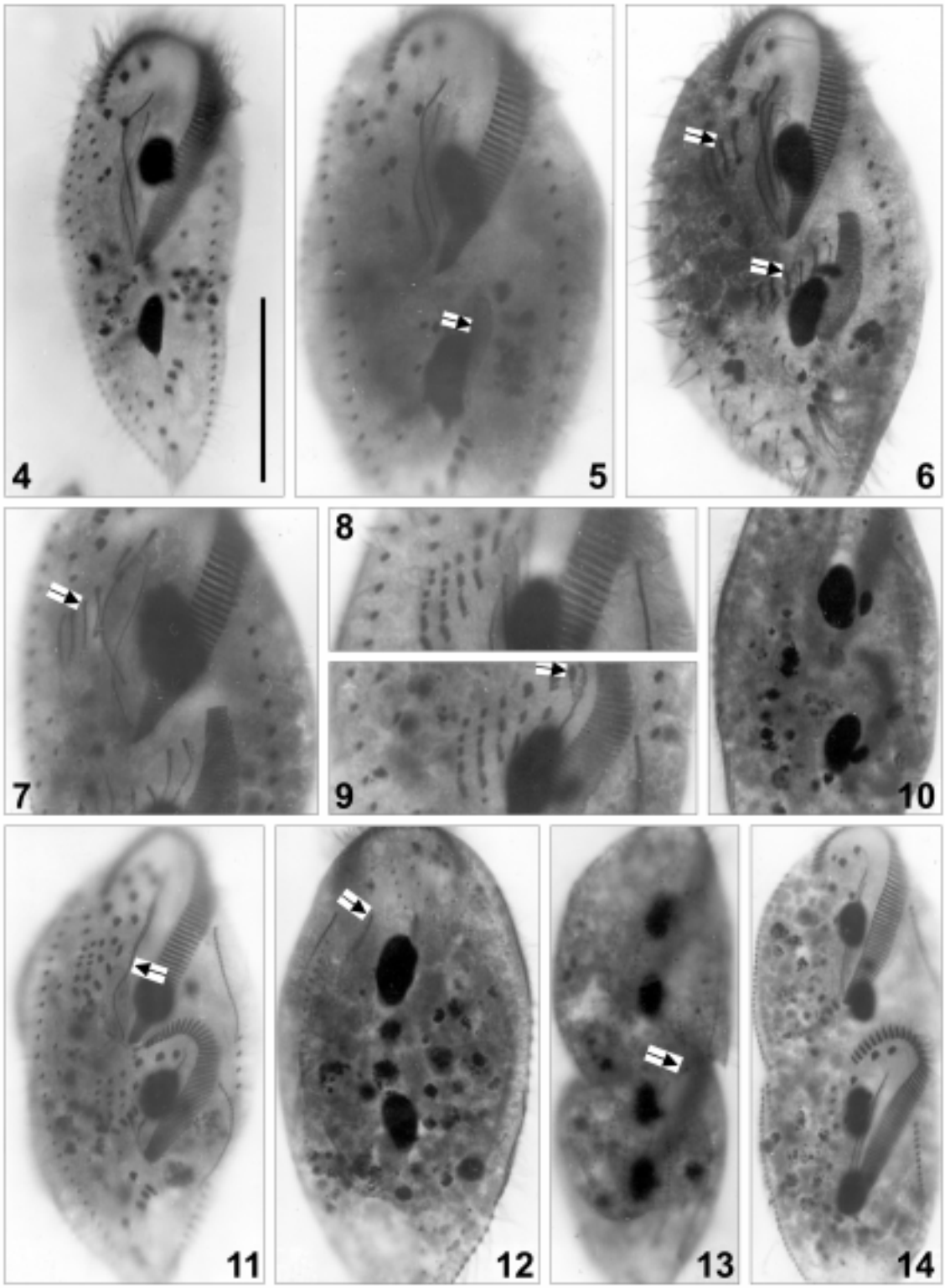
Stomatogenesis

In the opisthe. Morphogenesis commences with the proliferation of loosely arranged basal bodies to form the oral primordium (OP), which occurs *de novo* between the left marginal row and the ventral cirri in the middle portion of the cell. During its formation the adjacent cirri seem to remain intact (Figs 1d, 5). It should be noted, however, that the very first stage in the division process was not observed. The basal bodies of the oral primordium increase in number as its anterior portion elongates to reach the level of the parental cytostome. The adoral membranelles begin to organize in a posteriad direction, while the primordium for the undulating membranes, i.e. the opisthe's undulating membrane-anlage, is generated to the right of the oral primordium as a long streak (Figs 1e arrows, 6).

During the later stages, the anterior end of the newly built adoral zone of membranelles bends to the right and the differentiation of membranelles is completed forming the new oral structure for the opisthe (Figs 2a, c, e, 7, 9, 11, 14). Meanwhile, the undulating membrane-anlage splits longitudinally to form two streaks from which the endoral and paroral membranes derive. Initially these streaks lie close together in parallel (Figs 2e, 14) but later they separate, arch to the left and intersect at around their mid-point (Fig. 3). At this stage, the leftmost frontal cirrus is generated from the anterior end of the undulating membrane-anlage (Figs 2a, c, e, arrows, 9).

In the proter. As in most other oxytrichids, the parental adoral zone of membranelles is retained completely intact during the morphogenetic process, so changes to the oral structure in the proter are confined to the paroral and endoral membranes. The first sign of the formation of the undulating membrane-primordium is the dedifferentiation of the anterior part in the old paroral membrane (Figs 1e, 2a, 7, 8). Slightly later, all basal bodies within both paroral and endoral membranes dedifferentiate giving rise to the undulating membrane-anlage (Figs 2c, 11, arrow).

In the subsequent stages, the basic development of the undulating membrane-anlage follows a similar pat-



Figs 4-14. Photomicrographs of *Allotricha curdsi* (after protargol impregnation); **4** - ventral view, showing the general infraciliature; **5-7 -** ventral views of cell in early-middle stages of morphogenesis; arrow in Fig. 5 showing oral primordium, arrows in Figs 6-7 marking cirral anlagen in both dividers; **8, 9 - v**entral view of cell in late-middle stage of morphogenesis; arrow marking the first cirrus from undulating membrane-anlagen; **10 -** showing the macro- and micronuclei; **11 - v**entral view of cell in late stage in morphogenesis; arrow marks undulating membrane-anlage in the proter; **12 -** dorsal view of cell in middle stage of mophogenesis; arrow marks one of the new dorsal kineties which develop *de novo*, separate from the parental structure; **13, 14 -** dorsal (13) and ventral (14) views of the same individual in late stage of morphogenesis; arrow showing newly formed caudal cirrus. Scale bar - 80 µm.

tern to that of the opisthe: one frontal cirrus is produced from the anterior end and, during late phases, the paroral and endoral membranes are regenerated (Figs 1e, 2a, c, e, 14).

Development of the somatic ciliature

The development of the somatic ciliature begins with the formation of the frontoventral transverse cirral anlagen. The ventral cirri appear to contribute to the formation of these anlagen. Thus, 5 thread-like anlagen are formed in both proter and opisthe, which lie to the right of the parental undulating membranes (Fig. 1e). Evidently some old frontal cirri join in the proliferation of basal bodies of these primordia (possibly the new anlagen arise directly from the parental structure).

Subsequently, these cirral anlagen will develop independently in both dividing parts. As in other oxytrichids, after segregation and migration of the cirri developed from the 5 anlagen, a total of 19-21 cirri are formed: 8-9 frontal, 6-7 ventral and 5 transverse (Figs 2a, c, e, 7, 8, 11, 14). Meanwhile the leftmost frontal cirrus is formed from the undulating membrane-anlage (Figs 2a, c).

Thus, the number and the origin of the frontoventral-transverse cirri formed during morphogenesis can be summarized as follows:

	UM-anlage	Cirral anlagen				
		I	II	III	IV	V
Number of cirri	1	3	3	3	5 or 6	5 or 6

i.e. for each daughter cell 9 or 10 frontal cirri originate from the undulating membrane-anlage (1) and cirral anlagen I (2), II (2), III (1), V (3 or 4); 6 or 7 ventral cirri derive from anlage III (1), IV (4 or 5) and V (1); each of these cirral anlagen contributes one transverse cirrus.

The formation of the new marginal rows in this species exhibits a “neokinetal” pattern (Eigner 1997), i.e. the new structures are built by newly generated basal bodies which are closely connected with disaggregating (or resorbed) old marginal cirri. The left marginal row anlagen develop within the old structures (Fig. 1e) while the three thread-like right marginal row anlagen appear within, or near, the rightmost old marginal row (Figs 1e, 10). At the same time the neighbouring parental cirri gradually disaggregate but were not seen to contribute to the development of these anlagen. The new marginal cirri develop and migrate posteriad to replace the old ones (Figs 2a, c, e, 11, 14).

New dorsal kineties are formed by two groups of primordia: one group develops intrakinetally within the parental structure which at first consists of only three streaks in both opisthe and proter (Figs 1f, 2). In middle and late dividers, the rightmost streak fragments in the posterior region, usually producing 2 new anlagen. Thus, 4 rows of long dorsal kineties are formed (Figs 2b, d, f). The second group of anlagen involved in the formation of the dorsomarginal kineties develops *de novo* to the right of the rightmost cirral row anlage. It later migrates to the dorsal side and develops into two short dorsal kineties (Figs 2b, d, f). It should be noted, however, that the initial stage of this process was not observed. During the morphogenetic process, one caudal cirrus is formed at the posterior ends of each of the 1st, 2nd,and 4th dorsal kineties (Figs 2d, f, 13, arrow).

Summary of morphogenesis

The most significant events in morphogenesis in *Allotricha curdsi* can be summarized as follows: (1) Three rows of new right marginal cirri derive from 3 separate anlagen, which originate within or near the rightmost marginal row. The newly-formed left marginal row is derived from the left marginal row anlage which develops within the old structure. (2) Five frontoventral-transverse cirral anlagen, together with undulating-membrane-anlage, develop into 9 frontal, 6-7 ventral and 5 transverse cirri, rather than the “8:5:5” pattern which is typical of other oxytrichids. (3) As in most other oxytrichids, the parental adoral zone of membranelles is completely retained by the proter. (4) Three left dorsal and one dorsomarginal anlagen are formed initially as two groups in both proter and opisthe and give rise to all dorsal kineties, the rest of the anlagal streaks being formed following the fragmentation of these anlagen. (5) The caudal cirri derive from the posterior ends of the 1st, 2nd and 4th dorsal kineties; (6) Just before cytokinesis, the old undifferentiated cirri are resorbed.

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ACTA
PROTOZOOLOGICA

New Species of *Apolocystis* (Aseptatorina: Monocystidae) from the Coelom of *Microscolex dubius* (Oligochaeta: Acantodrillidae) in Los Talas, Buenos Aires, Argentina

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Summary. *Apolocystis janovyi*, a new species of acephaline monocystid gregarine is described from the coelomic cavity of the oligochaete *Microscolex dubius* Fletcher, 1887; *A. janovyi* is the first monocystid species described from this host. These parasites differ from previously described species of *Apolocystis* in having a larger size range, especially smaller forms in all life cycle stages, and many more sporocysts within the gametocysts.

Key words: *Apolocystis janovyi* sp. n., Monocystidae, coelom, *Microscolex dubius*, Argentina.

INTRODUCTION

Monocystid eugragarines are parasitic protozoa which are frequently found in seminal vesicles and coelomic cavities of terrestrial oligochaetes. Many papers have been published on these parasites, but almost all of them are taxonomic studies and lists of species and their hosts (Ruston 1959; Marek 1967; Segun 1971 a, b; Levine 1988; Pizl 1989 a, b). Rees (1961, 1962 and 1963) described new species and some phases of monocystid life cycle. The genus *Apolocystis* Cognetti de Martiis, 1923 was proposed to include those gregarines belonging to the genus *Monocystis* von Stein, 1848, but which had spherical trophozoites lacking polarity. *Apolocystis* was described as having spherical trophozoites without the principal axis marked by the presence of any special peripheral organ. Only three species of *Apolocystis* complete their life cycle in the host coelomic cavity: *A. catenata* Muslow, 1911, *A. michaelseni* Hesse, 1909 and *A. stammeri* Meier, 1956. The present study reports on a new species of *Apolocystis* found within the coelomic cavity of the terrestrial oligochaete *Microscolex dubius* Fletcher, 1887. This finding is also the first report of gregarines in this host. The aim of the present study is to introduce the new species and to describe the phases of its life cycle.

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MATERIALS AND METHODS

The terrestrial oligochaete *Microscolex dubius* was obtained on August 27, 1997, from a vegetal substrate, mainly formed by *Azolla filiculoides* Lam. in Los Talas, Buenos Aires, Argentina (34°53'S, 57°50'W). Examination of fresh material using stereoscopical microscope revealed white, opaque, oval structures immersed in the coelomic fluid in all somites behind the clitellum. This material was fixed in Bouin´s fluid, dehydrated, and embedded in paraffin for further study. Histological sections of 10 µm thickness from the host anterior half were made with a Minnot like micrometer and stained with either Meyer´s or Erlich´s hematoxylin and eosin. Life cycle stages found (intercellular and coelomic trophozoites, gametocysts, cysts containing zygotes and sporocysts, and sporocysts) were measured using optical microscope with micrometric ocular (n = 170) and then photographed.

RESULTS

Apolocystis janovyi sp. n.

Host: *Microscolex dubius* Fletcher, 1887

Localization: coelom.

Description: sub-spherical, oval, kidney-shaped intercellular trophozoites (9-36 µm in major diameter, 6-26 in minor diameter). Sub-spherical or oval coelomic trophozoites without polar differentiation and lacking of ectoplasmic processes (17-283 µm in major diameter, 17-250 µm in minor diameter). Gametocysts 116-206 µm in minor diameter, 193-369 in major diameter. Navicular sporocysts 2.7-6.8 µm in width and 5-15 µm in length.

Specimens deposited: intercellular trophozoites, trophozoites, gametocysts, cysts containing sporocysts, and sporocysts. Hapantotypes (7 slides, N 11) have been deposited in Colección División Zoología Invertebrados (Protozoa), Museo de La Plata, Argentina. Other five slides were deposited in the University of Nebraska State Museum Parasite Collection, School of Biological Sciences, University of Nebraska, USA.

Remarks: the different developmental stages were found in the following locations: esophageal epithelium (intercellular trophozoites), coelomic cavity (trophozoites, gametocysts, cysts containing zygotes and sporocysts, and sporocysts) and only sporocysts in seminal vesicle (Fig. 1). Intercellular trophozoites were located near the basal membrane as well as slightly distal to it. They showed spherical, oval, kidney-shaped forms (Figs 2 a, b) and their sizes varied from 9-36 µm in major diameter to 6-26 µm in minor diameter (Table 1). Coelomic trophozoites were spherical and oval without polar differentiation and lacked ectoplasmic processes. They were surrounded by a mesothelial layer and, in some cases, were attached to the parietal as well as to the visceral peritoneum. Once released to the coelomic

Table 1. Average size (µm) of the observed *Apolocystis janovyi* phases in esophagic epithelium and coelom of *Microscolex dubius*

Phase			Average	Standard deviation	n
Intercellular trophozoite		Major diameter	26.319	5.729	26
		Minor diameter	18.827	4.934	26
Trophozoite within coelomic cavity	Major diameter		158.222	63.578	37
	Minor diameter		128.484	51.778	37
	Nucleus	Major diameter	37.462	11.503	16
		Minor diameter	32.883	8.413	16
	Endosome		17.065	7.715	12
Gametocyst	Major diameter		252.854	45.666	18
	Minor diameter		183.01	24.89	18
	Nucleus	Major diameter	41.624	11.332	
		Minor diameter	30.445	4.045	7
	Endosome		13.984	2.524	5
Cyst containing sporocysts	Major diameter		262.122	44.301	25
	Minor diameter		148.875	41.222	25
Sporocyst		Length	9.996	2.201	55
		Width	4.187	0.886	55

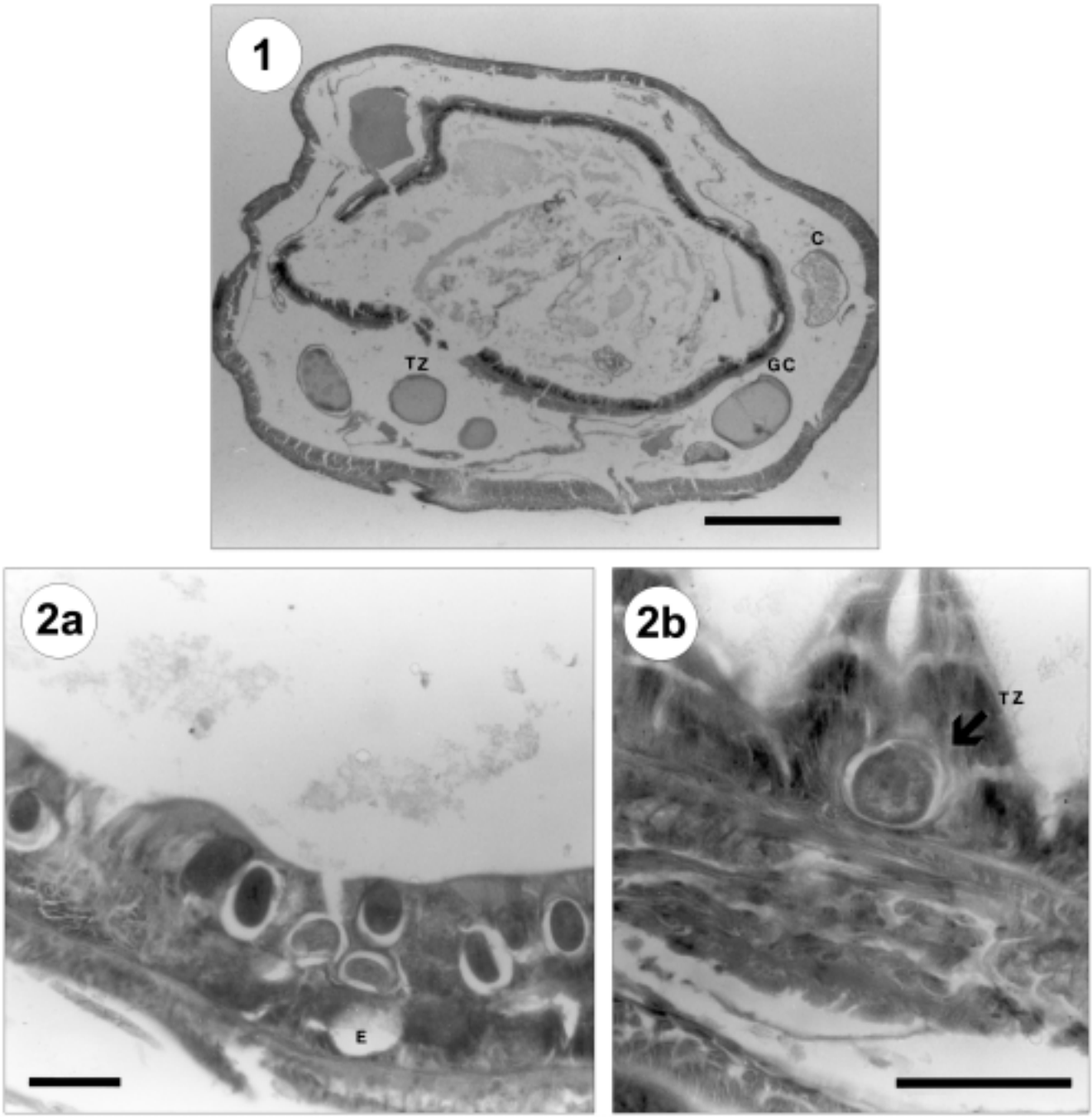
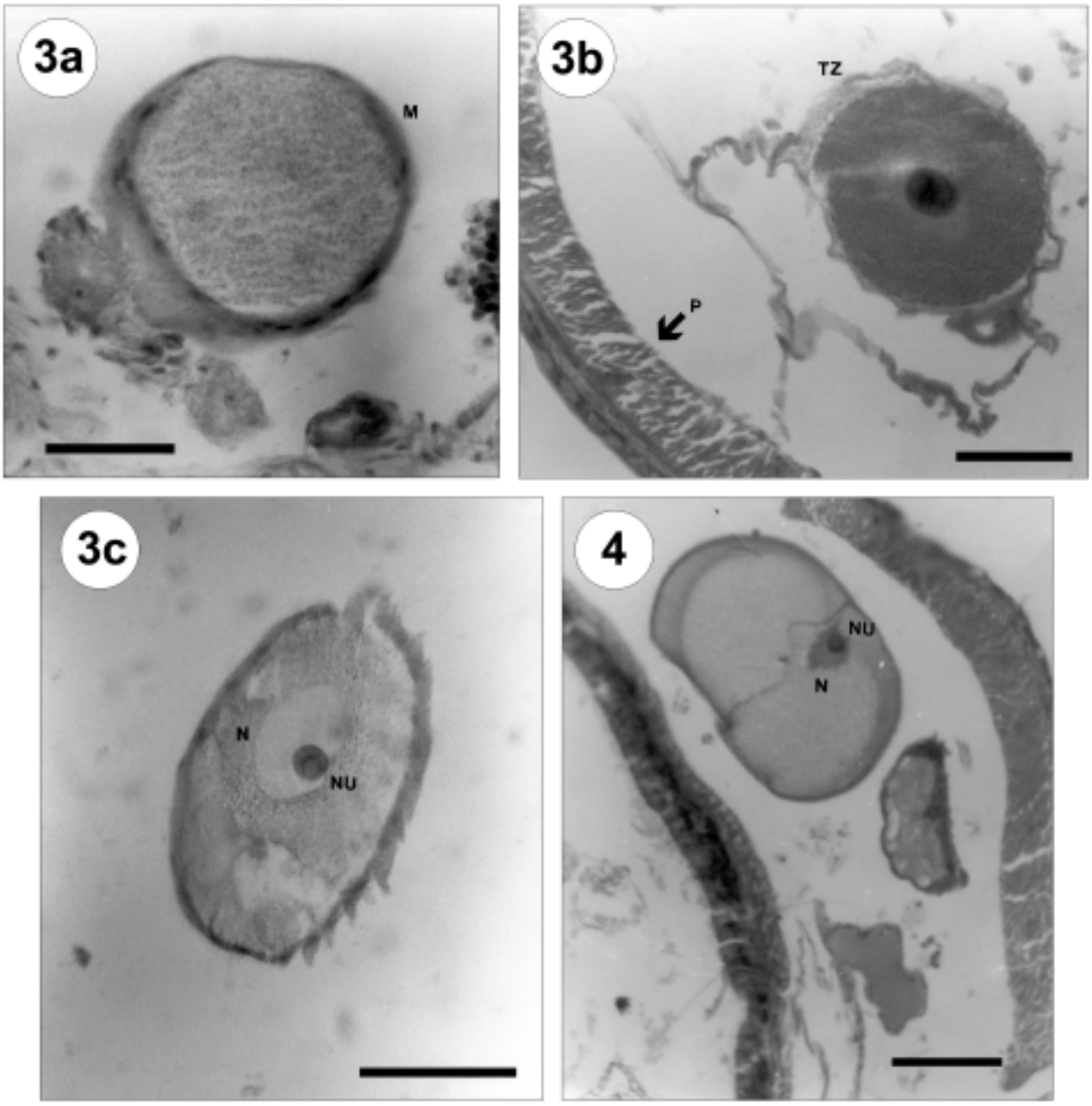
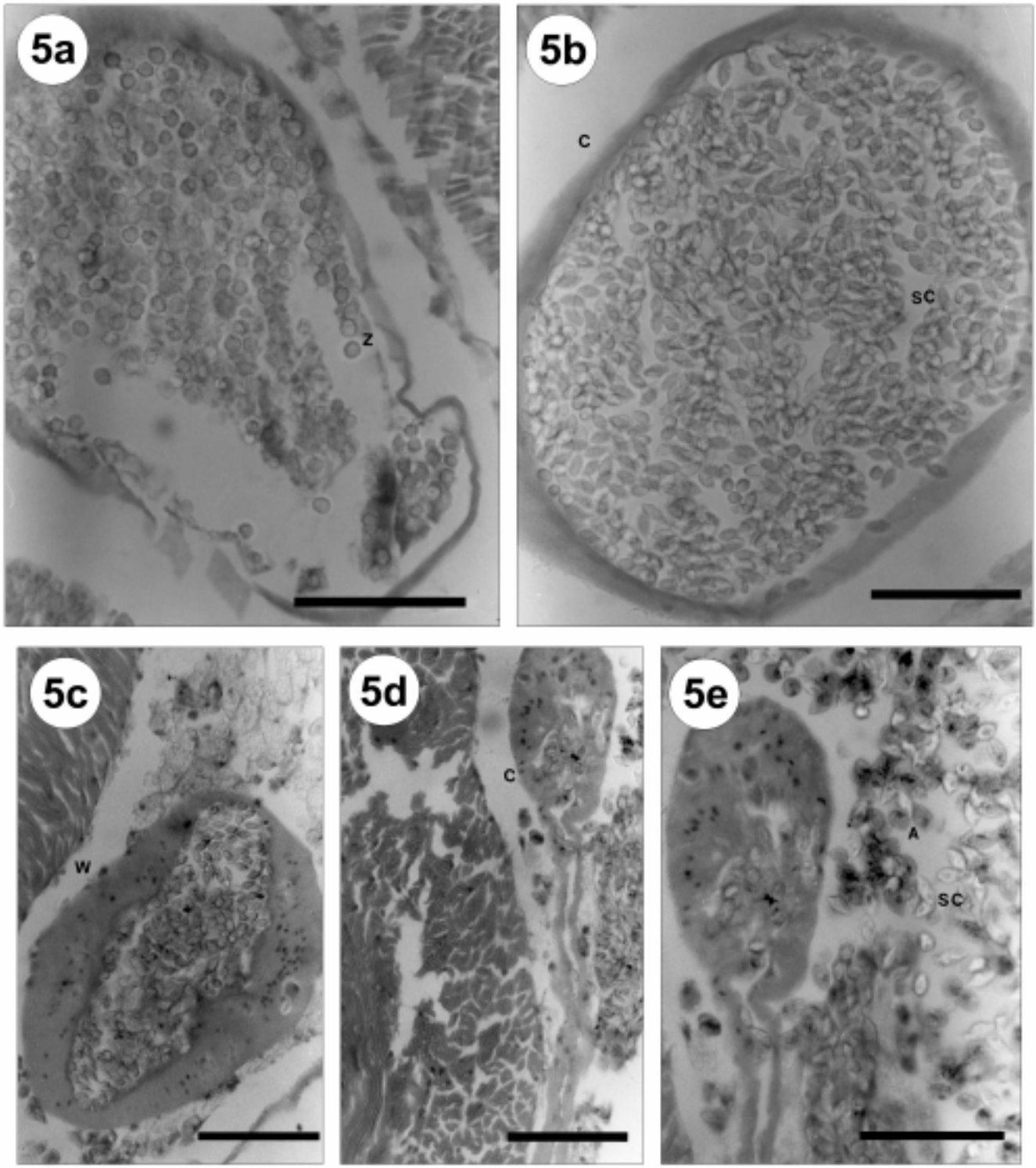


Fig. 1. Transverse section of *Microscolex dubius* in postclitellar segments; note trophozoites (TZ), gametocysts (GC) and cysts (C) in the coelom. Scale bar - 500µm
Figs 2 a, b. Intercellular trophozoites of *Apolocystis janovyi* in esophageal epithelium (E); **a** - different shapes and localizations can be seen; **b** - spherical trophozoite (TZ) in the basal membrane proximity. Scale bar - 50 µm



Figs 3 a-c. *Apolocystis janovyi* coelomic trophozoites; **a** - note the mesothelial cell cover (M); **b** - trophozoite (TZ) attached to the parietal peritoneum (P); **c** - free trophozoite within the coelomic cavity; note the irregular cover, the nucleus (N) and endosome (NU). Scale bar - 50 µm
Fig. 4. *Apolocystis janovyi* gametocyst inside coelomic cavity; note the star-shape nucleus (N) and endosome (NU). Scale bar - 100 µm



Figs 5 a-e. *Apolocystis janovyi* cysts inside coelom cavity; **a** - note the zygote (Z) inside; **b** - navicular sporocysts (SC) inside the cyst (C); **c** - note the multinuclear wall (W) of cyst; **d** - collapsed cyst (C); **e** - free sporocysts (SC) within the coelomic cavity surrounded by numerous amebocytes (A). Scale bar - 50 µm

cavity, this layer adopted an irregular aspect, showing cytoplasmic prolongations (Figs 3 a, b, c). An increase in the size of trophozoites to 17-283 µm in major diameter and 17-250 µm in minor diameter was observed (Table 1). The nucleus was spherical, occasionally star-shaped and showed an eccentric position, with a spherical, vesicular endosome. Basophilic granules were observed in cytoplasm, of about 0.62 to 1.87 µm, increasing in size towards the nucleus, where they appeared to be more dispersed. Likewise, gametocysts were observed in the coelomic cavity. They also showed a mesothelial layer and a star-shaped nucleus (Fig. 4). Their sizes varied between 116-206 µm in minor diameter and 193-396 µm in major diameter (Table 1). Both coelomic trophozoites and gametocysts were found within the coelomic cavity, which corresponds to the postclitellar region.

The morphology of the cysts having sporocysts varied (Figs 5 a, b). Sizes ranged from 76-239 µm in minor diameter, and 167-328 µm in major diameter (Table 1). These stages were found within the coelomic cavity of the preclitellar, clitellar and postclitellar region, and they showed a layer formed by a syncytial wall with spherical nuclei. The thickness of this wall varied according to the degree of cyst turgidity, being thinner in turgid cysts and turning into more noticeable multinuclear nature in the collapsed ones (Figs 5 c, d). Cysts that released sporocysts to the coelom showed the presence of abundant amebocytes (Fig. 5 e).

Sporocysts were navicular, and were 2.7-6.8 µm in width and 5-15 µm in length (Fig. 5 b). They were found within the coelom of preclitellar and clitellar segments, and in the seminal vesicle. Abundant amebocytes were observed surrounding the sporocysts in those segments as a probable host response to the presence of the parasite.

Microanatomic observation of the host gonads suggested an incipient maturation of the ovary and the seminal vesicle, showing different stages of the spermatogenesis with little presence of spermatozoids.

Etymology: dedicated to the eminent gregarinologist, Prof. John Janovy, Jr., Verner Professor of Biological Sciences, University of Nebraska, Lincoln, U.S.A.

DISCUSSION

Until this description, gregarines have never been described before from *M. dubius*, although this host has a cosmopolitan distribution (Righi 1979). There are only three species of *Apolocystis*, which complete their life cycle within the host coelomic cavity. The different phases of the life cycle of *A. michaelseni* Hesse, 1909 show a morphology, which can be compared to *Apolocystis janovyi* sp. n.

A. michaelseni has spherical or ovoid trophozoites, granular endoplasm, and a nucleus with a large, central endosome. Gametocysts and cysts are ellipsoidal, and sporocysts are navicular. Although the morphology of the mentioned stages have a correspondence with those described for the new species, the sizes measured for *A. janovyi* are more variable than the sizes observed by Hesse (1909) for *A. michaelseni* (225-295 µm trophozoites, gametocysts of 235-300 µm in major diameter and 170-220 µm in minor diameter, and sporocysts of 9 µm in width and 15 µm in length).

In opposition to *A. michaelseni*, in which trophozoites are particularly abundant in the clitellum proximity, the species here described has its trophozoites distributed along the worm. Both parasite species are frequently surrounded by host conjunctive cells, which constitute a pedicel that attaches them to the body wall. Occasionally, the trophozoites are found free in the coelom. Hesse (1909) observed in *A. michaelseni* gametocysts with more than two individuals, not knowing their destiny. An important difference between these two species must be highlighted in relation to the number of sporocysts contained inside a cyst. Hesse (1909) found up to a maximum of 16 ellipsoidal, very voluminous sporocysts inside the cyst, while in *A. janovyi* there are over than 100 ones in a cyst.

Despite the methodology used we could not find basophilic granules, their aspect in the *A. janovyi* trophozoite cytoplasm suggest its glycogenic nature (Hesse 1909; Rees 1963; Pizl 1989 a, b). As observed in other monocystid gregarine species, (*Cephalocystis singularis* and *Dendrocystis piriformis*), the two phases in the life cycle of *A. janovyi* are: a period of trophozoite growth inside the esophagical epithelium and a second phase when the trophozoite is free within the coelomic cavity of the worm (Rees 1962). The coelomic trophozoite is an important growth phase inside the host body cavity. Nevertheless, the existence of trophozoite growth in the digestive epithelium could not be denied. As Rees (1962) observed in *C. singularis,* after the association of trophozoites, a size contraction occurred in such a way that the gametocyst had a slightly bigger size than the trophozoite.

The variety or shapes found in cysts could be attributed to its liquid content. The sporocysts seem to be the

only phase of life cycle recognized as foreign by the host. This is because in no other phase amebocytes were observed.

The possible infection mode should have been the digestive one. Different episodes of infestation could have been happened due to the presence of distinct life cycles stages in diverse localities. As Hesse (1909) suggested, we consider that the release of sporocysts could occur in different ways: by coelomic liquid exudation, host fragmentation, tissue necrosis and its death. New cycles within the same host would not happen, since the sporocysts become infective only when they reach the digestive tract. The sporocysts can remain unchanged in the environment, even after a carnivore eats the host.

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ACTA
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Short Communication

A Free-Living Amoeba with Unusual Pattern of Mitochondrial Structure Isolated from Atlantic Salmon, *Salmo salar* L.

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Summary. A strain of non-encysting free-living amoeba (LOS7N) isolated from the gills of *Salmo salar* L. was characterised morphologically and by the sequence of SSU rRNA gene. High level of sequence similarity (99.58%) with the strain F-13 (ATCC 30942) denominated as *Saccamoeba limax* Page, 1974 was at variance with results of comparative ultrastructural study. Strain LOS7N differed from the previous and thus far the only ultrastructural description of *S. limax* (Page 1985) in having regularly arranged, straight tubular non-branching cristae of mitochondria.

Key words: free-living amoeba, mitochondrial cristae, *Salmo salar*, SSU rRNA gene.

INTRODUCTION

The lack of concordance between morphological similarity of organisms and their relatedness inferred from SSU rDNA sequence-based phylogenetic analyses mentioned for many protistan groups has been recognised also in amoebae (Amaral Zettler *et al*. 2000, Bolivar *et al*. 2001). Variable and changeable morphological features used to describe amoeboid protists gradually loose the character of taxonomic criteria although some of them still are of considerable descriptive value discriminating, e.g., so called limax amoebae from acanthamoebae (Page 1974). Of the major ultrastructural features used for identification, the form of mitochondrial cristae is considered paramount (e.g., for discrimination of the family Vahlkampfiidae or Acrasida). The morphology of cristae has long been recognised to be very conservative and of great phylogenetic value (Cavalier-Smith 1996/97). Recent papers by Sims *et al*. (1999), Amaral Zettler *et al*. (2000) and Bolivar *et al*. (2001) clearly indicate phylogenetic diversity of amoeboid organisms and evidence the need to re-evaluate criteria for their classification. Despite examples of incongruence between morphological and molecular characteristics, the attempts to integrate structural and molecular approaches are most desirable. Since cultures of type species of amoebae are not always available,

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well-described and morphologically documented strains should be included in sample sets of taxa used for molecular analyses.

The aim of this paper is to characterise a free-living amoeba isolated from the gills of Atlantic salmon, *Salmo salar* L., both morphologically and on molecular level and draw attention to inconsistency of our data with literature.

MATERIALS AND METHODS

Methods of isolation of amoebae, their culturing on agar plates and cloning procedures, as well as methods of light and electron microscopic examination, were as reported in previous papers (Dyková *et al*. 1997, 1998, 1999).

DNA isolation, amplification and sequencing

Total genomic DNA was isolated from trophozoites of cloned strain LOS7N/I using standard phenol/chloroform extraction technique with ethanol precipitation (Sambrook *et al*. 1989). Universal eukaryotic primers 5´AYCTGGTTGATYYTGCCAG-3´ and 5´TGATCCATCTGCAGGTTCACCT-3´ reported by Medlin *et al*. (1988) were used for amplification of the SSU rRNA gene. PCR reaction was carried out in a volume of 25 µl using a standard technique with 1 unit of Taq polymerase (TaKaRa, Japan), 250 µM of each dNTP, 10 pmol of each primer and 2.5 µl of 10x Taq polymerase buffer. Conditions for PCR were as follows: initial denaturation temperature 95°C for 5 min followed by 30 amplification cycles at 95°C for 1 min, 52°C for 1.5 min and 72°C for 2 min; final extension 72°C for 10 min. PCR product was purified from an 1% agarose gel and cloned into pCR® 2,1 TOPO Cloning vector using the TOPO-TA Cloning Kit (Invitrogen). Sequencing was carried out on an automatic sequencer CEQ™ (Beckman Coulter) using CEQ Dye Terminator Cycle Sequencing Kit (Beckman Coulter) according to the manufacturer's protocol.

Phylogenetic analyses

The SSU rRNA gene of the strain under study was aligned together with sequences of Gymnamoebia *sensu stricto* according to Bolivar *et al*. (2001) and *Hexamita inflata* as an outgroup. The alignments were performed using Clustal X program (Thompson *et al*. 1997) with various alignment parameters and corrected by eye in the program BioEdit sequence alignment editor (Hall 1999). Ambiguously aligned regions were removed. The phylogenetic relationships between taxa were determined using maximum parsimony (MP) and distance method carried out in the program package PAUP*, Version 4,0b10 (Swofford 2001). MP analysis was done using the heuristic search with random addition of taxa and transversion/transition (Tv/Ts) ratios of 1:1 and 1:2. Gaps were treated as missing data. The distance method was performed using heuristic search and minimum evolution (ME) as an objective setting with Kimura two-parameter substitution model. Genetic distances were calculated using K2P algorithm. Clade support was assessed by bootstraping (1000 replicates).

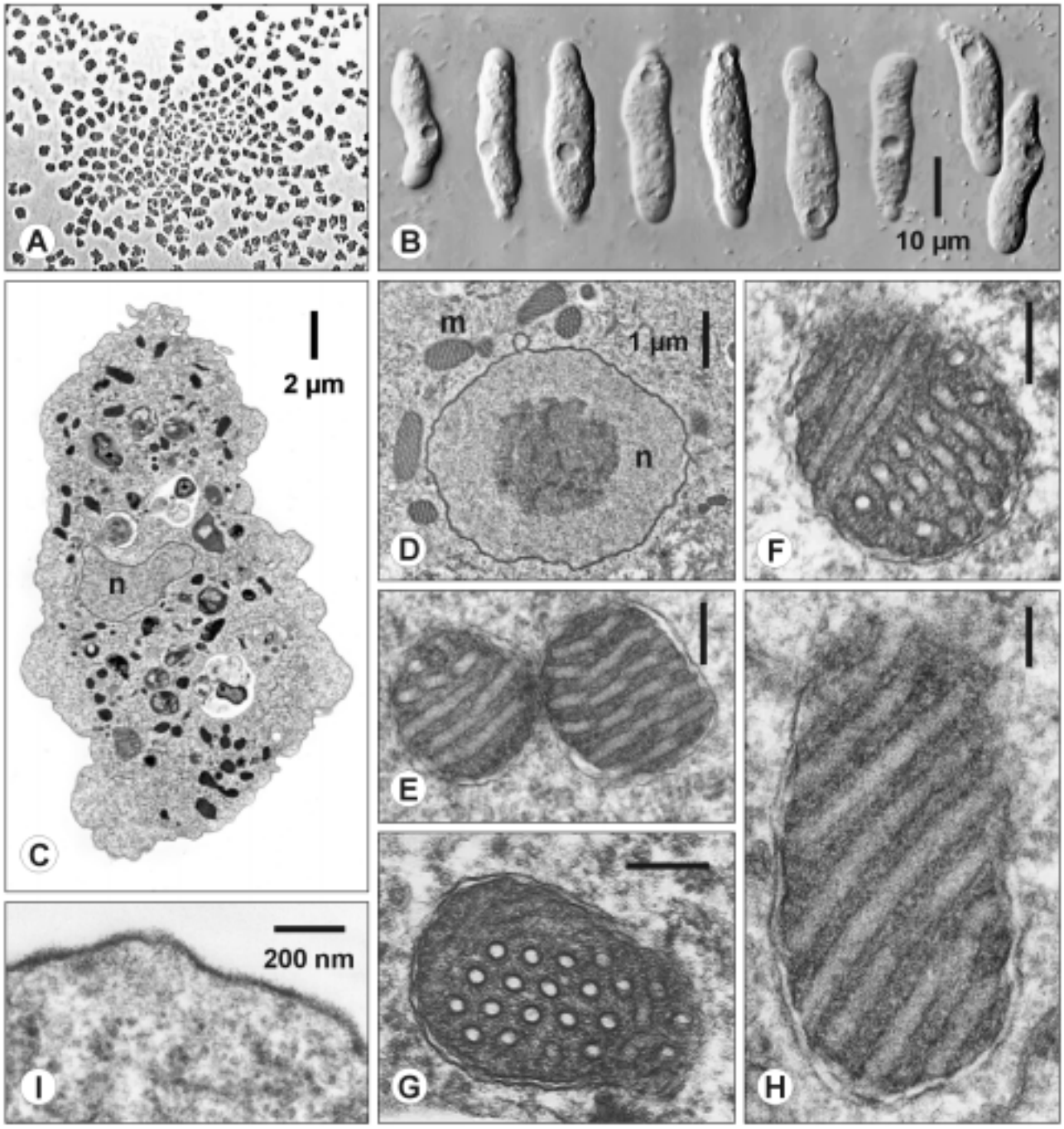
RESULTS

The gills of *Salmo salar* L. fry (size category 2.4-2.8 cm) that was used for salmon restocking of the river Elbe basin in the Czech Republic were found infected with free-living amoebae. An amoeba strain (LOS7N) was isolated in July 2000 and a clone LOS7N/I was derived from this strain in passage No. 9 in October 2000.

Description of the cloned strain LOS7N/I

Trophozoites cultured on agar plates (Fig. 1A) were mostly polymorphic (when attached to agar surface). Locomotive forms observed in hanging drop preparations had monopodial, cylindrical or subcylindrical shape with a short anterior hyaline zone (Fig. 1B). They possessed conspicuous contractile vacuole and small knob-like uroid. The length of limax-like locomotive forms was 34.5 (30-40) µm; the breadth did not exceed 10 µm. Encystment was not observed during the six month period of clonal culturing. Strain morphology as observed in the light microscope resembled, to some extent, that of species of the genus *Saccamoeba* Frenzel, 1892, emend. Bovee, 1972 and *Trichamoeba* Fromentel, 1874. Crystal structures recorded in the cytoplasm of species of both these genera were not observed in trophozoites of our strain.

Transmission electron microscopy (Figs 1C-H) revealed a conspicuous feature of ultrastructure: a highly characteristic organisation of mitochondria. Smooth outer membrane-bounded mitochondria were oval or elliptical in sections. The interiors of mitochondria were crowded with tubular cristae of uniform diameter (40-45 nm). The tubular form of cristae was evident when cut transversely (Fig. 1G). The other planes of sectioning evidenced that cristae were straight, not branched, and packed in close parallel arrays (Figs 1E, F). They extended all the way across the organelle, maintaining the same distances (Fig. 1H). The surface coat of amoeba trophozoites was rather amorphous; glycostyles or other surface structures were not discernible (Fig. 1I). The cisternae of endoplasmic reticulum, encountered rather rarely, had the form of flattened tracks. The Golgi apparatus consisted of flat saccules piled up



Figs 1 A-I. Light micrographs of living amoebae of the cloned strain LOS7N/I and the features of their ultrastructure; **A** - cultured trophozoites attached to agar surface as seen through Petri dish; **B** - trophozoites in hanging drop preparation (Nomarski differential interference contrast); **C** - general structure with part of nucleus in the plane of section, numerous densely stained mitochondria and rests of phagocytized material in the cytoplasm; **D** - nucleus surrounded by mitochondria; **E, F** - straight, non-branching cristae of mitochondria in various planes of sectioning; **G** - tubular form of mitochondrial cristae as seen in transverse section; **H** - parallel arrangement of tubular cristae; **I** - cell surface. Scale bars: E-G, I - 200 nm; H -100 nm

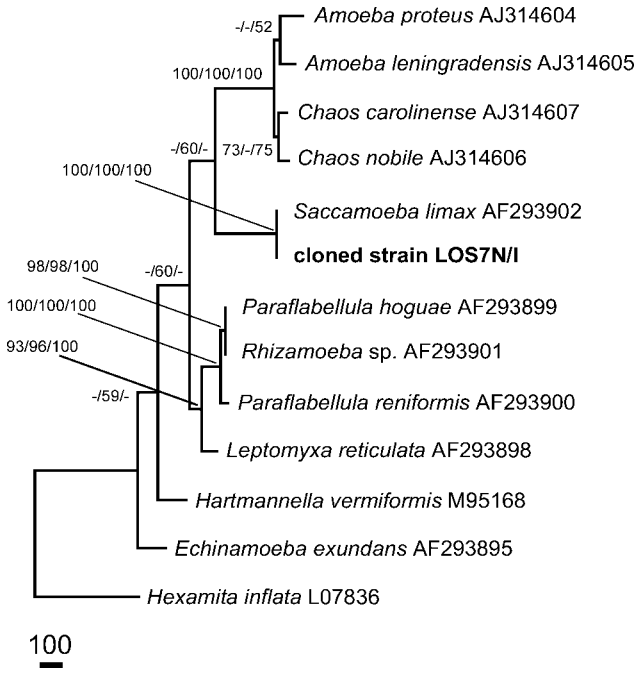


Fig. 2. Maximum parsimony phylogenetic tree of the SSU rRNA sequences rooted at *Hexamita inflata* (one most parsimonious tree, Tv/Ts = 1:1, 1287 steps, CI = 0.74, RI = 0.72). Bootstrap values (MP Tv/Ts = 1:1, MP Tv/Ts = 1:2, distance K2P method) are indicated for the nodes gaining more than 50% support. The scale is given under the tree. The accession numbers are in parentheses

in close parallel arrays. The cytoplasm of trophozoites contained remnants of phagocytized material, mainly of bacterial origin (Fig. 1C).

SSU rRNA data and phylogenetic analysis

The length of SSU rRNA gene of the strain under study (LOS7N/I) was 1902 nucleotides with a G+C content 37.7%. The sequence has been deposited in the EMBL/GenBank database under Accession Number AY145442. The analyses were based on sequences of 13 taxa. The final alignment consisted of 1426 nucleotide sites (776 sites were excluded). The number of parsimony informative characters was 413. LOS7N/I clustered in all analyses performed with strain F-13 (ATCC 30942) denominated in Amaral Zettler *et al.* (2000) as *Saccamoeba limax* Page, 1974. The similarity of sequences of these two strains (computed from the alignment) was 99.58%. The comparison of entire length of sequences revealed eleven nucleotide changes. Both strains branched with *Amoeba-Chaos* clade with low bootstrap support (60%) in maximum parsimony (MP), Tv/Ts = 1:2; under 50% in MP, Tv/Ts = 1:1 and minimum evolution (ME). The topology of the phylogenetic tree is shown in Fig. 2.

DISCUSSION

More than 70 cloned strains of fish-infecting amoebae that we have isolated thus far have been characterised at the cellular and ultrastructural levels. Among them, the strain LOS7N/I is unique in having regularly arranged tubular non-branching mitochondrial cristae. The level of similarity in sequences of LOS7N/I strain and F-13 (ATCC 30942) strain denominated as *Saccamoeba limax* (Amaral Zettler *et al*. 2000) was high enough to suggest species identity. However, the comparison of ultrastructural data available calls in question the assignment of our strain (LOS7N/I) to the genus *Saccamoeba sensu* Page (1985). The strain F-13 (ATCC 30942) denominated as *S. limax* and included in the set of strains sequenced in the study of Amaral Zettler *et al*. (2000) was analysed also by Bolivar *et al*. (2001). Its sequence is deposited in GenBank under accession number AF 293902. To the best of our knowledge, ultrastructural study of this strain has not been published to date. Two strains of *S. limax* (CCAP 1534/6 and CCAP 1572/3) stored in the UK National Culture Collection (UKNCC, formerly CCAP) were described by Page (1985) as having tubular but seldom branching mitochondrial cristae. Their appearance, documented with electron micrographs as irregularly twisted and, most probably, blindly ending crists in the interior of mitochondrion matrix, differs substantially from that of our strain (LOS7N/I). Similarly, another species of the genus *Saccamoeba* (*S. stagnicola*) was characterised by Page (1985) as having tubular mitochondrial cristae of twisting appearance that branch rather rarely.

Our comparisons that focused on the organisation of tubular mitochondrial cristae included also species of non-vahlkampfiid genera forming limax-like monopodial trophozoites (Page 1980). Although in the re-diagnosis of the genus *Trichamoeba* Fromentel, 1874 tubular cristae of mitochondria were characterised as occasionally branched, an electron micrograph of *T. sinuosa* (Siemensma and Page 1986, Fig. 12) shows mitochondria almost identical with those of *Saccamoeba* spp. documented by Page (1985, Fig. 35). The genus *Rhizamoeba*, separated from *Trichamoeba* by Page (1972) on the basis of differences in uroidal structures, as well as *Hydramoeba* Reynolds and Looper, 1928, separated from *Trichamoeba* due to parasitic way of

life of the only species of the former genus, were not sufficiently described to compare their mitochondria with the strain under study.

Our data have shown that neither morphology-based systematics (Page 1988, Page and Siemensma 1991) nor molecular data available can immediately solve the generic assignment of our strain LOS7N/I. Nevertheless, combined with new data on other strains and species, they can contribute in the future to a better understanding of phylogenetic and taxonomic relationships of limax amoebae and amoebae in general. Continued interest in taxonomic studies correlating morphological and molecular characters of amoeboid organisms will undoubtedly be the basis for this.

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