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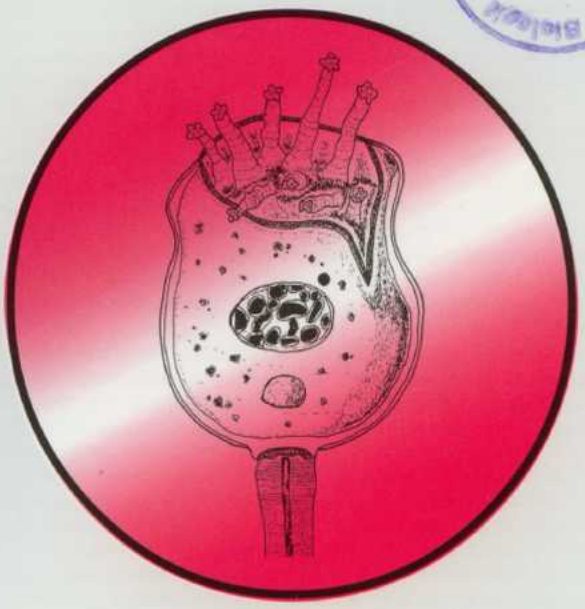

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Phylogenetic Positions of Two Ciliates, *Paranophrys magna* and *Mesanophrys carcini* (Ciliophora: Oligohymenophorea), within the Subclass Scuticociliatia Inferred from Complete Small Subunit rRNA Gene Sequences

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Summary. The complete small subunit rRNA gene sequences of two scuticociliates, *Paranophrys magna* Borror, 1972 and *Mesanophrys carcini* Grolière & Leglise, 1977, were determined. The results show that each comprises 1759 nucleotides. The phylogenetic positions of both species within the subclass Scuticociliatia were deduced using distance matrix and maximum parsimony methods. The trees indicate that the order Philasterida is probably a monophyletic group, within which *Mesanophrys carcini* is allied in a clade with *Anophryoides haemophila* that branches basally to other four species: *Paranophrys magna*, *Uronema marinum*, *Pseudocohnilembus marinus* and *Cohnilembus verminus*, while the clade including *Paranophrys magna* and *Uronema marinum* is grouped with that of *Pseudocohnilembus marinus* and *Cohnilembus verminus*.

Key words: Ciliophora, *Mesanophrys carcini*, *Paranophrys magna*, phylogeny, scuticociliates, SSrRNA.

INTRODUCTION

The subclass Scuticociliatia is regarded by most taxonomists as a monophyletic group within the phylum Ciliophora (Corliss 1979, Lynn 1979, Puytorac *et al.* 1984, Lynn and Sogin 1988, Lynn and Small 1997, Strüder-Kypke *et al*. 2000). According to Lynn and Small (1997) this subclass, which belongs to the class Oligohymenophorea, is divided into three orders: Philasterida, Pleuronematida and Thigmotrichida. Taxonomic and systematic studies on scuticociliates are traditionally based morphological and morphogenetic characters. Over the past two decades, numerous studies have been carried out in this field. Nevertheless, there is still some confusion concerning the phylogenetic relationships among many taxa within the group (Borror 1972; Grolière and Leglise 1977; Grolière 1980; Song 1993, 2000; Morade and Small 1994; Hu *et al.* 1996; Song and Wei 1998; Song and Wilbert 2000).

Molecular methods, in particular the determination of small subunit rRNA (SSrRNA), have been used to re-evaluate the systematics of various ciliate groups in recent years. However, sequence data for scuticociliates remains comparatively rare and incomplete. To date, SSrRNA gene sequences have been determined for only

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about 10 species within the subclass Scuticociliatia (Greenwood *et al.* 1991, Ragan *et al*. 1996).

Paranophrys magna and *Mesanophrys carcini* are two common scuticociliates that are frequently found in coastal or saprobic mariculture environments near Qingdao, China. Their morphologies were redescribed recently by Song (2000). The aims of the present paper were to sequence the SSrRNA gene of both species and to compare these with sequences of other ciliates in order to gain a better understanding of the phylogenetic relationships among various taxa within the Scuticicoliatia.

MATERIALS AND METHODS

Ciliate collection and culture

Paranophrys magna Borror, 1972 and *Mesanophrys carcini* Grolière & Leglise, 1977 were collected from two off-shore mariculture ponds near Qingdao, China. Clonal cultures were established and maintained in sterilized seawater at room temperature with rice grains as food source to enrich bacteria.

Identification of species

Observations on living cells were carried out using Nomarski differential interference contrast microscopy. Protargol and Chatton-Lwoff silver impregnation techniques were used in order to reveal the infraciliature and the silverline system respectively. Terminology is based mainly on Song (2000).

Extraction of genomic DNA

Cells were starved overnight, rinsed three times with sterile artificial marine water and then pelleted by centrifugation. 0.5 ml lysis buffer (10mM Tris-HCl, pH 8.3; 50mM KCl; 2.5mM $MgCl_2$; 0.6% Tween 20; 0.6% Nonidet P40; 60µg/ml Proteinase K) was added and the cells were incubated at 56°C for 2 h. After incubation, DNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with 70% alcohol. DNA was stored at -20 °C (Kusch and Heckmann 1996, Chen *et al.* 2000).

PCR amplification

Amplifications by PCR were carried out in a total volume of 100 ml containing 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 0.1% Triton X-100; 3 mM $MgCl_2$; 0.2 mM dNTP; 0.5 mM of each oligonucleotide primer (16s-like F:5'AACCTGGTTGATCCTGCCAGT-3';16s-like R:5'-TGATCCTTCTGCAGGTTCACCTAC-3'); 50 ng of genomic DNA and 5U Taq Ex DNA polymerase (TaKaRa, Japan). The reaction mixtures were denatured at 94°C for 5 min before the polymerase was added (hot start), followed by the first 5 cycles consisting of denaturation for 1 min at 94°C, primer annealing for 2 min at 56°C, and extension for 2 min at 72°C. In the subsequent 35 cycles, the annealing temperature was increased to 62°C. This was followed by a final extension step for 5 min at 72°C (Elwood *et al.* 1985, Medlin *et al.* 1988, Chen and Song 2001).

Cloning and sequencing the SSrRNA gene

The amplified products were extracted with UNIQ-5 DNA Cleaning Kit (Sangon Bio. Co., Canada) and inserted into a pUCm-T vector. The plasmid mini-prep spin column kit (Sangon Bio. Co., Canada) was used to harvest and purify plasmid DNA. DNA sequencing for *Paranophrys magna* and *Mesanophrys carcini* was accomplished using the ABI Prism 377 Automated DNA Sequencer (Applied Biosystems Inc.) with three forward and three modified reverse 16S sequencing primers (Elwood *et al.* 1985, Medlin *et al.* 1988) as well as the RV-M and M13-20 primers. All sequences were confirmed from both strands.

Sequence availability

The nucleotide sequences used in this paper are available from the GenBank/EMBL databases under the following accession numbers: *Anophyroides haemophila* U51554, *Cyclidium plouneouri* U27816, *Cohnilembus verminus* Z22878, *Cyclidium glaucoma* Z22879, *Cyclidium porcatum* Z29517, *Ichthyophthirius multifiliis* U17354, *Obertrumia georgiana* X65149, *Ophryoglena catenula* U17355, *Paramecium bursaria* AF100314, *Paramecium tetraurelia* X03772, *Paramecium nephridiatum* AF100317, *Platyophrya vorax* AF060454, *Pseudomicrothorax dubius* X65151, *Pseudocohnilembus marinus* Z22880, *Pseudoplatyophrya nana* Af060452, *Tetrahymena corlissi* U17356, and *Uronema marinum* Z22881. A karyorelictid ciliate, *Loxodes striatus* L24248 was selected as the outgroup species.

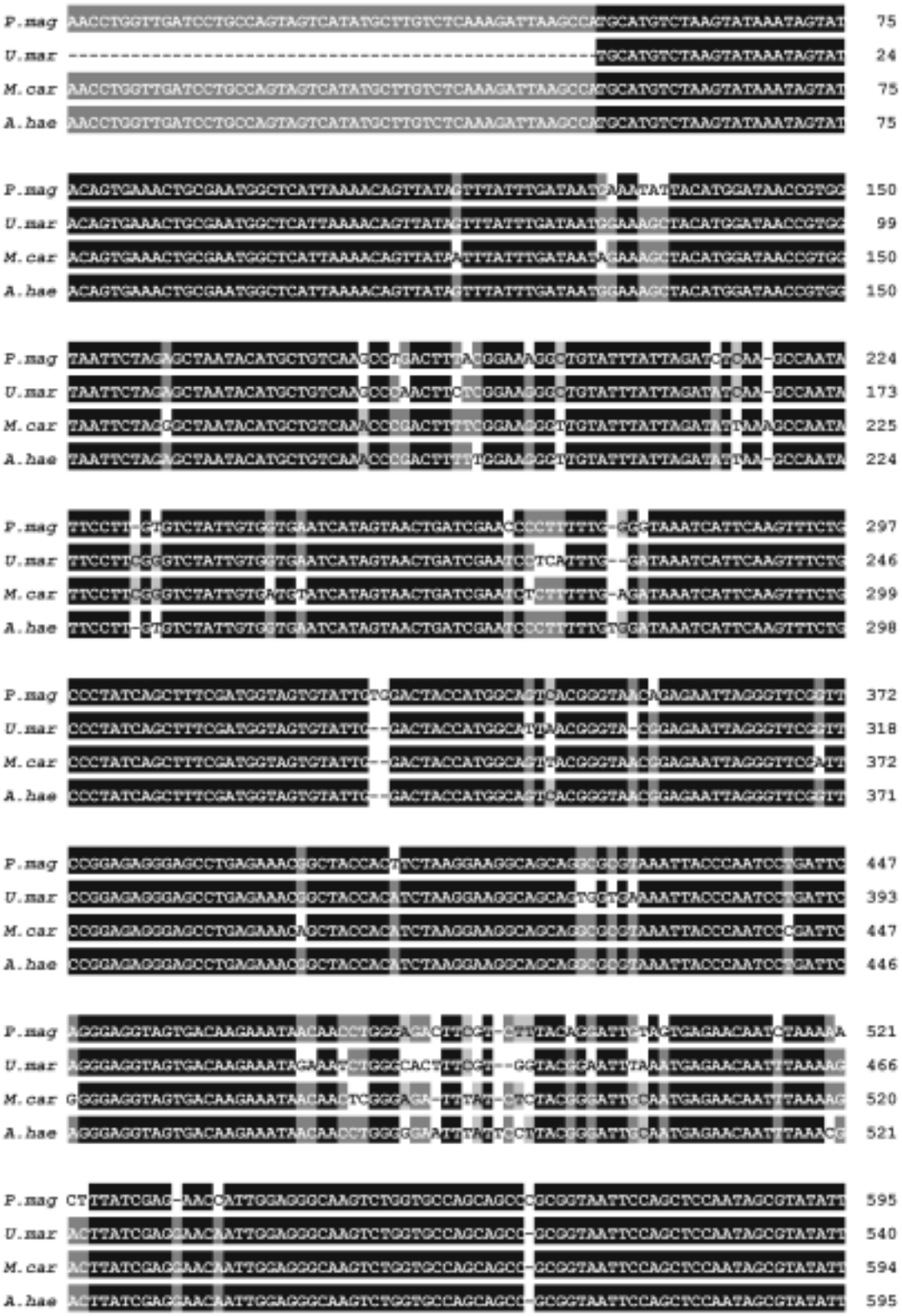
Phylogenetic analyses

The sequences were aligned with other SSrRNA gene sequences using a computer assisted procedure, Clustal W, ver. 1.80 (Thompson *et al.* 1994), and refined by considering the conservation of primary structures. PHYLIP package, version 3.57c (Felsenstein 1995) was used to calculate the sequence similarity and evolutionary distances between pairs of nucleotide sequences using the Kimura (1980) two-parameter model. Distance-matrix trees were then constructed using the least-squares [LS] and the neighbor-joining [NJ] methods (Fitch and Margoliash 1967, Saitou and Nei 1987). The DNAPARS program in PHYLIP was used to find the most parsimonious tree (Kluge and Farris 1969). Both parsimony and distance data were bootstrap resampled 1,000 times (Felsenstein 1985).

RESULTS

Sequences and comparisons (Fig. 1)

The complete SSrRNA gene sequences of *Paranophrys magna* (GenBank/EMBL accession number AY103191) and *Mesanophrys carcini* (GenBank/EMBL accession number AY103189) are the same length at 1759 nucleotides. The GC contents (*Paranophrys magna* 43.89%, *Mesanophrys carcini* 43.15%) are in the same range as most other ciliates



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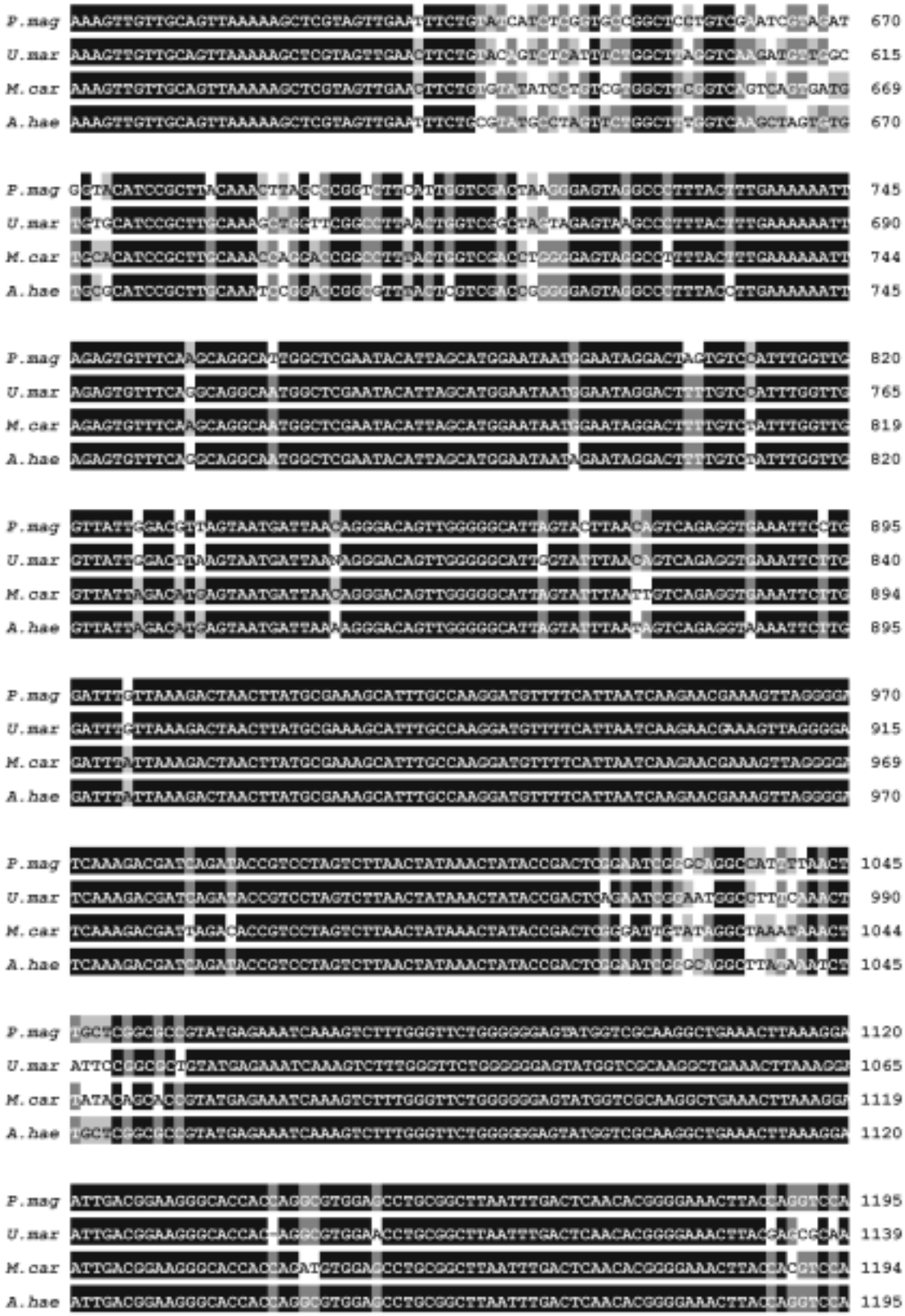

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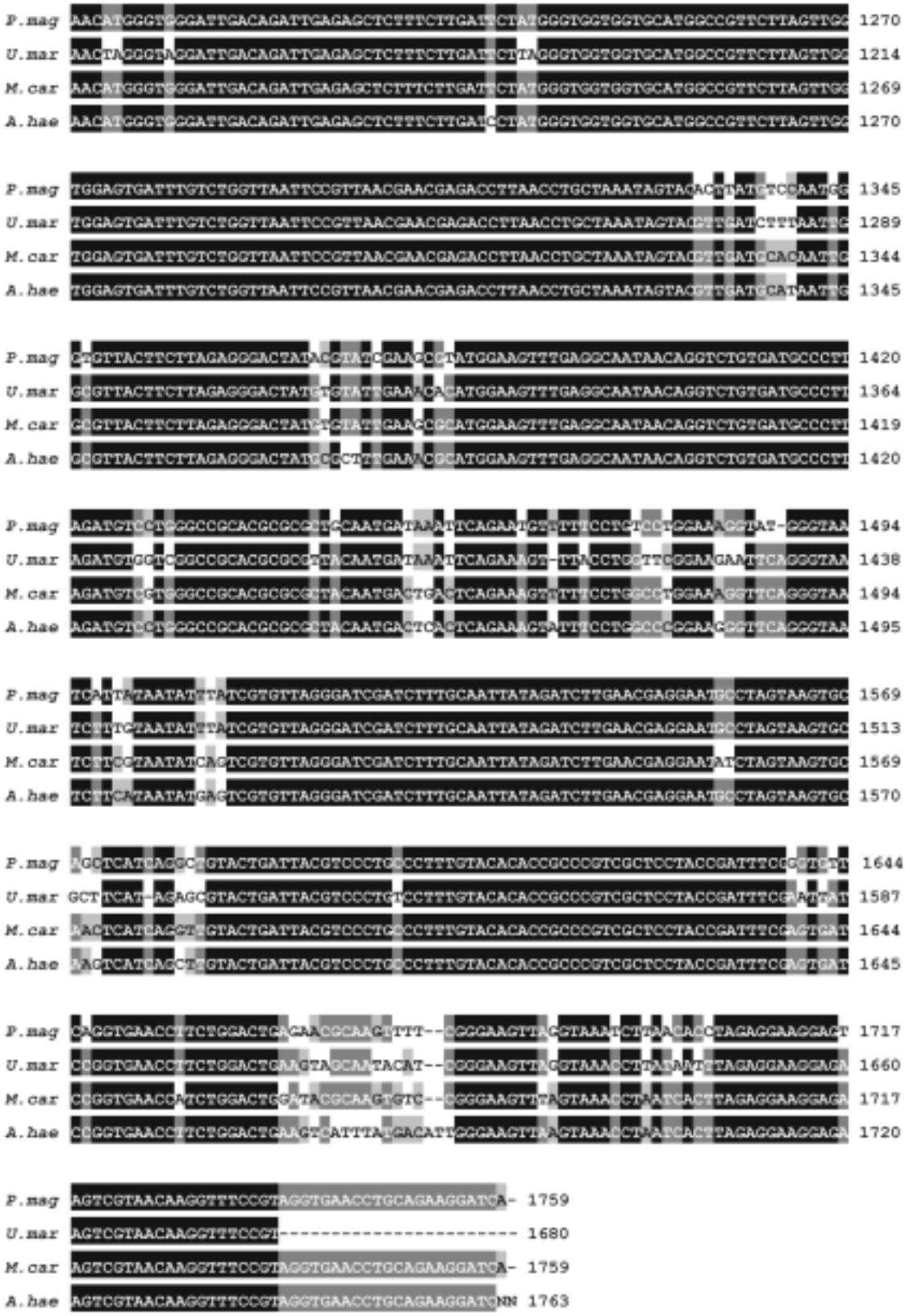

Fig. 1. Small subunit ribosomal RNA gene sequences of the scuticociliates *Paranophrys magna* (*P. mag*) and *Mesanophrys carcini* (*M. car*) aligned with the sequences from *Uronema marinum* (*U. mar*) and *Anophyroides haemophila* (*A.hae*). Numbers at the end of lines indicate the number of nucleotides. The differences in sequence length were compensated for by introducing alignment gaps (-) in the sequences. Matched sites are highlighted in black.

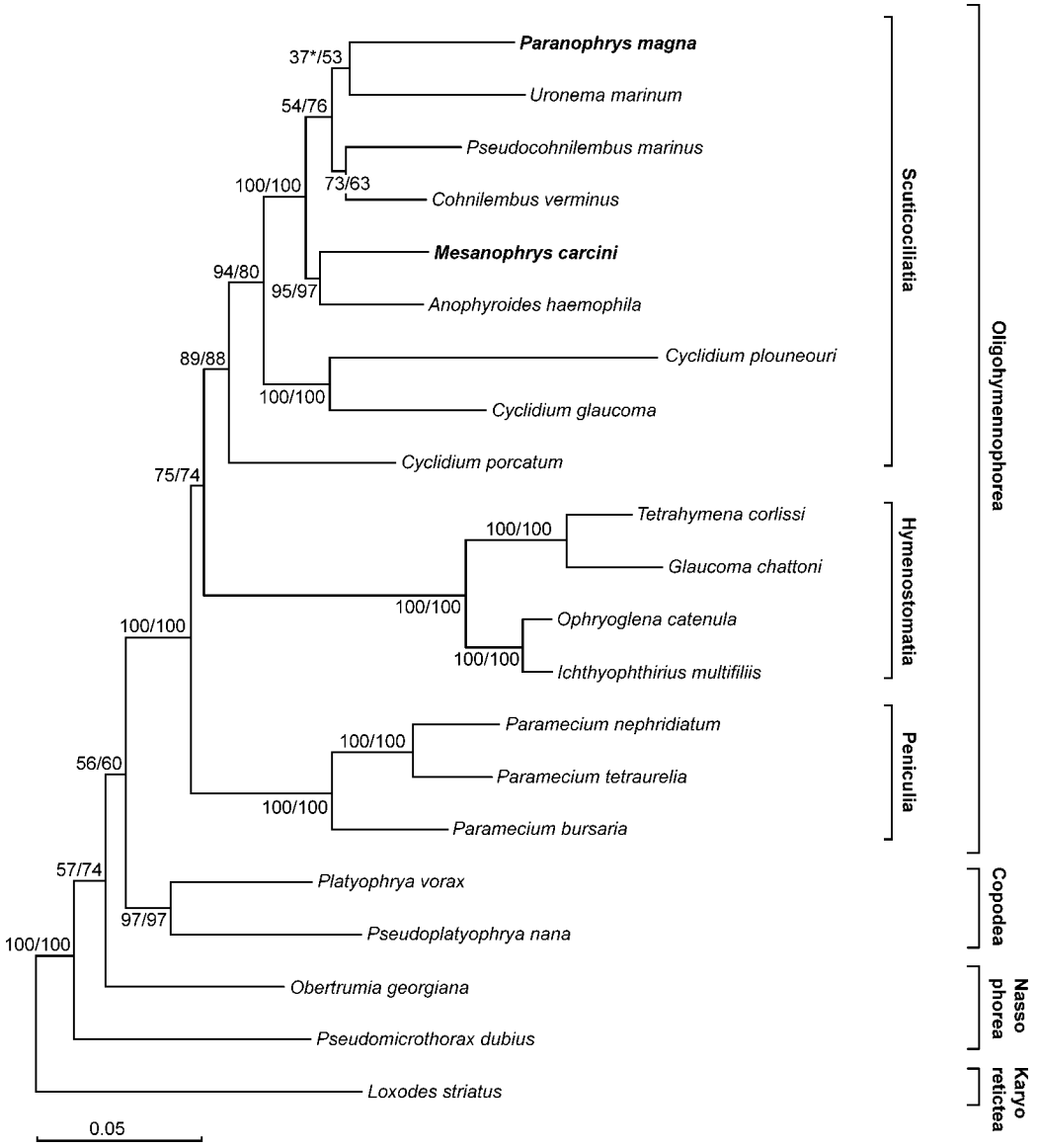

Fig. 2. A small subunit ribosomal RNA tree derived from evolutionary distances showing the phylogenetic positions of *Paranophrys magna* and *Mesanophrys carcini*. The consensus tree of 1000 bootstrap resamplings of the data set was constructed using the Fitch and Margoliash (1967) least-squares [LS] method. The numbers at the nodes represent the bootstrap percentages of 1000 for the LS method followed by the bootstrap values for the neighbor-joining [NJ] method of Saitou and Nei (1987). Asterisks indicate bootstrap values less than 50%. Evolutionary distance is represented by the branch length to separate the species in the figure. The scale bar corresponds to five substitutions per 100 nucleotide positions. The new sequences are represented in boldface.

(Elwood *et al.* 1985, Sogin and Elwood 1986). The sequence of *P. magna* differed in 181 nucleotides from that of *M. carcini* with the similarity 89% between them. After the removal of ambiguous sites in the alignment, a total of 1712 nucleotides remained for the subsequent analysis (Fig. 1). Sequence data were reduced from 1712 to 809 phylogenetically informative sites for the maximum parsimony (MP) analysis.

Table 1 shows the structural similarity and evolutionary distance values that were calculated pairwise for 20 ciliate species including the two scuticociliates treated in the present work. From these data it can be seen that the evolutionary distance value for *Mesanophrys carcini* and *Anophyroides haemophila* is only 0.0611, suggesting that these two species are closely related, while the most closely related species to *Paranophrys magna* is

Table 1. 16s-like SSrRNA structural similarity (upper half) and evolutionary distance (lower half) for scuticociliates and ciliates from other classes for which relevant sequence data are available. Data analysed by the Elwood *et al.* (1985) and Jukes and Cantor (1969) formulas for conversion of structural similarity.

	P. mag	*P. mar*	*U. mar*	***C. ver***	***M. car***	*A. hae*	*C. plo*	*C. gla*	*C. por*	*T. cor*	*G. cha*	*O. cat*	*I .mul*	*P. nep*	*P. tet*	*P. bur*	*P. vor*	*P. nan*	*O. geo*	*P. dub*	*L. str*
P.mag	-	0.9056	0.8916	0.9072	0.8969	0.9032	0.8126	0.8588	0.8490	0.8061	0.7951	0.8218	0.8139	0.8240	0.8293	0.8448	0.8418	0.8347	0.8393	0.8110	0.7775
P. mar	0.0944	-	0.8900	0.9334	0.9131	0.9227	0.8303	0.8734	0.8659	0.7933	0.7821	0.8116	0.8039	0.8300	0.8244	0.8492	0.8499	0.8361	0.8398	0.8211	0.7705
U. mar	0.1084	0.1100	-	0.9159	0.8916	0.9064	0.8252	0.8555	0.8537	0.8062	0.7968	0.8109	0.8164	0.8115	0.8146	0.8256	0.8353	0.8236	0.8388	0.8065	0..7618
C. ver	0.0928	0.0666	0.0841	-	0.9215	0.9283	0.8374	0.8830	0.8820	0.8135	0.8051	0.8267	0.8282	0.8364	0.8409	0.8370	0.8598	0.8424	0.8610	0.8321	0.7837
M .car	0.1031	0.0869	0.1084	0.0785	-	0.9389	0.8322	0.8779	0.8827	0.8220	0.8164	0.8374	0.8366	0.8271	0.8416	0.8548	0.8340	0.8446	0.8464	0.8174	0.7749
A. hae	0.0968	0.0773	0.0936	0.0717	0.0611	-	0.8416	0.8840	0.8765	0.8138	0.8038	0.8308	0.8354	0.8331	0.8368	0.8507	0.8381	0.8517	0.8525	0.8303	0.7861
C. plo	0.1874	0.1697	0.1748	0.1626	0.1678	0.1584	-	0.8567	0.7234	0.7694	0.7662	0.7801	0.7867	0.7835	0.7783	0.7910	0.7906	0.7720	0.8058	0.7682	0.7314
C. gla	0.1412	0.1266	0.1445	0.1170	0.1221	0.1160	0.1433	-	0.8689	0.8042	0.7949	0.8160	0.8114	0.8278	0.8269	0.8425	0.8365	0.8194	0.8439	0.8214	0.7803
C. por	0.1510	0.1341	0.1463	0.1180	0.1173	0.1235	0.1766	0.1311	-	0.8101	0.8048	0.8233	0.8210	0.8324	0.8392	0.8416	0.8530	0.8398	0.8548	0.8356	0.7696
T. cor	0.1939	0.2067	0.1938	0.1865	0.1780	0.1862	0.2306	0.1958	0.1899	-	0.9630	0.9385	0.9337	0.7974	0.8016	0.8079	0.7928	0.7780	0.7908	0.7900	0.7249
G. cha	0.2049	0.2179	0.2032	0.1949	0.1846	0.1962	0.2338	0.2051	0.1952	0.0370	-	0.9326	0.9285	0.7753	0.7804	0.7894	0.7915	0.7790	0.7837	0.7745	0.7137
O. cat	0.1782	0.1884	0.1891	0.1733	0.1626	0.1692	0.2199	0.1840	0.1767	0.0615	0.0674	-	0.9815	0.8013	0.8110	0.8123	0.8104	0.7997	0.8004	0.8053	0.7370
I. mul	0.1861	0.1861	0.1836	0.1718	0.1634	0.1646	0.2133	0.1886	0.1790	0.0663	0.0715	0.0185	-	0.8012	0.8099	0.8089	0.8080	0.7996	0.8004	0.8012	0.7338
P. nep	0.1760	0.1700	0.1885	0.1636	0.1729	0.1669	0.2165	0.1722	0.1676	0.2026	0.2247	0.1987	0.1988	-	0.9425	0.9111	0.8362	0.8265	0.8232	0.8094	0.7703
P. tet	0.1707	0.1756	0.1854	0.1591	0.1584	0.1632	0.2217	0.1731	0.1608	0.1984	0.2196	0.1890	0.1901	0.0575	-	0.9132	0.8315	0.8225	0.8293	0.8132	0.7642
P. bur	0.1552	0.1508	0.1744	0.1430	0.1452	0.1493	0.2090	0.1575	0.1584	0.1921	0.2106	0.1877	0.1911	0.0889	0.0868	-	0.8378	0.8294	0.8340	0.8244	0.7795
P. vor	0.1582	0.1501	0.1647	0.1402	0.1460	0.1419	0.2094	0.1635	0.1470	0.2072	0.2085	0.1896	0.1920	0.1638	0.1685	0.1622	-	0.8126	0.8823	0.8608	0.7953
P. nan	0.1653	0.1639	0.1764	0.1576	0.1554	0.1483	0.2280	0.1806	0.1602	0.2220	0.2210	0.2003	0.2004	0.1735	0.1775	0.1706	0.1074	-	0.8736	0.8473	0.7933
O. geo	0.1607	0.1602	0.1612	0.1390	0.1546	0.1475	0.1942	0.1561	0.1452	0.2092	0.2163	0.1996	0.1996	0.1768	0.1707	0.1660	0.1177	0.1264	-	0.8725	0.8034
P. dub	0.1890	0.1789	0.1935	0.1679	0.1726	0.1697	0.2318	0.1786	0.1644	0.2100	0.2255	0.1947	0.1988	0.1906	0.1868	0.1756	0.1392	0.1527	0.1275	-	0.8048
L. str	0.2225	0.2295	0.2382	0.2163	0.2251	0.2139	0.2686	0.2197	0.2304	0.2751	0.2863	0.2630	0.2662	0.2297	0.2358	0.2205	0.2047	0.2067	0.1966	0.1952	-

Abbreviation: *P. mag - Paranophrys magna*; *P. mar - Pseudocohnilembus marinus*; *U. mar - Uronema marinum*; *C. ver - Cohnilembus verminus*; *M. car - Mesanophrys carcini*; *A. hae - Anophyroides haemophila*; *C. plo - Cyclidium plouneouri*; *C. gla - Cyclidium glaucoma*; *C. por - Cyclidium porcatum*, *T. cor - Tetrahymena corlissi*; *G. cha - Glaucoma chattoni*; *O. cat - Ophryoglena catenula*; *I. mul - Ichthyophthirius multifiliis*; *P. nep - Paramecium nephridiatum*; *P. tet - Paramecium tetraurelia*; *P. bur - Paramecium bursaria*; *P. vor - Platyophrya vorax*; *P. nan - Pseudoplatyophrya nana*; *O. geo - Obertrumia georgians*; *P. dub - Pseudomicrothorax dubius*; *L. str - Loxodes striatus*

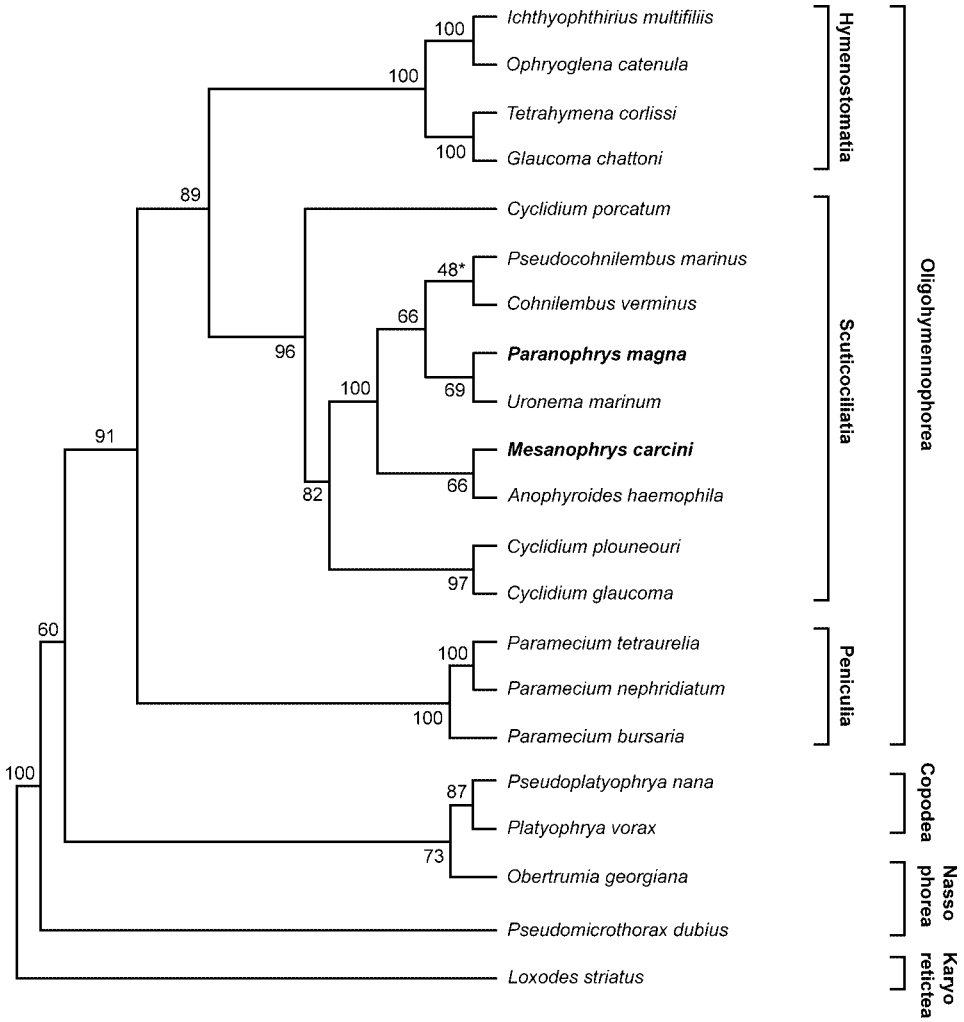

Fig. 3. A consensus bootstrap tree based on maximum parsimony analysis constructed from small subunit ribosomal RNA sequences. The systematic positions of *Paranophrys magna* and *Mesanophrys carcini*, and phylogenetic relationships among those scuticociliates for which small subunit ribosomal RNA sequences are available, are shown. The numbers at the forks give the percentage of times the group occurred out of the 1,000 trees. No significance is placed on branch lengths connecting the species. The new sequences are represented in boldface.

Uronema marinum (evolutionary distance value 0.1084). *Pseudocohnilembus marinus* and *Cohnilembus verminus*, which are members of the same clade, are both more closely related to *P. magna* (evolutionary distance values 0.0944 and 0.0928 respectively) than is *U. marinum* (0.1084).

Distance matrix analysis (Fig. 2)

Both least-squares (LS) and neighbor-joining (NJ) analyses give strong bootstrap support for the monophyly of the class Oligohymenophorea (100% LS, 100% NJ, Fig. 2). The monophyly of the class Colpodea is also well supported (100% LS, 100%NJ), whereas according to these analyses the class Nassophorea is probably paraphyletic (Figs 2, 3). Within the class Oligohymenophorea, the subclass Peniculia branches basally with a maximum bootstrap support (100% LS, 100% NJ); the clades for the Hymenostomatia and the Scuticociliatia branch later and appear to be sister groups with moderate support (75% LS, 74% NJ).

As shown in Fig. 2, the subclass Scuticociliatia is monophyletic (89% LS, 88% NJ). The species within Philasterida also form a monophyly with maximum bootstrap support (100% LS, 100% NJ) and are separate from the three *Cyclidium* species which belong to the order Pleuronematida. Within the Philasterida,

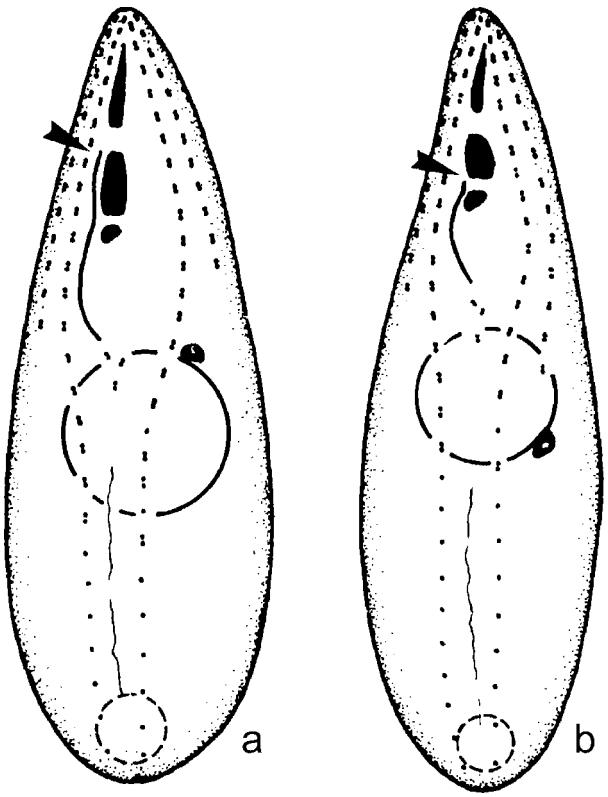

Fig. 4. Schemetic comparison of buccal apparatus of *Paranophrys magna* (a) and *Mesanophrys carcini* (b). Arrowhead marks the anterior end of paroral membrane (after Song and Wilbert 2000).

Mesanophrys carcini and *Anophyroides haemophila* branch as a monophyletic clade (95% LS, 97% NJ), while *Paranophrys magna* forms a clade with *Uronema marinum* (53% LS, 37% NJ) which is a sister group to a lineage that includes *Pseudocohnilembus marinus* and *Cohnilembus verminus* (54% LS, 76% NJ).

Maximum parsimony analysis (Fig. 3)

The maximum parsimony tree (MP), as shown in Fig. 3, is generally similar to that inferred from the distance matrix analysis. The only significant difference is the position of *Obertrumia georgiana*, which groups with *Pseudoplatyophrya nana* and *Platyophrya vorax* in the MP tree (Fig. 3).

DISCUSSION

Comparisons of the complete SSrRNA gene sequences supports the monophyly of the Scuticociliatia which is consistent with other recent molecular studies (Bernhard *et al.* 1995, Strüder-Kypke *et al.* 2000). Our results also confirm that the Scuticociliatia and Hymenostomatia are sister taxa, as has been previously suggested following analysis both of morphological and molecular data (Lynn 1979, 1981; Bardele 1981; Beran 1990; Strüder-Kypke *et al.* 2000).

According to the SSrRNA gene sequence data obtained in the present study, the monophyly of the order Philasterida is confirmed with maximum bootstrap support (100% LS, 100% NJ, 100% MP).

Corliss (1979) placed *Paranophrys*, *Mesanophrys* and *Uronema* in the family Philasteridae on account of their similar morphologies. The molecular data reported here, however, suggest that *Paranophrys* and *Uronema* form a clade, the sister group of which includes *Pseudocohnilembus* and *Cohnilembus* of the family Cohnilembidae (according to the Corlissian system). Nevertheless, it should be noted that these four taxa exhibit at least three distinct patterns of morphogenesis and arrangements of the buccal apparatus; one for *Paranophrys* and *Uronema*, a second one for *Pseudocohnilembus* and a third for *Cohnilembus* (Song and Wilbert 2000, Ma *et al.* 2003). *Mesanophrys*, by contrast, groups with *Anophyroides* rather than with *Paranophrys* and *Uronema*.

Song and Wilbert (2000) suggested that the genus *Anophyroides* should be synonymized with *Paranophrys*. However, in the present study, *Anophyroides haemophila* converged with a morphospecies of the genus *Mesanophrys* (*M. carcini*), which differs from *Paranophrys* only in the terminal position of the anterior end of the paroral membrane (i. e. adjacent to to the posterior end of M_2 *vs*. ajacent to the anterior end of M_2) (Fig. 4). The SSrRNA of *M. carcini* differs by only 107 nucleotides from that of *A. haemophila* with a similarity 93.89%, while the SSrRNA of *Paranophrys magna* differs by 170 nucleotides from that of *Anophyroides haemophila* (similarity 90.32%), indicating that *Anophyroides* might be phylogenetically closer to *Mesanophrys* than to *Paranophrys*. Previous studies of scuticociliates and other ciliate groups have similarly reported that phylogenetic trees based on molecular data may be incongruent with those based on morphological data (Ragan *et al.* 1996, Chen *et al.* 2000, Strüder-Kypke *et al*. 2000). As noted recently, there at least two reasons for this phenomenon: (a) the morphological and morphogenetic characters do not necessarily reflect evolutionary relationships between taxa since

some morphological similarities might be due to phenetic adaptation; (b) the information from the SSrRNA gene sequence does not always accurately reflect the actual phylogeny (Bernhard *et al.* 1995, Hirt *et al.* 1995, Wright and Lynn 1995, Strüder-Kypke *et al*. 2000).

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ACTA
PROTOZOOLOGICA

Gymnophrys cometa and *Lecythium* sp. are Core Cercozoa: Evolutionary Implications

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Summary. Recent phylogenetic analyses based on different molecular markers have revealed the existence of the Cercozoa, a group of protists including such morphologically diverse taxa as the cercomonad flagellates, the euglyphid testate filose amoebae, the chloroplast-bearing chlorarachniophytes, and the plasmodiophorid plant pathogens. Molecular data also indicate a close relationship between Cercozoa and Foraminifera (Granuloreticulosea). Little is known, however, about the origin of both groups and their phylogenetic relationships. Here we present the complete small-subunit ribosomal RNA (SSU rRNA) sequence of *Gymnophrys cometa*, formerly included in the athalamid Granuloreticulosea, as well as that of the test-bearing filose amoeba *Lecythium* sp. Our study shows that the two organisms clearly belong to the Cercozoa, and indicates that *Gymnophrys* is not closely related to Foraminifera, supporting the view that Granuloreticulosea *sensu lato* do not form a natural assemblage. Phylogenetic analyses including most available SSU rRNA sequences from Cercozoa suggest that a rigid, external cell envelope appeared several times independently during the evolution of the group. Furthermore, our results bring additional evidence for the wide morphological variety among Cercozoa, which now also include protists bearing granular pseudopodia and exhibiting mitochondria with flattened cristae.

Key words: Cercozoa, Granuloreticulosea, *Gymnophrys cometa*, *Lecythium* sp., molecular phylogeny, SSU rRNA.

Abbreviations used: ML - maximum likelihood, MP - maximum parsimony, NJ - neighbor joining, SSU rRNA - small-subunit ribosomal RNA.

INTRODUCTION

Gymnophrys cometa (Cienkowski, 1876) is a fresh-water protist, which due to its thin, grossly granular reticulopodia and absence of a test was placed by Cash (1905) in the family Reticulosa and later by De Saedeleer (1934) in the suborder Athalamia, order Granuloreticulosa (order Athalamida, class Granuloreticulosea *sensu* Bovee 1985b). The cells of *G. cometa* are solitary and move with the help of short lobose pseudopodia, which may be found in addition to the reticulopodia. Its complex life cycle includes an amoeboid stage, a cyst and a motile zoospore bearing two heterodynamic flagella lacking mastigonemes (Mikrjukov and Mylnikov 1996, 1997). Its mitochondria contain flat, plate- or ribbon-like cristae,

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and the reticulopodia contain a longitudinal bundle of 2-6 microtubules. Complex extrusive organelles (microtoxicysts) are present, which give their granular aspect to the reticulopodia. Two reduced flagella arise from a pair of conventional, almost parallel kinetosomes. Based on these particular features, Mikrjukov and Mylnikov (1997, 1998) proposed a new class Gymnophrea for *Gymnophrys* and the closely related genus *Borkovia*, but their position in the eukaryotic tree remains enigmatic. Cavalier-Smith (1998) tentatively placed *Gymnophrys* in the newly erected phylum Cercozoa (see below), whereas a recent catalogue of protists places *Gymnophrys* among heterotrophic flagellates of uncertain affinities (Patterson *et al*. 2000b), leaving open the question of its relation to Granuloreticulosea and other amoeboid protists.

Lecythium (Hertwig *et* Lesser, 1874) is a freshwater, filose amoeba which in culture can be found in groups of two to four cells. It possesses a flexible test and was placed by De Saedeleer (1934) in the suborder Testaceafilosa, order Filosa (order Gromiida, class Filosea *sensu* Bovee 1985a). Recent studies based on small-subunit ribosomal RNA (SSU rRNA) sequences revealed a close relationship between some members of the Testaceafilosa (i.e., the Euglyphida, *Pseudodifflugia* and *Gromia*) and a heterogeneous assemblage which includes the cercomonad flagellates, the chloroplast-bearing chlorarachniophytes, some marine nanoflagellates like *Cryothecomonas*, and the plasmodiophorid plant pathogens (Bhattacharya *et al*. 1995, Cavalier-Smith and Chao 1997, Atkins *et al*. 2000, Bhattacharya and Oliveira 2000, Kühn *et al*. 2000, Bulman *et al*. 2001, Burki *et al*. 2002, Vickerman *et al*. 2002). A new phylum Cercozoa was created to accommodate this assemblage (Cavalier-Smith 1998). This taxon is also supported by some protein-coding genes, including tubulin, actin and ubiquitin (Keeling *et al*. 1998, Keeling 2001, Archibald *et al*. 2003). Although the monophyly of Cercozoa is robust in most analyses, the internal relationships among cercozoan lineages are yet unclear. Notably, available data suggest the polyphyly of Testaceafilosa (Burki *et al*. 2002, Wylezich *et al*. 2002).

In order to examine the relationships between Cercozoa and other amoeboid protists, we sequenced the complete SSU rRNA gene of *G. cometa* and *Lecythium* sp. Our phylogenetic analyses clearly show that both genera belong to the Cercozoa, confirming the heterogeneous character of this group.

MATERIALS AND METHODS

Cell cultures and DNA extraction, amplification, cloning, and sequencing

The cultures of *G. cometa* and *Lecythium* sp. were taken from the culture collection of IBIW RAS (Russia). They were isolated from samples collected in waste treatment plants of Borok, Yaroslavskaya oblast, Russia. Both cultures were maintained on the artificial Pratt medium (KNO_3 0.1 ‰, K_2HPO_4 x $3H_2O$ 0.01 ‰, $MgSO_4$ x $7H_2O$ 0.01 ‰, $FeCl_3$ x $6H_2O$ 0.001 ‰; pH 6.5-7.5) with the addition of *Aerobacter aerogenes* as the source of food. DNA was extracted using the DNeasy Plant Minikit (Qiagen, Basel, Switzerland). The complete SSU rRNA gene of *G. cometa* and *Lecythium* sp. was amplified using the universal primers sA (5' ACCTGGTTGATCCTGCCAGT 3') and sB (5' TGATCCTTCTGCAGGTTCACCTAC 3'). PCR amplifications were done in a total volume of 50 µl with an amplification profile consisting of 40 cycles with 30 s. at 94 °C, 30 s. at 50 °C, and 2 min. at 72 °C, followed by 5 min. at 72 °C for the final extension. The amplified PCR products were purified using the High Pure PCR Purification Kit (Roche, Rotkreuz, Switzerland), then ligated into pGEM-T Vector System (Promega, Wallisellen, Switzerland), cloned in XL-2 Ultracompetent Cells (Stratagene, Basel, Switzerland), sequenced with the ABI-PRISM Big Dye Terminator Cycle Sequencing Kit, and analyzed with an ABI-377 DNA sequencer (Perkin-Elmer, Rotkreuz, Switzerland), all according to the manufacturer's instructions. The length of the amplified sequences of SSU rRNA of *G. cometa* and *Lecythium* sp. were 1814 and 1767 nucleotides, respectively.

Phylogenetic analyses

The complete SSU rRNA gene sequences from *G. cometa* and *Lecythium* sp. were manually aligned with sequences from diverse eukaryotes using the Genetic Data Environment software (Larsen *et al.* 1993), following the secondary structure model proposed by Neefs *et al*. (1993) and Wuyts *et al*. (2000). Preliminary analyses revealed the approximate phylogenetic position of both species (data not shown). An alignment of 43 sequences was constructed, including the two sequences obtained in this study, as well as 28 sequences from Cercozoa and 13 sequences from other eukaryotes. 1364 unambiguously aligned positions were used in the phylogenetic analyses, of which 748 were constant and 474 were parsimony informative. A second "Granuloreticulosea" dataset was designed, including sequences from *Reticulomyxa filosa* and 4 other Foraminifera, a sequence from the so-called granuloreticulosean *Diplophrys*, 8 sequences from Cercozoa and sixteen sequences from other eukaryotes. Because of the high divergence of foraminiferan SSU rRNA sequences (see, e.g., Pawlowski *et al*. 1996), only 1116 positions could be kept for phylogenetic analyses, of which 593 were constant and 404 were parsimony informative.

Phylogenetic trees were inferred using the neighbour-joining (NJ) method (Saitou and Nei 1987), the maximum parsimony (MP) method, and the maximum likelihood (ML) method (Felsenstein 1981). The reliability of internal branches was assessed using the bootstrap method (Felsenstein 1985) with 1000 replicates for NJ analyses,

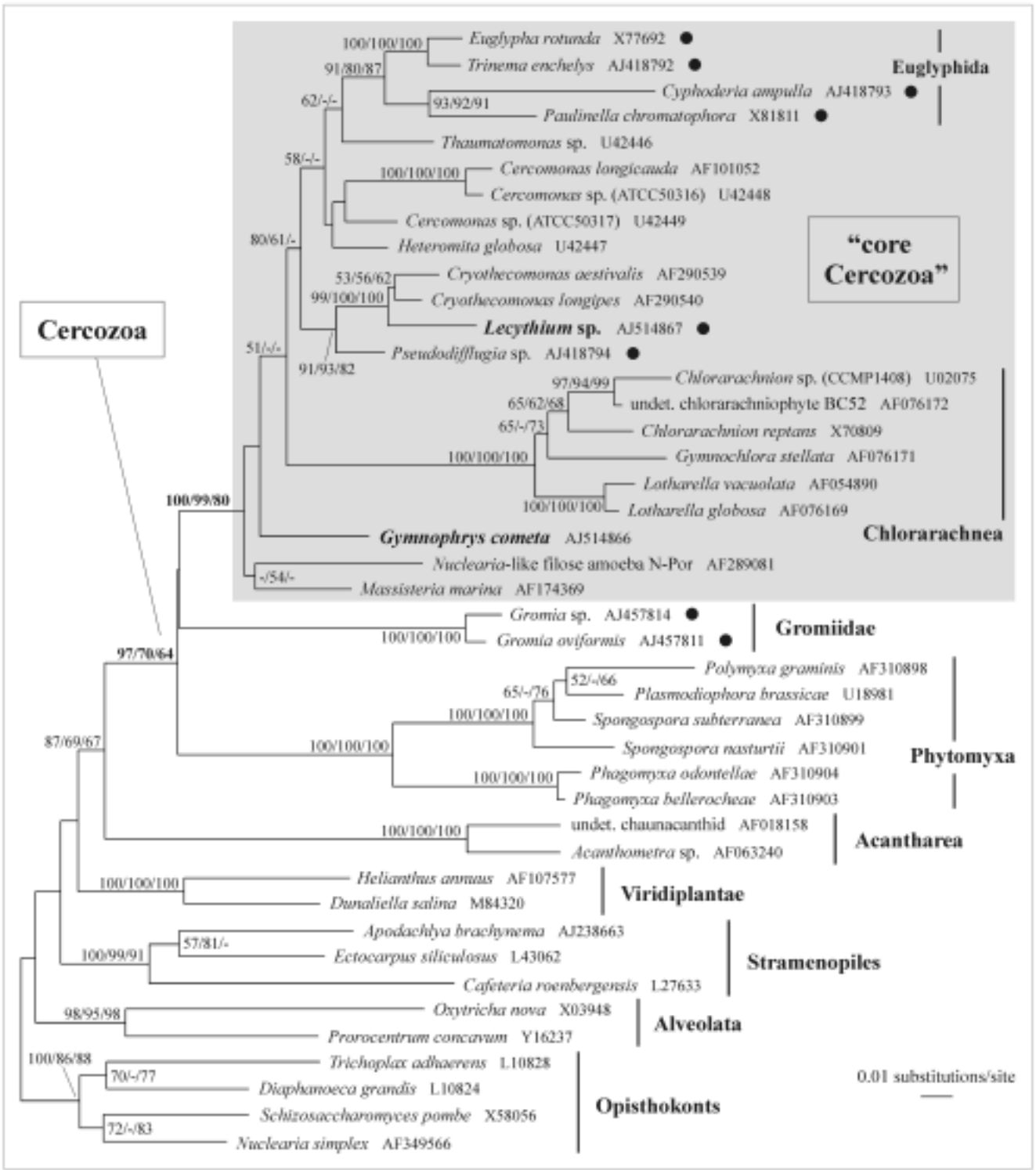

Fig. 1. Phylogenetic position of *Gymnophrys cometa* and *Lecythium* sp. among eukaryotes, inferred using the maximum likelihood method with the GTR + G + I model. Both organisms clearly belong to Cercozoa, and appear in a strongly supported clade called here "core Cercozoa" (grey box). Filose amoebae with a proteinaceous, agglutinated, or siliceous test are marked with a black circle. Numbers at nodes represent percentages of bootstrap support greater than 50% following 100 (ML), 1000 (NJ), or 500 (MP) data resamplings. All branches are drawn to scale. The tree was rooted using four sequences of opisthokonts, following a recent hypothesis on the position of the eukaryotic root (Stechmann and Cavalier-Smith 2002).

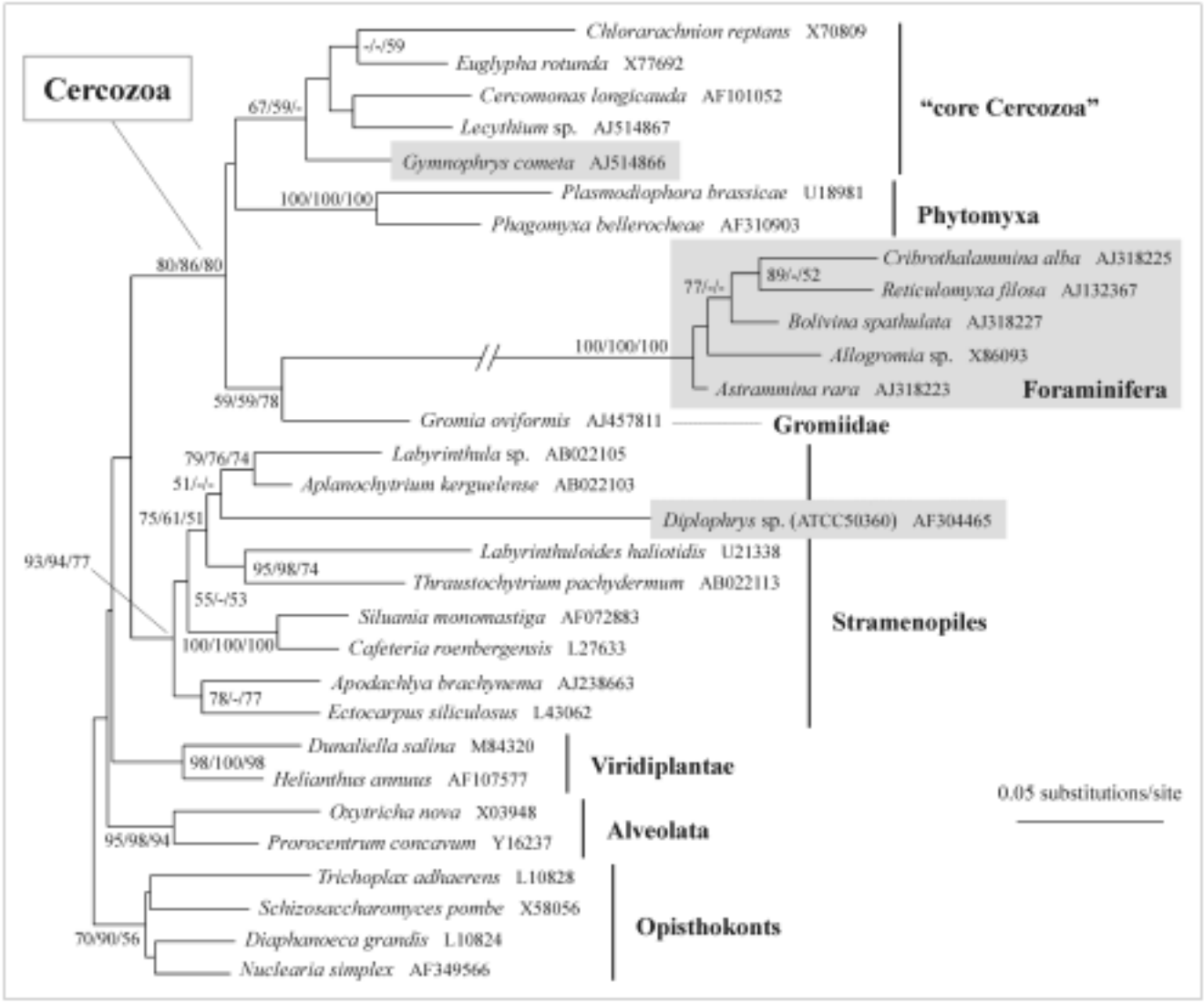

Fig. 2. Phylogenetic relationships among some eukaryotes inferred using the maximum likelihood method with the GTR + G + I model, emphasizing the polyphyly of the Granuloreticulosea *sensu lato* (grey boxes). The "athalamid" *Gymnophrys cometa* appears as a basal lineage among "core Cercozoa", while the "athalamid" *Reticulomyxa filosa* and the Foraminifera form an independent lineage branching with the marine, testate filosean *Gromia oviformis*, and the "monothalamid" *Diplophrys* sp. is closely related to the labyrinthulids and thraustochytrids (stramenopiles). Numbers at nodes represent percentages of bootstrap support greater than 50% following 100 (ML), 1000 (NJ), or 500 (MP) data resamplings. All branches are drawn to scale, except the stem branch leading to Foraminifera, which was reduced to a fourth of its actual size. The tree was rooted as in Fig. 1.

100 replicates for ML analyses, and 500 replicates for MP analyses. The PHYLO_WIN program (Galtier *et al*. 1996) was used for distance computations and NJ trees building and bootstrapping, using the HKY85 model of substitution (Hasegawa *et al*. 1985). MP and ML analyses were performed using PAUP* (Swofford 1998). The most parsimonious trees for each MP bootstrap replicate were determined using a heuristic search procedure with 20 random addition sequence replicates and tree bisection-reconnection branch-swapping. All characters were equally weighted and the transversion cost was set to twice the transition cost. ML analyses were performed using the GTR model of evolution (Lanave *et al*. 1984, Rodriguez *et al*. 1990), taking into account a proportion of invariables sites, and a gamma distribution of the rates of substitution for the variable positions, with 8 rate categories (GTR+G+I). All parameters were estimated from the dataset using Modeltest (Posada and Crandall 1998). Starting trees were obtained via 20 random addition sequence

replicates, and then swapped using the tree bisection-reconnection algorithm. In order to reduce computational time, starting trees for the ML bootstrap analysis were obtained via NJ.

RESULTS

Morphology

The morphological features of *Lecythium* sp. are characteristic for this genus. The cells are ovoid, 8 to 10 µm in length and 5 to 8 µm in width. The cell body is rigid and a flexible test is tightly adjacent to the cell surface. Flattened or narrow, needle-shaped, branched pseudopodia arise from a portion of cytoplasm extruding from the lower part of the cell, which are motile and drive the cell along the substrate. The nucleus is in the center of the cell, the contractile vacuole is lateral. Floating specimens, cysts, plasmodia, or zoospores have not been observed. *Lecythium* sp. multiplies by binary fission, and sometimes forms aggregations of three cells. The isolate we studied closely ressembles the type species, *Lecythium hyalinum* (Hertwig *et* Lesser, 1874), as illustrated by Cash and Wailes (1915; plates 49 and 51). However, the determination could not be ascertained, so we named it *Lecythium* sp., while further examination of the isolate is in progress (Mylnikov, in preparation).

The cultured strain of *G. cometa* used in this study is the same that was examined in two previous ultrastructural works (Mikrjukov and Mylnikov 1997, 1998), which provided the detailed description of the species.

Phylogenetic analyses

Analysis of our data shows that both *G. cometa* and *Lecythium* sp. clearly belong to Cercozoa, and the clade is supported by high bootstrap values (Fig. 1). Inside Cercozoa, *Gromia* and the Phytomyxea form two early diverging lineages with all methods of tree reconstruction. *G. cometa* and *Lecythium* sp. branch within a strongly supported clustering of the rest of the cercozoan taxa, called here "core Cercozoa", and which consists of the cercomonads, the chlorarachniophytes, some nanoflagellates, and some filose testate and naked amoebae. The branching order within "core Cercozoa" is not well resolved. Only three strongly supported monophyletic groups can be distinguished with all methods of tree reconstruction: the Chlorarachnea, the Euglyphida, and a group including *Lecythium* sp., *Pseudodifflugia* sp., and the nanoflagellate *Cryothecomonas*. *G. cometa* occupies a relatively basal, independent position, branching immediately after the divergence of the cercomonad *Massisteria marina* and the *Nuclearia*-like filose amoeba "N-Por", as a sister group to all other "core Cercozoa" species. NJ and MP trees are broadly congruent with the ML tree shown in Fig. 1, the main differences lying in the branching order between the most basal members of the "core Cercozoa": in the NJ tree, the Chlorarachnea are the first diverging lineage, followed by a badly supported group consisting of *G. cometa*, *M. marina* and "N-Por", whereas in the MP tree, "N-Por" is the first diverging species, followed by *M. marina*, then *G. cometa* (data not shown).

In order to test the relationships between members of the Granuloreticulosea for which SSU rRNA sequences are available, a second dataset was analysed, including *G. cometa*, *Diplophrys* sp. and five species of Foraminifera. Analysis of this dataset shows that the Granuloreticulosea *sensu lato* do not form a natural assemblage (Fig. 2). *G. cometa* branches among "core Cercozoa" with all methods of tree reconstruction, as indicated by the analysis of the larger dataset (see Fig. 1), although the bootstrap support for this clade is only moderate with a reduced number of sites. The Foraminifera branch as a sister group to the marine, testate, filose amoeba *Gromia*, whereas *Diplophrys* sp. branches among stramenopiles as a sister-group to *Labyrinthula* sp. and *Aplanochytrium kerguelense* (Fig. 2), a result confirmed by NJ and MP analyses (data not shown).

DISCUSSION

When the phylum Cercozoa was erected (Cavalier-Smith 1998), *Gymnophrys* was tentatively placed in it, and this position was later supported by a distance tree including unpublished sequences (Cavalier-Smith 2000). As a member of the Testaceafilosa, *Lecythium* could also be argued to be a member of this group. Our results allow us to refine the position of both organisms. By placing *Gymnophrys* and *Lecythium* within Cercozoa, we confirm the wide range of morphological and ultrastructural characters of this phylum (Cavalier-Smith 2000). Although the monophyly of Cercozoa is strongly supported by molecular data, no satisfying morphological definition exists for the group yet. Our study shows that such characters as the presence of a test, the form of

mitochondrial cristae or the structure of pseudopodia are poor markers for the cercozoan phylogeny.

First, our phylogenetic analyses including most available sequences from Cercozoa support the idea that a rigid, external cell envelope appeared or was lost several times independently during the evolution of the phylum. The Cercozoa include all testate, filose amoebae for which molecular data exist yet, i.e. *Gromia*, *Lecythium*, *Pseudodifflugia*, and the Euglyphida. These organisms were conveniently grouped together in the Testaceafilosa by De Saedeleer (1934), a classification scheme followed by some other authors (see, e.g., Bovee 1985a). Previous molecular data showed that the monophyletic Euglyphida (with secreted, siliceous scales) group within Cercozoa (Wylezich *et al*. 2002), but suggested the polyphyly of the Testaceafilosa as a whole, with *Gromia* and *Pseudodifflugia* appearing as independent lineages within the phylum (Burki *et al*. 2002, Wylezich *et al*. 2002). Our results are congruent with this view, and confirm that the testate, filose amoebae lacking siliceous scales (the Gromiina *sensu* Bovee 1985a) do not form a natural group. This is in agreement with the great ultrastructural differences between the tests of these organisms (see, e.g., Meisterfeld 2000), suggesting that they are not homologous features. SSU rRNA data also point out at *Cryothecomonas* to be closely related to *Lecythium* (Fig. 1). Both genera have an external covering and produce pseudopodia. In *Cryothecomonas*, however, the pseudopodia are small and produced only for the capture of prey (Thomsen *et al*. 1991), whereas the pseudopodia in *Lecythium* are bigger and present constantly, and are also used for locomotion. Besides, the covering in *Cryothecomonas* resembles an envelope outside the cell, and cannot be considered as a true test (Thomsen *et al*. 1991). Further ultrastructural studies of these two genera will be necessary to confirm their possible relationship.

By including *G. cometa* among "core Cercozoa", we also show that the shape of mitochondrial cristae cannot be used as a criterion to include or exclude a species into/from the Cercozoa. The mitochondrial cristae of *G. cometa* are flattened, whereas all other known representatives of the "core Cercozoa" have tubular cristae (Patterson 1999). At the level of the phylum Cercozoa, the only possible other exceptions are the Phytomyxa, because the plasmodiophorid plant pathogens display mitochondrial cristae of ambiguous shape, appearing either flat or sacculate (Patterson 1999). The shape of cristae in mitochondria is traditionally considered as one of the most important ultrastructural features for general protist phylogeny (Cavalier-Smith 1997; Karpov 2000; Taylor 1976, 1999), and there are only few examples of monophyletic groups, which include both tubulocristate and lamelocristate taxa (Patterson 1999). Our results indicate that the "core Cercozoa" are one of such groups.

Finally, our results show that the Cercozoa not only include protists with filose pseudopodia, but also species with granular reticulopodia, such as *Gymnophrys* (Fig. 1). Bovee (1985a) conveniently grouped all organisms presenting fine, more or less granular pseudopodia that can form more or less complex anastomosing networks in the class Granuloreticulosea, which is divided in three groups, the naked Athalamida and the testate Monothalamida and Foraminifera. Although the monophyly of Foraminifera is strongly supported by both morphological and molecular data (see, e.g., Bock *et al*. 1985, Pawlowski 2000), it is generally accepted that Athalamida and Monothalamida are heterogeneous and need taxonomic revision (see, e.g., Lee *et al*. 2000). The only previously sequenced athalamid, *Reticulomyxa filosa*, was shown to be a naked, freshwater foraminiferan (Pawlowski *et al*. 1999), confirming the profound similarities in structure and motility between the pseudopodia of *R. filosa* and foraminifers. However, our data show that this is not the case for all athalamids. Foraminifera (including *R. filosa*) are related to Cercozoa on the basis of actin (Keeling 2001), ubiquitin (Archibald *et al*. 2003) and revised SSU rRNA analysis (Berney and Pawlowski, in press), but the position of *G. cometa* within "core Cercozoa" in SSU rRNA trees is clearly distant from the position of Foraminifera, which branch with *Gromia* among the early diverging cercozoan lineages (Berney and Pawlowski, in press; see Fig. 2). This is in agreement with morphological studies that clearly distinguish the branching, rarely anastomosing filopodia of *G. cometa*, whose granular aspect is due to the presence of numerous extrusomes (Mikrjukov and Mylnikov 1998), from the "true" granuloreticulopodia of Foraminifera, which exhibit a typical bidirectional streaming of particles, are not reinforced with geometrically arrayed microtubules, and form complex anastomosing networks (Lee *et al*. 2000). The polyphyletic nature of the Granuloreticulosea *sensu* Bovee (1985a) is also confirmed by available data on another granuloreticulosean, the monothalamid *Diplophrys* sp., which is related to labyrinthulids and thraustochytrids (stramenopiles) in SSU rRNA analyses (Fig. 2), in agreement with ultrastructural studies (Patterson 1989, Patterson *et al*. 2000a). Our results support the need for

a redefinition of the Granuloreticulosea, which might ultimately be reduced to the Foraminifera alone.

According to SSU rRNA sequences, *G. cometa* has a relatively basal position within the "core Cercozoa", and appears not to be closely related to any other known member of the phylum. This is in agreement with the ultrastructural peculiarities of this genus, which prompted Mikrjukov and Mylnikov (1997, 1998) to place *Gymnophrys* in a new class Gymnophrea. Interestingly, two of the most basal "core Cercozoa", *G. cometa* and *M. marina* share some important morphological features (Patterson and Fenchel 1990, Mikrjukov and Mylnikov 1998). Both organisms are sessile and possess thin and branching filopodia, with internal bundles of microtubules, which can form chain aggregations in culture. Characteristic concentric extrusomes of similar structure, termed kinetocysts (Mylnikov 1988) are located in the filopodia and next to the body surface (Patterson and Fenchel 1990). *G. cometa* and *M. marina* have two smooth heterodynamic flagella, and an amoeboid outline of the rear part of the cell. Given the absence of bootstrap support for the branching order among basal "core Cercozoa", it is plausible that *G. cometa*, *M. marina* and possibly "N-Por" might form a monophyletic lineage. Indeed, the likelihood of the best tree where this lineage was constrained was not significantly inferior to the likelihood of the tree shown in Fig. 1 (data not shown). Alternatively, if the topology shown in Fig. 1 is correct, then the similarities between *G. cometa* and *M. marina* suggest that an amoeboid, but flagellated state might be ancestral for the "core Cercozoa". Subsequent reduction of the flagella or loss of the capacity to produce filopodia in some lineages might account for the chaotic distribution of these features along the cercozoan tree. This may explain the difficulty of finding a satisfying morphological definition for the Cercozoa, from which highly specialized lineages such as the Foraminifera might be derived. Additional protein data will be needed to test further the relationships among Cercozoa and closely related amoeboid protists.

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ACTA
PROTOZOOLOGICA

Antigenic Relationships Between *Aggregata octopiana* and *A. eberthi* Two Parasites of Cephalopods

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Summary. Proteins from soluble extracts of sporocysts of the two protozoan parasites of cephalopods *Aggregata octopiana* Schneider, 1875 and *A. eberthi* Labbé, 1895 (Apicomplexa: Aggregatidae) were compared by electrophoresis and immunoblotting using specific antisera and the immunologic cross-reactivity between both species analyzed. Both species showed a considerable cross-reactivity in ELISA and immunoblotting. However, each species showed a characteristic electrophoretic pattern and species-specific antigens which makes species differentiation possible in SDS-PAGE and immunoblotting.

Key words: *Aggregata octopiana*, *Aggregata eberthi*, antigens, Apicomplexa, cross-reactivity.

INTRODUCTION

The genus *Aggregata* has a two-hosts life cycle, with asexual stages in the intestines of crustaceans and sexual stages in the digestive tracts of cephalopods. In cephalopods, the merozoites migrate through the epithelium of the digestive tract causing degeneration and death of the parasitized cells and leading to detachment of necrotic fragments of the intestine (Hochberg 1990).

Since the first references to the genus *Aggregata* (Schneider 1875, Labbé 1899, Dobell 1925) were published until the present, one of the main controversies of the genus *Aggregata* has been its taxonomy. Problems related to synonymy have arisen due to the fact that the *Aggregata* species have traditionally been differentiated on the basis of the final host and morphological characters (Poynton *et al.* 1992, Estévez *et al*. 1996). For these reasons, several authors have suggested that most species of *Aggregata* in Europe require a new description to determine taxonomic validity (Sprague and Couch 1971; Levine 1985, 1988).

Because of the problems with *Aggregata* spp. systematics, we decided to undertake the biochemical and immunological characterization of two of the most common species of *Aggregata* in natural cephalopod populations in the Ria of Vigo: *A. octopiana* and *A. eberthi*, infecting *Octopus vulgaris* and *Sepia officinalis* with

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prevalences of 100% and 87% respectively (Pascual *et al*. 1996). We analyzed the protein patterns of both species and the possibility of a cross-reactivity between them.

MATERIALS AND METHODS

Parasites

Oocysts of *Aggregata octopiana* Schneider, 1875 and *A. eberthi* Labbé, 1895 were isolated from the digestive tracts of molluscan cephalopods *Octopus vulgaris* Cuvier, 1798 and *Sepia officinalis* L., 1758, respectively, naturally infected in the Ría de Vigo, NW of Spain.

Purification of sporocysts and sporocyst extracts

Sporocysts of both species *A. octopiana* and *A. eberthi* were purified from oocysts, as described previously Estévez *et al*. (1992). Briefly, the sporocysts were obtained by maceration of oocysts in phosphate-buffered saline (PBS). The resulting suspension was then filtered through increasingly fine meshes to remove tissue fragments. The filtrate was centrifuged at 2,000 *g* for 15 min; this filtration-centrifugation process was repeated various times until a pure sample of sporocysts was obtained. Sporocysts were washed twice in PBS, then resuspended in phosphate buffer containing 3% sodium dodecyl sulphate (SDS), and 1 mM ethylenediaminetetraacetic acid (EDTA; Sigma Chemical Co., St. Louis, USA), 10 mM iodacetamide (Merck, Darmstadt, Germany) and 50 µg/ml phenylmethylsulphonyl fluoride (PMSF; Sigma) as protease inhibitors. Sporocysts were lysed by sonication on ice (60 W in 1 min pulses for 45 min). The lysed sporocysts were centrifuged at 10,000 *g* for 30 min at 4°C and the supernatant, being used in all analyses, was exhaustively dialysed against PBS and stored at - 30°C until use (Leiro *et al*. 1993).

Immunization and sera

Ten BALB/c mice were immunized by subcutaneous injection of 0.2 ml mixture of Freund's complete adjuvant (Sigma) and PBS containing about 5 x 10^6 sporocysts of *A. octopiana* and re-immunized 30 days later by intraperitoneal (i.p.) injection of 0.2 ml of PBS without adjuvant containing the same number of sporocysts. On day 45, a third dose (without adjuvant) was administered by i.p. injection. Mice were bled 60 days after primary immunization through the retroorbital route. Serum (anti-*A. octopiana*) were separated by centrifugation at 2,000 *g* for 10 min, mixed 1:1 with glycerol, and stored at -30°C until use.

Another 10 mice were immunized with sporocysts of *A. eberthi* following the same protocol for obtaining anti-*A. eberthi* serum.

Enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA was carried out as described previously by Estévez *et al*. (1994). The corresponding sporocyst extracts (1µg/well) were bound to PVC microtitre plates (Costar, Massachusetts) in 50 µl/well of carbonate/bicarbonate buffer, pH 9.6, overnight at 4°C. Plates were then washed 3 times with Tris-buffered saline (TBS: 50 mM Tris, 0.15 M NaCl; pH 7.4) and active sites remaining on the plates were blocked, for 2h at 37°C, with a solution of 5% non-fat dry milk in TBS containing 0.2% Tween 20 (TBS-T_1). Antigen-coated plates were incubated for 2h at 37°C with 50 µl of a dilution 1:20,000 of mouse anti-*A. octopiana* or anti-*A. eberthi* serum (both polyclonal sera showed similar titer in ELISA), in TBS-T_1 containing 1% non-fat dry milk, washed five times with TBS containing 0.05% Tween 20 (TBS-T_2) and incubated for 1h at 37°C with 50 µl peroxidase conjugated rabbit anti-mouse immunoglobulin polyclonal antibody (Dakopatts A/S, Glostrup, Denmark) diluted 1:2,000 in TBS-T_1 containing 1% non-fat dry milk and 3% polyethyleneglycol (PEG) 6,000. After 5 washes with TBS, 50 µl of substrate containing 0.04% *o*-phenylendiamine (Sigma) and 0.001% H_2O_2 in phosphate/citrate buffer (pH 5.0) were added to the wells. The reaction was stopped after 20 min by adding 25 µl of 3N H_2SO_4. Optical density at 492 nm was measured with a microtitre plate reader (Titertek Multiskan, Labsystems, Finland).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The components of soluble extracts of sporocysts were separated electophoretically by SDS-PAGE. Electrophoresis was carried out using linear gradient polyacrylamide gels (5-15% under non-reducing and 10-20% under reducing conditions), using a Mini-Protean II cell (BioRad, Richmond, USA), as described previously by Estévez *et al*. (1993). Reduction and denaturalization were carried out in 62 mM Tris-HCl buffer, pH 6.8, containing 2% SDS, 10% glycerol, 0.004% bromophenol blue and 0.02 M dithiothreitol (DTT), for 4 min at 95-100°C. Samples (8 µg/lane) were run for 45 min at 200 V in electrode buffer containing 25 mM Tris, 190 mM glycine and 1% SDS; pH 8.3. Bands were revealed with silver staining (Sambrook *et al*. 1989). Molecular weights were estimated using a calibration curve [log Mw vs log polyacrylamide concentration (%T) (Hames 1981)] constructed with the low molecular weight markers phosphorylase B (97.4 kDa), bovine serum albumine (66.2 kDa), ovoalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa) as standards (BioRad).

Immunoblotting analysis

Following electrophoresis as above, proteins were immunoblotted at 15 V for 45 min onto a 0.45 µm pore size nitrocellulose membrane using a Trans-Blot SD semi-dry transfer cell (BioRad) with electrode buffer recommended by the supplier (48 mM Tris, 39 mM glycine, 0.037% SDS and 20% methanol, pH 9.2). The membrane was washed with TBS and stained with Ponceau S to verify transfer and to locate molecular weight markers, then dried, and cut into strips. Each strip was incubated for 2 h at room temperature (RT) with TBS-T_1 containing 5% non-fat dry milk. Immunorecognition was performed by incubation for 2 h at RT with the corresponding antibodies (anti-*A. octopiana* or anti-*A. eberthi* serum) diluted 1:200 in TBS-T_1 containing 1% non-fat dry milk. Each strip was then washed five times with TBS-T_2 and incubated for 1h at RT with a 1:800 dilution of peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts) in TBS-T_1 containing 4% PEG-6,000. After washing five times with TBS-T_1 and other five with TBS, the recognized bands were stained by adding TBS containing 0.003% H_2O_2, 0.06% diaminobenzidine tetrahydrochloride (Sigma) and 0.03% $NiCl_2$.

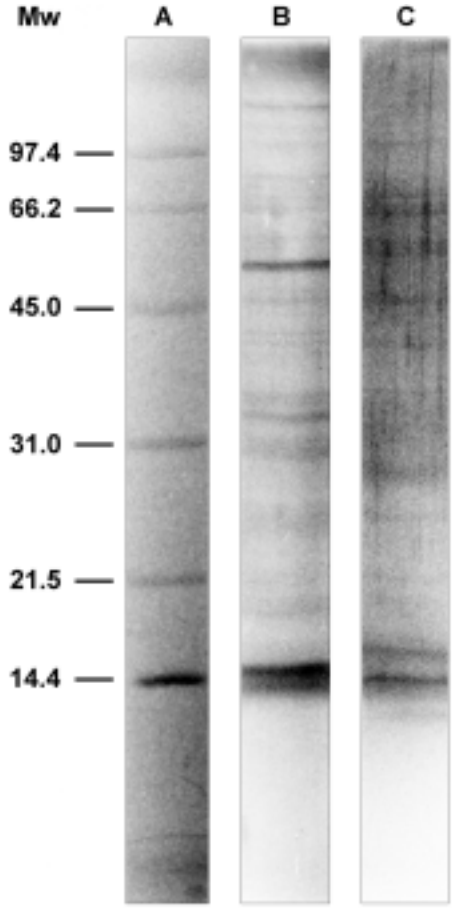

Fig. 1. SDS-PAGE of extracts of sporocysts of the two coccidian species under reducing conditions using a 10-20% linear gradient and silver staining. Lane **A:** Molecular weight markers (kDa). Lane **B:** Sporocysts extract of *Aggregata octopiana*. Lane **C:** Sporocysts extract of *A. eberthi*.

The reaction was stopped after approximately 3 min by exhaustive washing with TBS, and the stained lanes were recorded photographically (Estévez *et al.* 1993).

Protein determination

Protein concentration of all preparations were estimated using the Bio-Rad Protein Assay (BioRad) with bovine serum albumin (Sigma) as standard.

RESULTS

SDS-PAGE characterization of sporocyst extracts

Soluble extracts of sporocysts of *Aggregata* species, *A. octopiana* and *A. eberthi*, were characterized by SDS-PAGE under reducing conditions (Fig. 1). The banding patterns of the two extracts were completely different, noting the presence of four dominant bands of 13, 15, 34 and 57 kDa (Fig. 1, lane B) in the *A. octopiana* extract and the presence of six dominant bands of 13, 16, 45, 48, 60 and 66 kDa in the *A. eberthi* extract (Fig. 1, lane C).

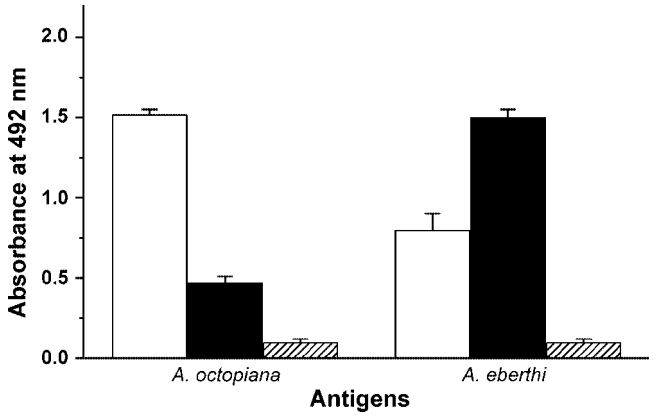

Fig. 2. Reactivity in ELISA of anti-*A. octopiana* mouse sera (white), anti-*A. eberthi* mouse sera (black) and normal mouse sera (ruled), with sporocysts extracts of *Aggregata octopiana* and *A. eberthi*. The values shown are the means ± standard error of assays of sera from ten mice.

Cross-reactivity between *Aggregata octopiana* and *A. eberthi*

In indirect ELISA, the polyclonal antibodies obtained in mice immunized with purified sporocysts of *A. eberthi* and *A. octopiana* showed cross-reactivity with heterologous antigenic extracts (Fig. 2). This cross-reactivity was greater when *A. eberthi* antigens were assayed.

Immunoblotting under reducing conditions (Fig. 3) confirmed the cross-reactivity between the two species observed in ELISA. However, a group of bands migrating between 28 and 34 kDa were specifically recognized by the anti-*A. octopiana* serum on the homologous antigenic extract. Some species-specific antigenic bands at the 21-24 kDa region were also detected on the *A. octopiana* extract in immunoblottings under non-reducing conditions (Fig. 4).

DISCUSSION

The results obtained showed that sporocyst extracts from *A. octopiana* and *A. eberthi* gave totally different profiles with SDS-PAGE, confirming the potential value of this technique for distinguishing between these two

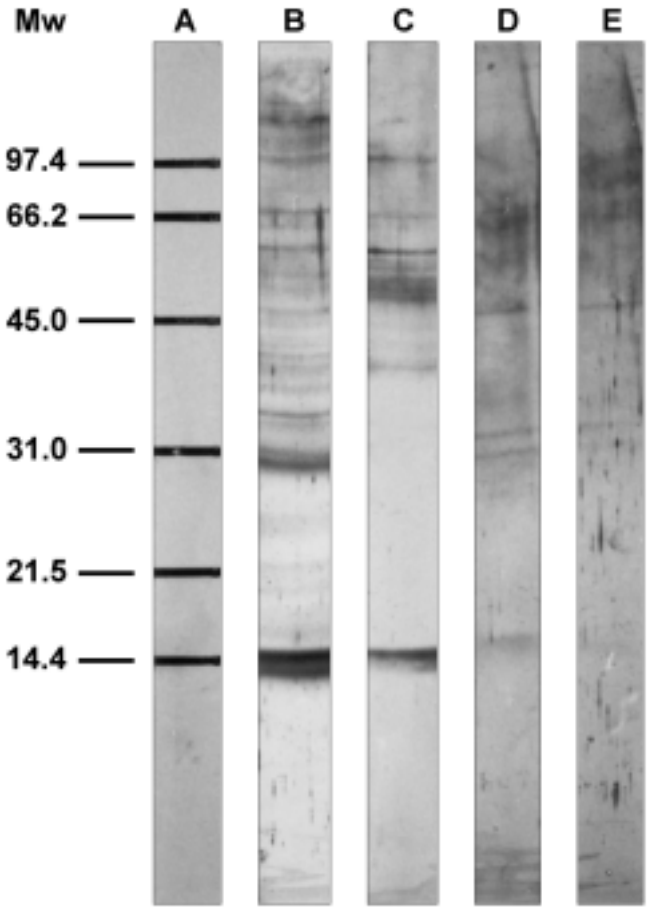

Fig. 3. Immunoblots of extracts of sporocysts of *Aggregata octopiana* and *A. eberthi* under reducing conditions using an 10-20% linear gradient, with anti-*A. octopiana* mouse serum or anti-*A. eberthi* mouse serum. Lane A: Molecular weigh markers (kDa). Lane B: *A. octopiana* sporocysts extract with anti-*A. octopiana* serum. Lane C: *A. octopiana* sporocysts extract with anti-*A. eberthi* serum. Lane D: *A. eberthi* sporocysts extract with anti-*A. eberthi* serum. Lane E: *A. eberthi* sporocysts extract with anti-*A. octopiana* serum.

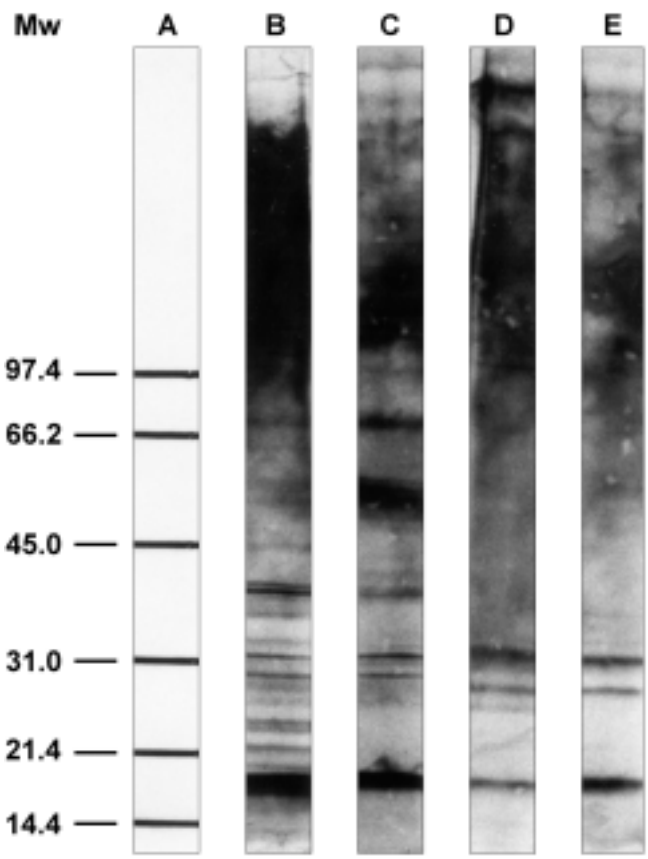

Fig. 4. Immunoblots of extracts of sporocysts of *Aggregata octopiana* and *A. eberthi* under non-reducing conditions using an 5-15% linear gradient, with anti-*A. octopiana* mouse serum or anti-*A. eberthi* mouse serum. Lane A: Molecular weigh markers (kDa). Lane B: *A. octopiana* sporocysts extract with anti-*A. octopiana* serum. Lane C: *A. octopiana* sporocysts extract with anti-*A. eberthi* serum. Lane D: *A. eberthi* sporocysts extract with anti-*A. eberthi* serum. Lane E: *A. eberthi* sporocysts extract with anti-*A. octopiana* serum.

species. The electrophoretic techniques have also been applied to distinguish between the spores of microsporidian species (Street and Briggs 1982, Leiro *et al*. 1994).

Our ELISA and immunoblotting studies revealed the existence of a considerable cross-reactivity between *A. octopiana* and *A. eberthi* species although some species-specific antigenic bands were detected in immunoblotting. Thus three low weight antigens migrating between 21 and 31 kDa were specifically stained on the *A. octopiana* extract. This result suggests that monoclonal antibodies raised against these antigens or other species-specific antigens may be helpful tools to differentiate species belonging to the genus *Aggregata*, where a great intraspecific morphologic variability exists (Hochberg 1990). On the other hand, in studies on other coccidian species, similar low weight bands have been identified as membrane or surface proteins (Jenkins and Dame 1987, Wisher and Rose 1987), some of which vary from one species to another in the same genus (Wisher 1986, Tomavo *et al*. 1989, Tilley and Upton 1990), and even between the different life cycle stages of the same species (Kawazoe *et al*. 1992). At the moment, we do not know if the low weight specific antigens detected in our study are expressed on the *A. octopiana* sporozoite surface but this possibility stimulates more exhaustive characterization studies focused on the exact location and functionality of these

antigens. Knowledge of the *A. octopiana* biology is very important because this coccidian parasite could become a serious octopus pathogen under culture conditions (at present, experiences on nutrition and reproduction of *Octopus vulgaris* under captivity are very advanced; Villanueva 1995, Iglesias *et al.* 2000).

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High Light-induced Sign Change of Gravitaxis in the Flagellate *Euglena gracilis* is Mediated by Reactive Oxygen Species

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Summary. *Euglena gracilis* responds to abiotic stress factors (high light, salinity, heavy metals) with a sign change of its gravitactic behavior. This phenomenon is oxygen dependent and can be suppressed by the application of the reductant dithionite. It is not mediated by the photoreceptor since also blind mutants change their movement behavior upon high light exposure. It is also not mediated by the chloroplasts since the gravitactic sign change was also found in white, chloroplast-free mutants. The NO radical donor SNAP and the NO cleaver carboxy-PTIO had no obvious effects on gravitaxis or gravitactic sign change, respectively, indicating that NO radicals are not likely involved in gravitactic sign change. Gravitactic sign change was suppressed when oxygen was removed by flushing the cell suspension with nitrogen. Also, the addition of the radical scavengers Trolox, ascorbic acid or potassium cyanide abolished or reduced gravitactic sign change. Quantification of reactive oxygen species (ROS) in the cells indicated that these treatments reduced the evolution of ROS. Furthermore, addition of hydrogen peroxide induced gravitactic sign change in the absence of external stress factors. These results indicate that gravitactic sign change is triggered by ROS (most likely hydrogen peroxide) which are probably produced by cytochrome-*c*-oxidase in the mitochondria. The clear responses of *Euglena* to abiotic stress factors suggest that these cells are probably interesting model systems in the study of stress signaling.

Key words: *Euglena gracilis*, gravitaxis, hydrogen peroxide, reactive oxygen species, sign change, UV.

Abbreviations: Carboxy-PTIO - 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, DCF - 2',7'-dichlorofluorescein diacetate, DCFH(-DA) - 2',7'-dichlorodihydrofluorescein diacetate, GSC - gravitactic sign change, H_2O_2 - hydrogen peroxide, NO• - nitric oxygen radical, NOS - Nitrogen oxygen species, PAR - photosynthetic active radiation, ROS - reactive oxygen species, SNAP - S-nitroso-N-acetylpenicillamine, Trolox - 6-hydroxy-2,5,7,8-tetramethylchroman-2-carbonic acid.

INTRODUCTION

Euglena gracilis orients itself in the water column mainly by means of phototactic and gravitactic orientation (Häder and Griebenow 1988). Normally the cells are guided by negative gravitaxis towards the surface, while negative phototaxis helps the cells to avoid regions with deleterious solar radiation, because they are relatively sensitive against excessive UV exposure (Brodhun and Häder 1995). Recently, it was found that *Euglena gracilis* reverses gravitaxis when exposed to certain environmental stressors. The effects of high light exposure and increased salinity on gravitactic sign change (GSC) was demonstrated (Richter *et al.* 2002a, b) Probably sudden changes in temperature also induce gravitactic sign change, but this was not explicitly proven yet. It was clearly demonstrated that the gravitactic sign

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change is not correlated with the photoreceptor or the chloroplasts, because this behavior also occurs in mutants, which lack these organelles. The gravitactic sign change is oxygen dependent, because in the presence of dithionite (removes oxygen chemically) the gravitactic sign change was completely suppressed. This led to the assumption that reactive oxygen species (ROS) or other radicals trigger gravitactic sign change.

ROS are unavoidably produced among others during photosynthesis or during respiration in the mitochondrion. Because of their high reactivity, ROS can exert deleterious effects on nearly every cellular level, like on the membranes, proteins or DNA. For this reason the ROS concentration is controlled by an arsenal of cellular detoxification mechanisms (e.g. catalase, superoxide dismutase). Recently it was found that ROS play an important role in cell signaling. ROS have been shown to be important transducer molecules in many organisms, which among others signal biotic and abiotic stresses, like UV (Mackerness *et al.* 2001), drought (Smirnoff 1998), increased salinity (Rout and Shaw 2001), chilling stress (Lee and Lee 2000) or heavy metals (Piqueras *et al.* 1999). Among others ROS were described to be produced by NADPH oxidase activity, the activity of which is probably regulated by intracellular calcium; (Bowler and Fluhr 2000, Yang, *et al.* 2002) or *via* a phospolipase pathway (Chapman 1998).

In the unicellular ciliates *Paramecium* and *Loxodes* a strong oxygen dependency of gravitaxis was reported, (Fenchel and Finley 1986, Hemmersbach-Krause and Briegleb 1991). In *Loxodes* probably cytochrome-*c*-oxidase is the oxygen receptor, because incubation with KCN led to a loss of gravitactic orientation (Finley and Fenchel 1986). Singlet oxygen generated in high light has been suggested to trigger the reversal for phototactic reaction in *Anabaena* (Nultsch and Schuchart 1985).

The experiments presented in this paper were carried out to reveal in detail the role of ROS in the switching mechanism of gravitaxis in *Euglena gracilis*.

MATERIAL AND METHODS

Organisms and growth conditions

The experiments presented in this study were performed with *Euglena gracilis* Z and the colorless mutant *Euglena gracilis* 1F. Both strains were obtained from the algal culture collection of the University of Göttingen (Schlösser 1994). *Euglena gracilis* Z cells were grown in a modified (contains no EDTA) mineral medium (sodium acetate, $(NH_4)_2HPO_4$, KH_2PO_4, $MgSO_4$, $CaCl_2$, $FeCl_3$, trace elements and vitamin B12 and B6) as described earlier (Starr 1964, Checcucci 1976) in stationary cultures in 100-ml Erlenmeyer flasks at about 20°C under continuous light of about 18 W m^{-2} from mixed cool white and warm tone fluorescent lamps. *Euglena gracilis* 1F was grown in complex medium, which consists of sodium acetate, $CaCl_2$, tryptone, peptone and yeast extract (Starr 1964), in 100-ml Erlenmeyer flasks at about 20°C in the dark. If not otherwise indicated, the culture age was about 3 weeks, the cell density about 1 million cells per milliliter.

Chemicals

Potassium cyanide (KCN) was purchased from Merck, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carbonic acid) from Aldrich (Taufkirchen, Germany), ascorbic acid from Sigma (Deisenhofen, Germany). The NO• donor S-nitroso-N-acetylpenicillamine (SNAP) and the NO• scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline- 1-oxyl-3-oxide (Carboxy-PTIO) were from Tocris (Bristol, UK). The 2',7'-dichlorodihydrofluorescein diacetate (DCFH) used for detection of reactive oxygen species was obtained from Molecular Probes (Leiden, The Netherlands). Hydrogen peroxide (30 % solution) was from Aldrich and hypochloride from Roth (Karlsruhe, Germany).

Exposure to artificial radiation

All samples used for a single experiment were taken from the same culture. 30 ml of cell suspension (each 5 ml in the case of experiments with SNAP and carboxy-PTIO) were transferred into small black plastic boxes (at least 3 parallel samples with inhibitors, control samples and corresponding dark controls) and placed into a temperature-controlled water bath (20°C). The samples were covered with a 295 nm cut-off filter foil (transmits UV-B, UV-A and PAR, Digefra, Munich, Germany) and irradiated with 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 W m^{-2}, UV-A 67 W m^{-2} and UV-B 1.9 W m^{-2} (total photon flux: 1079.6 µmol*s^{-1}*m^{-2}, PAR: 813 µmol*s^{-1}*m^{-2}, UV-A: 260 µmol*s^{-1}*m^{-2}, UV-B: 6.6 µmol*s^{-1}*m^{-2}) at a distance of 65 cm from the lamp. The exposure time is indicated separately for each presented experiment. The cells were filled into a cuvette and subsequently analyzed with the image analysis software WinTrack 2000 (see below). The observed sign change phenomenon persisted for several hours (Richter *et al*. 2002a), so that the time, necessary to prepare and to perform the measurements, was sufficient. Each experiment was repeated at least three times.

Motion analysis

After irradiation some drops of *Euglena* cell suspension were sealed between two glass slides by means of silicon (Bayer Silone, high viscous, Bayer, Leverkusen, Germany) and immediately analyzed. Motion analysis was performed with a recently developed cell tracking system (Lebert and Häder 1999b, WinTrack 2000). 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 Θ indicates the mean movement direction of a cell culture. The increment of the angle is clockwise (see one of the circular histograms in Fig. 6).

The measurements were performed in darkness (infrared observation of the cells) to avoid any phototactic or photophobic effects 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 control data acquisition of the obtained cell tracks by the software.

Gassing with nitrogen

Oxygen was removed by nitrogen flushing of the samples during the whole light exposure. The corresponding controls were flushed with air. Oxygen was not completely removed by this method but it was less than 2 mg/l (measurement with DO-5509 oxymeter, Conrad electronics, Hirschau, Germany).

Detection of reactive oxygen species

The ROS concentration was estimated with the fluorescence probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) according to He and Häder (2002a). The lipophilic DCFH-DA passes the cell membrane and is cleaved inside the cell to the non fluorescent 2',7'-dichlorodihydrofluorescein (DCFH). Upon reaction with intracellular reactive oxygen species (ROS) DCFH is oxidized to the highly fluorescent 2',7'-dichlorodifluorescein (DCF). The fluorescence of the samples was measured with a spectrofluorometer (RF-5000, Shimadzu, Kyoto, Japan) at room temperature (excitation wavelength: 485 nm, emission band between 500 and 600 nm). The integral of the signal (500-600 nm) was used for analysis. DCFH-DA was added from a 2 mM methanolic stock solution to an end concentration of 5 µM. As DCFH-DA is sensitive against light, the samples were incubated directly after the exposure experiment (1 h incubation time in the darkness). Each one milliliter of cells was transferred into a cuvette (1.5 ml semi-micro disposable cuvettes, Brand, Wertheim, Germany), after gentle mixing of the cell culture. At least three independent fluorescence signals were measured from each sample.

RESULTS

Effects of the physical replacement of oxygen with nitrogen

Samples from which oxygen was removed physically, showed a pronounced reduction of GSC as compared to the untreated controls (Fig. 1). The effect was not as

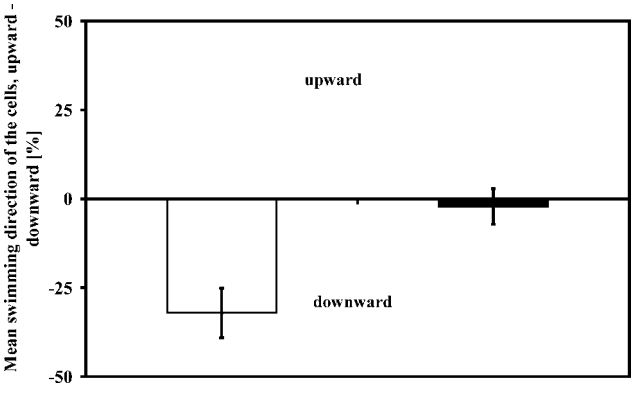

Fig. 1. Effect of nitrogen flushing of the cell suspension on the movement direction of light-exposed *Euglena gracilis* 1F mutant cells. The cells have been irradiated for 120 min with artificial solar radiation in order to induce a sign change of negative gravitaxis. The pronounced gravitactic sign change of light-exposed control cells (white column) is considerably inhibited, when oxygen pressure is decreased by nitrogen (black column). The diagram shows the percentage of upward swimming cells minus the percentage of downward swimming cells. Positive values indicate upward, negative values downward mean orientation of the cells in a culture. Bars indicate SD of each four parallel control and nitrogen treated samples. The experiment was repeated three times with similar results.

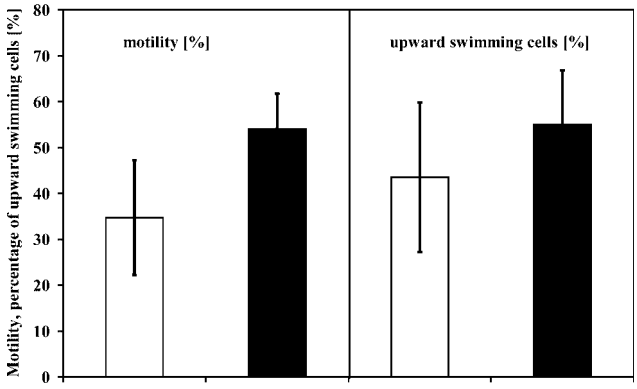

Fig. 2. Effect of carboxy-PTIO on motility and upward oriented movement in a *Euglena gracilis* culture after 210 min exposure to artificial solar radiation. The data indicate a slight protective role of carboxy-PTIO. White columns: control cells, black columns: samples plus carboxy-PTIO (200 µM). Bars indicate SD of each four parallel control and carboxy-PTIO treated samples. The experiment was repeated several times, but the results were not consistent (see text).

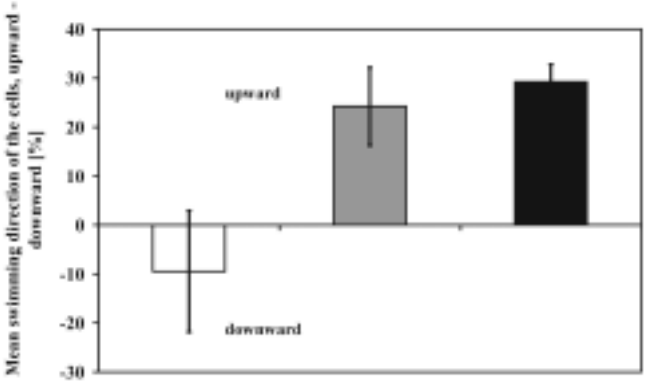

Fig. 3. Inhibitory effect of Trolox and ascorbic acid on the light-induced gravitactic sign change in a *Euglena gracilis* strain Z. The cells were exposed to artificial solar radiation (340 min). Trolox (gray column) and ascorbic acid (black bar) considerably decreased the extent of gravitactic sign change compared to the control (white column). Considerably more cells were swimming upward in the presence of Trolox (black column) and ascorbic acid (gray column) compared to the control (white column). Bars indicate SD of four controls and each three Trolox or ascorbic acid treated samples. The experiment was repeated four times with similar results.

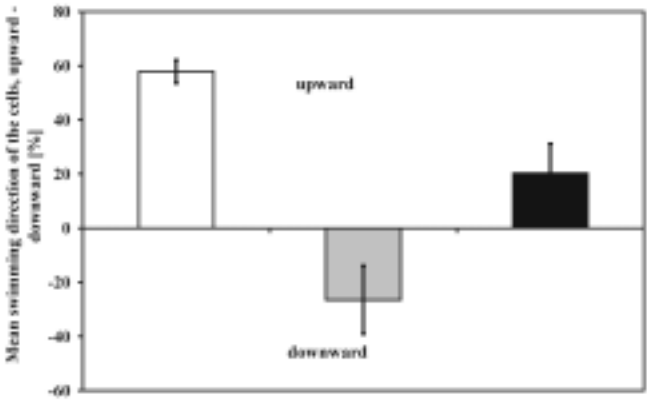

Fig. 5. Influence of potassium cyanide on gravitactic sign change in *Euglena gracilis* mutant 1F exposed to artificial solar radiation (120 min). In the presence of KCN (500 µM) gravitactic sign change was considerably inhibited, while the control cells (light only) showed a pronounced reversal of gravitaxis. White column: dim light control; gray column: light exposed control; black column: light exposure plus 500 µM KCN. Error bars indicate SD of each four parallel dim light controls, high light controls and KCN-treated samples. The experiment was repeated three times with similar results.

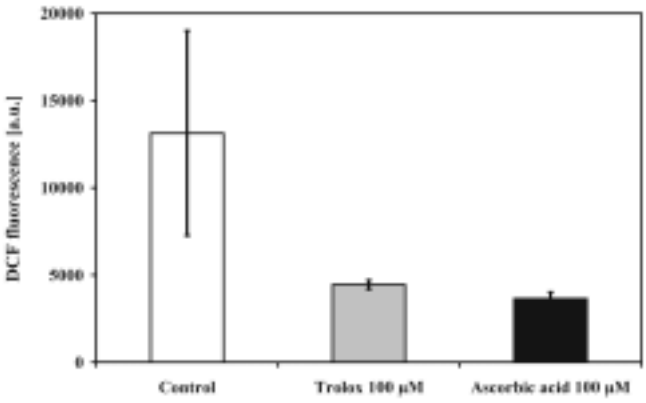

Fig. 4. ROS scavenging effect of Trolox and ascorbic acid determined with the fluorescence indicator DCFH-DA. Higher fluorescence values indicate higher ROS generation (oxidation of the non-fluorescent DCFH to highly fluorescent DCF). The addition of Trolox and ascorbic acid reduces the amount of DCF considerably. The samples shown in this diagram were exposed to artificial solar radiation (120 min) and subsequently incubated with DCFH-DA. Error bars indicate SD.

pronounced as in experiments performed in the presence of dithionite probably because the experimental setup did not allow removing intracellular oxygen completely.

Experiments with carboxy-PTIO and SNAP

Carboxy-PTIO is a water-soluble and stable free radical molecule that reacts stoichiometrically with NO• (NO• scavenger). SNAP releases NO• radicals under physiological conditions. SNAP (200 µM) induced no gravitactic sign change compared to the controls (neither in darkness nor in light). Carboxy-PTIO (200 µM) did not clearly suppress GSC. The data indicate only a marginal protective effect. The proportion of motile and possibly upward swimming cells is higher in the presence of carboxy-PTIO upon light exposure than in the controls (Fig. 2), but this was not seen in all experiments. Currently, the role of NO• in GSC can not clearly be stated.

Effects of radical scavengers on gravitactic sign change

In the presence of the radical scavengers ascorbic acid and Trolox a pronounced reduction in GSC was detected at low concentrations (Fig 3.). Additionally, a decrease in the DCF-fluorescence in scavenger-treated cells was obvious indicating a loss in ROS concentration (Fig. 4). High Trolox concentrations (> 500 µM) impair the cells upon high light exposure, while dark controls were not affected (data not shown). Trolox is most likely cleaved by UV and blue light, and the products have a toxic effect on the cells at high concentrations, superimposing the positive effect of radical scavenging.

Effects of potassium cyanide on gravitactic sign change

Potassium cyanide considerably reduced GSC in *Euglena gracilis*. Although gravitactic orientation was

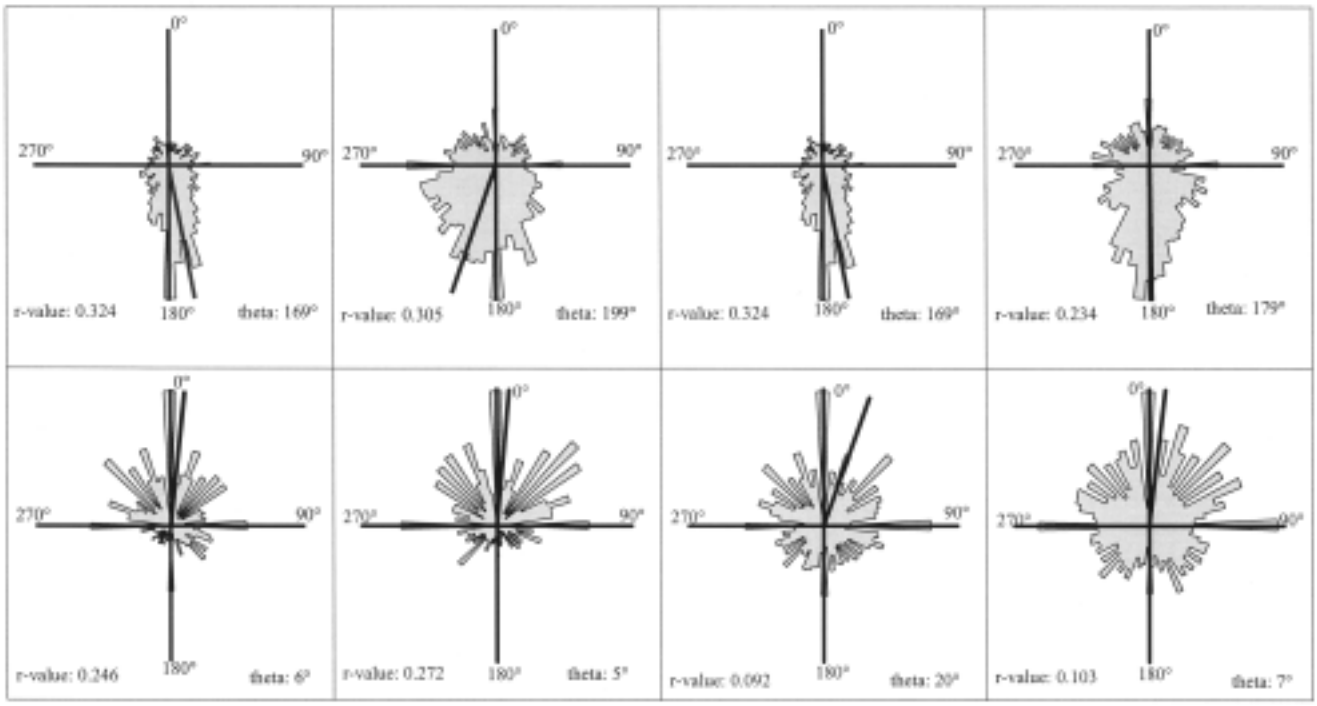

Fig. 6. Circular histograms of the movement behavior of the cells in Fig. 5. Upper row: untreated light-exposed controls; lower row: light-exposed samples plus 500 µM KCN. The length of a single sector indicates the fraction of cells swimming in this direction (details see text). The r-value indicates the precision of orientation, theta (solid black line) indicates the mean movement direction of cell culture (details see text).

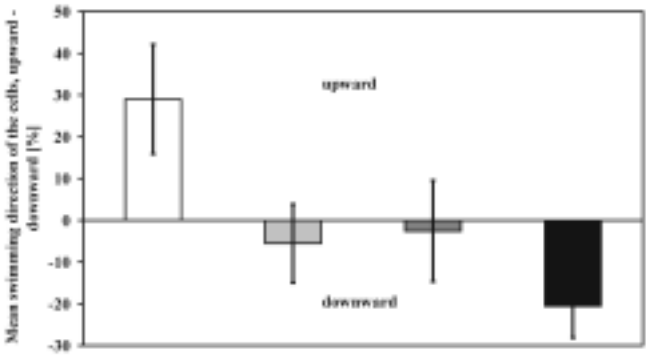

Fig. 7. Influence of hydrogen peroxide on gravitactic sign change in *Euglena gracilis* mutant 1F. The cells were incubated in H_2O_2 without external stresses. In the presence of hydrogen peroxide gravitactic sign change was induced, while the control cells showed negative gravitaxis. White column: untreated control; light gray column: 0.43 mM H_2O_2; dark gray column: 0.61 mM H_2O_2; black column 0.84 mM H_2O_2. Incubation time about 30 min. Bars indicate SD of each six independent samples (control and H_2O_2-treated).

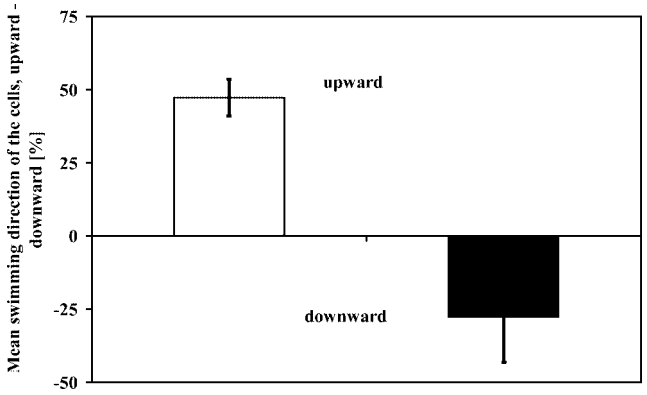

Fig. 8. Influence of hydrogen peroxide on gravitactic sign change in *Euglena gracilis.* In the presence of hydrogen peroxide gravitactic sign change was induced, while the control cells showed negative gravitaxis. White column: untreated control; black column 2.1 mM H_2O_2. Incubation time about 30 min. Bars indicate SD of six independent controls and four H_2O_2-treated samples.

impaired compared to dark or dim light (presented data) controls, the mean direction of the cells in the culture was still upward (Figs 5, 6). In contrast, untreated light controls (no KCN added) showed GSC or at least a loss of gravitactic orientation (Figs 5, 6).

Effects of hydrogen peroxide and hypochloric acid

The addition of hydrogen peroxide at low concentrations induced GSC or at least increased the percentage of downward swimming cells in *Euglena gracilis* cul-

tures in the absence of other external stimuli. Colorless strains are more sensitive against H_2O_2. In the *Euglena gracilis* mutant 1F the addition of 0.8 mM H_2O_2 to the medium induced a pronounced GSC after about 30 min of incubation (Fig. 7). In the green strain Z the addition of 2 mM of hydrogen peroxide to the medium was found to invert gravitaxis (Fig. 8). In old cultures of strain Z (>3 month) in which the cells had a drop-like cell shape GCS was not inducible, although a loss of gravitactic orientation was detected (data not shown).

In the presence of hypochloric acid (various concentrations tested, from non-effective to lethal concentrations) no GSC was detected (data not shown).

DISCUSSION

Recent experiments revealed that *Euglena gracilis* changes the sign of gravitaxis upon abiotic stress. This effect is oxygen-dependent (see introduction). The question was which mechanism triggers this phenomenon. Samples in which oxygen was reduced by nitrogen flushing, the extent of gravitactic sign change was remarkably decreased, which confirms results with dithionite obtained in a previous study (Richter *et al.* 2002a). With the present study, where oxygen was removed physically, a non-specific effect of dithionite leading to GSC can be ruled out and the oxygen dependency of GSC can be clearly stated. The suppression of GSC in nitrogen-flushed cells was not complete, most likely due to the fact that some oxygen remained within the cells, which gives rise to the evolution of oxygen radicals.

Recent studies revealed that NO• radicals and other nitrogen species (NOS) play, in combination with ROS, an important role in mitochondrial signaling (Brookes *et al.* 2002). Among others, NO• radicals interact with the cytochrome-c-oxidase and hereby control the evolution of H_2O_2, which probably plays an important role in cell signaling (see below). To elucidate a possible role of NO• radicals in GSC, experiments with SNAP and carboxy-PTIO were performed, which did not clearly indicate an involvement of NO• radicals. Neither the production of NO• radicals by SNAP nor the scavenging of NO• radicals by carboxy-PTIO had any clear effect on GSC except a slightly inhibitory effect on GSC under high light exposure in the presence of carboxy-PTIO.

To test whether radicals or hydrogen peroxide are possibly involved in GSC, the known radical scavengers Trolox and ascorbic acid were applied. Among others, these chemicals have been shown to have a protective effect against UV-B exposure. For example Trolox was found to decrease intracellular hydrogen peroxide generation in human keratinocytes in the presence or absence of UV-B radiation (Peus *et al.* 2001). This study clearly demonstrates that Trolox incubation significantly increases cell survival upon UV-B. Ascorbic acid was shown to reduce the ROS concentration in cyanobacteria and to increase their survival (He and Häder 2002a). In *Euglena* Trolox and ascorbic acid clearly reduce GSC, and in parallel, the amount of ROS was significantly reduced. This indicates that probably ROS trigger GSC.

A very important observation was that hydrogen peroxide induces GSC in the absence of external stressors. The GSC-inductive effect of hydrogen peroxide makes its involvement as key signaling molecule of abiotic stress in *Euglena* very likely. It can not be excluded, that hydrogen peroxide itself acts as stressor, so that the observed GSC is a more indirect effect. But hypochloric acid, which is chemically similar to hydrogen peroxide, does not induce GSC. This indicates that the effect obtained in the presence of hydrogen peroxide is specific. The important role of hydrogen peroxide and other ROS in cell signaling was detected in the recent years (reviewed by Neill *et al.* 2002). Intracellular hydrogen peroxide levels are reported to be controlled by regulation of antioxidant enzymes like ascorbate peroxidase or catalase, which decrease the hydrogen peroxide concentration (Neill *et al.* 2002, He and Häder 2002b). A variety of H_2O_2 downstream signaling events were described, among others membrane channel activation or modulation of gene expression (probably via oxidation of H_2O_2-sensitive transcription factors) and modulation of other signaling molecules (Neill *et al.* 2002 and literature cited therein). Among others the role of hydrogen peroxide in root gravitropism was demonstrated (Joo *et al.* 2001).

The suppressive role of KCN, a known blocker of the cytochrome-c-oxidase in mitochondria, is a hint that mitochondria are a possible source of hydrogen peroxide upon abiotic stress in *Euglena*.

But KCN also interacts with other cellular metalloproteins, like e.g. catalase. The blockage of the mitochondrial respiratory chain by KCN did not kill the cells, because they also survive for hours under hypoxia (as seen in the nitrogen and dithionite experiments, which both led to microaerobic conditions). However, dramatic metabolic changes do occur even in these hypoxia-tolerant cells and may have an influence on the experiment: e.g. reduced protein synthesis and ion pump

activities, and arrest of membrane channels (see review by Boutilier and St-Pierre 2000).

It is unknown in which way ROS may induce GCS, because up to now the physiological mechanisms of gravitaxis are still not fully understood. According to a present model, gravitaxis is most likely triggered by mechano-sensitive membrane channels followed by a series of subsequent physiological mechanism, among others membrane potential changes, increased calcium and cAMP concentration, involvement of calmodulin (Lebert and Häder 1999a, Streb *et al.* 2002). An additional physical alignment component caused by body asymmetry is possible (Roberts and Deacon 2002, Richter *et al.* 2002c).

In earlier experiments performed with fluorescent calcium indicators increased intracellular calcium was observed upon cell stress (drought, high light exposure). But because this has not been the focus of these experiments the observations were not quantified or studied in more detail. From these observations it is not possible to say if calcium acts as stress signal in *Euglena gracilis* or if there is any interaction between calcium and the presumed ROS. In other systems an interaction between calcium and intracellular H_2O_2 level was described (Yang and Poovaiah 2002).

The clear response of *Euglena* cells to unfavorable environmental factors makes these cells potential model organisms in the study of stress and redox signaling.

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PROTOZOOLOGICA

Plasmodium and *Leucocytozoon* (Sporozoa: Haemosporida) of Wild Birds in Bulgaria

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Summary. Three species of parasites of the genus *Plasmodium* (*P. relictum, P. vaughani, P. polare*) and 6 species of the genus *Leucocytozoon* (*L. fringillinarum, L. majoris, L. dubreuili, L. eurystomi, L. danilewskyi, L. bennetti*) were found in the blood of 1332 wild birds of 95 species (mostly passerines), collected in the period 1997-2001. Data on the morphology, size, hosts, prevalence and infection intensity of the observed parasites are given. The total prevalence of the birds infected with *Plasmodium* was 6.2%. *Plasmodium* was observed in blood smears of 82 birds (26 species, all passerines). The highest prevalence of *Plasmodium* was found in the family Fringillidae: 18.5% (n=65). A high rate was also observed in Passeridae: 18.3% (n=71), Turdidae: 11.2% (n=98) and Paridae: 10.3% (n=68). The lowest prevalence was diagnosed in Hirundinidae: 2.5% (n=81). *Plasmodium* was found from March until October with no significant differences in the monthly values of the total prevalence. Resident birds were more often infected (13.2%, n=287) than locally nesting migratory birds (3.8%, n=213). Spring migrants and fall migrants were infected at almost the same rate of 4.2% (n=241) and 4.7% (n=529) respectively. Most infections were of low intensity (less than 1 parasite per 100 microscope fields at magnification 2000x). *Leucocytozoon* was found in 17 wild birds from 9 species (n=1332). The total prevalence of *Leucocytozoon* was 1.3%.

Key words: haemosporidians, *Leucocytozoon*, *Plasmodium,* prevalence, wild birds.

INTRODUCTION:

Haematozoa of the genera *Plasmodium* and *Leucocytozoon* are common among wild birds in the Northern Hemisphere. Their fauna and morphology are relatively well known in Western and Northern Europe and North America (Pierce and Mead 1977,1978; Kučera 1981a; Bennet *et al.* 1982; Bishop and Bennet 1992; Valkiunas 1997; Krone *et al.* 2001). It is known that vectors of *Plasmodium* are some species of mosquitoes (Culicidae) and the vectors of *Leucocytozoon* are dipterans of family Simuliidae (Valkiunas 1997). Until now little has been known about the distribution and the ecology of *Plasmodium* and *Leucocytozoon*, especially in South-Eastern Europe and the neighbouring areas of the Middle East (Valkiunas 1997, Valkiunas *et al.* 1999). From the Balkans data on these parasites were published for Macedonia (Wülker 1919), Greece (Wenyon 1926) and Bulgaria (Valkiunas *et al.* 1999). In the cited publications the blood parasites found were determined by genus and only in a few occasions by species level. The number of the investigated birds was not high. Only

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3 species of these 2 genera were found in the blood of the wild birds from the Balkans, namely: *Plasmodium matutinum* (Valkiunas *et al.* 1999), *Plasmodim praecox (=Plasmodium relictum)* (Wülker 1919), and *Leucocytozoon fringillinarum* (Valkiunas *et al.* 1999). *Plasmodium* was found in the blood of 5 bird species and *Leucocytozoon* in 10 bird species from the Balkan Peninsula (Wülker 1919, Wenyon 1926, Valkiunas *et al.* 1999).

The aim of this article is to present data on the haematozoan fauna of wild birds in Bulgaria, especially on the parasites of genera *Plasmodium* and *Leucocytozoon* and their prevalence in birds of different species, age, sex and migratory status.

MATERIALS AND METHODS

Blood smears of 1332 wild birds of 95 species (35 families and 12 orders) were studied. The birds were caught during the whole year from 1997 through 2001, mainly in 5 localities in Bulgaria: the Kalimok Biological Station, close to the village of Nova Cherna, Silistra District (933 birds caught); the Chelopechene Fishponds (109); Vrana Campsites (76) and Dragoman Lake (48), Sofia District; Durankulak Lake, Dobrich District (79).

Eighty-seven birds were caught in other regions of Bulgaria (Rupite, Blagoevgrad District; Sofia; Nissovo, Russe District and Atanassovsko Lake, Burgas District). The birds were caught in vertical mist nets, and blood was taken by cutting the longest claw of each specimen. Sex and age determination of the birds was made using the field guide by Svenson (1992). All the birds were ringed and released. The families and the species of birds studied and the prevalence of haematozoa infections in different hosts are shown in Table 9.

From each bird caught, 3 (rarely 2) blood smears were prepared, fixed in methanol for 5 min and stained with Giemsa solution for 50 min. The smears were examined by means of Zeiss microscope under 200, 400 and 2000 magnification. Identification of haemoproteids was performed using the descriptions of Valkiunas (1997). In most of the cases *Plasmodium* parasites were identified to subgenus level and in only 8 cases to species level. The difficulties in species identification of *Plasmodium* were due to the low intensity rates of infection, so the number of the erythrocyte meronts found was low. Bird classification followed Svensson and Grant (1999). To calculate the intensity of invasion, all parasites in 100 microscope fields at magnification 2000x were counted (approximately 4000 erythrocytes). All measurements in the text and the tables are given in micrometers.

To verify the degree of reliability of the data, Fisher's Criterion (F-test) was used (Plochinsky 1970). In these comparisons, the degree of probability (p) and the number of the birds investigated (n) were stated in the text. Fisher's Criterion (φ-method) was chosen because it gives better results than the chi-square test in cases when comparing small percentages (less than 20%) (Plochinsky 1970).

According to their migration status, the birds were divided in 3 groups: (i) long-distance migrants, birds species that spend the winter in Africa, South of the Sahara desert or in India; (ii) close migrants, birds species that spend the winter mainly in the Mediterranean zone, however some populations in Europe are partly resident; (iii) non-migratory (resident) birds spend the winter predominantly in their breeding range. Only some northern populations could move during the autumn and winter periods.

RESULTS

A total of 6 species of *Leucocytozoon* and 3 species of *Plasmodium* were found in the blood of the birds studied. *Plasmodium* was found in 82 birds from 26 species (in 27.3% of all species studied). *Leucocytozoon* was found in 17 birds of 9 species (in 9.5% of all species studied).

Leucocytozoon majoris Laveran, 1902 (Table 1)

Morphology: only round gametocytes were seen. More than half of the parasite's perimeter adheres to the host cell nucleus. The nucleus of the parasite is often diffuse, ellipsoid, but sometimes is clear-cut. The nucleolus could be seen in some cases. Small pieces of host cell protoplast were often visible.

Table 1. Measurements (in μm) of *Leucocytozoon majoris* (n=20).

	Range	Mean	Standard deviation
Length of gametocytes	9.0-5.0	12.3	1.7
Width of gametocytes	8.0-13.0	10.2	1.6
Length of erythrocyte nucleus	20.0-38.0	30.2	5.2
Length of gametocyte nucleus	3.0-6.0	4.2	0.9
Width of gametocyte nucleus	1.5-3.0	2.6	0.9

Hosts: *Acrocephalus schoenobaenus, Turdus merula, Oriolus oriolus.*

Intensity of infection: found in 4 birds. Three of the infections were of very low intensity (below 1.0), and in the fourth an intensity of 2.0 parasites per 100 microscope fields was found.

Localities: Nova Cherna and Vrana. At Nova Cherna it was found in the blood of 2 *A. schoenobaenus* (fall migrants, caught in September 2001) and in *O. oriolus* (local breeding bird, May 1999). At Vrana the parasite was observed in the blood of a local young *T. merula* (June 2001).

Notes: the species is widespread in a great number of passerines from all zoogeographic zones, except Neotropical (Valkiunas 1997).

Leucocytozoon fringillinarum Woodcock, 1910 (Table 2)

Morphology: only round gametocytes could be seen. Less than half of the parasite's perimeter adheres to the host cell nucleus. The width of the host cell nucleus is greater in comparison with the other species of passerine's leucocytozoids, so sometimes it is like a small "cap". The parasite's nucleus is round or ellipsoid, with clear nucleolus.

Table 2. Measurements (in μm) of *Leucocytozoon fringillinarum* (n=6).

	Range	Mean	Standard deviation
Length of gametocytes	9.0-10.5	9.8	0.5
Width of gametocytes	8.0-10.0	9.1	0.9
Length of erythrocyte nucleus	12.0-19.0	14.1	2.6
Length of gametocyte nucleus	2.5-5.0	3.9	0.7
Width of gametocyte nucleus	2.0-3.0	2.3	0.4

Hosts: *Phylloscopus trochilus, Sylvia atricapilla, Anthus trivialis, Turdus merula*.

Intensity of infection: usually lower than 1.0 parasite per 100 microscope fields.

Localities: Nova Cherna and Chelopechene. At Nova Cherna it was found in the blood of fall migrants: *Ph. trochilus* (8.3%, n=36) and *A. trivialis* (4.0%, n=24) and only twice in spring in *T. merula* and *A. trivialis.* At Chelopechene the parasite was found once in spring (May 2001) in the blood of *S. atricapilla.*

Notes: species was found in Bulgaria in *Acrocephalus schoenobaenus* by Valkiunas *et al.* (1999). Distributed in all zoogeographical zones (Valkiunas 1997).

Leucocytozoon dubreuili Mathis et Leger, 1911 (Table 3)

Morphology: only round gametocytes were seen. The host cell nucleus is wider in the ends and narrow in the centre. More than half of the parasite's perimeter adheres to the host cell nucleus. The nucleus of the parasite is clear-cut and ellipsoid in shape.

Hosts: *Turdus philomelos, Turdus merula.*

Intensity of infection: found once in the blood of *T. philomelos* with intensity of 3.0 and once in *T. merula* with intensity of 4.0 parasites for 100 microscope fields.

Localities: Nova Cherna (in *T. philomelos*, spring migrant, April 2000) and Rupite - in a local breeding *T. merula* (May 2000).

Table 3. Measurements (in μm) of *Leucocytozoon dubreuili* (n=6).

	Range	Mean	Standard deviation
Length of gametocytes	10.0-13.5	11.5	0.6
Width of gametocytes	8.5-12.0	10.9	0.9
Length of erythrocyte nucleus	21.0-28.0	24.2	2.9
Length of gametocyte nucleus	2.5-4.0	3.3	0.7
Width of gametocyte nucleus	1.5-3.0	2.3	0.3

Notes: species is common in the Holarctic, Indomalayan and Ethiopian zoogeographical zones and very rare in the Neotropic and Australian zones (Valkiunas 1997).

Leucocytozoon danilewskyi Ziemann, 1898 (Table 4)

Morphology: only oval to irregular in shape gametocytes were observed in a single case of infection. Approximately half of the parasite's perimeter adheres to the host cell nucleus. Small volutine granules are present. The parasite's nucleus is elongated in shape with a clear nucleolus situated close to the nuclear membrane. Pieces of the host cell cytoplasm were often observed.

Table 4. Measurements (in μm) of *Leucocytozoon danilewskyi* (n=6).

	Range	Mean	Standard deviation
Length of gametocytes	12-14.5	13.5	0.5
Width of gametocytes	11.5-4.0	12.8	0.9
Length of erythrocyte nucleus	11-15.5	13.7	3.1
Length of gametocyte nucleus	4.0-5.0	4.4	0.6
Width of gametocyte nucleus	3.0-4.5	4.0	0.4

Host: *Asio otus.*

Intensity of infection: found only once with low intensity (below 1.0).

Locality: Nova Cherna. Found in a local breeding bird (April 2001).

Notes: Elongated gametocytes were described for this species (Valkiunas 1997, Krone *et al*. 2001), but we did not find any. Wülker (1919) found a leucocytozoid parasite in the blood of *Athene noctua* from Macedonia. Probably it was *L. danilewskyi,* because it is the only leucocytzoid described until now in owls. The species is distributed in all zoogeographical zones (Valkiunas 1997).

Leucocytozoon eurystomi Kerandel, 1913 (Table 5)

Morphology: only elongated gametocytes were observed in a single case of infection. The host cell is elongated with sharp ends. Its length varies considerably. The host cell nucleus adheres to the parasite. The parasite's nucleus is oval with a clear nucleolus.

Table 5. Measurements (in μm) of *Leucocytozoon eurystomi* (n=6).

	Range	Mean	Standard deviation
Length of gametocytes	24-27	25.7	2.7
Width of gametocytes	6.0-9.5	8.3	1.1
Length of erythrocyte nucleus	10.5-19	14.6	2.5
Length of gametocyte nucleus	3.0-4.5	3.5	0.9
Width of gametocyte nucleus	2.0-3.0	2.2	0.6

Host: *Coracias garrulus.*

Intensity of infection: found only once with an intensity of 5.0 parasites per 100 fields.

Locality: Nova Cherna (May 1999).

Notes: the species is found in Central and Southern Palearctic, Indomalayan and Ethiopian zoogeographic zones (Valkiunas 1997).

Leucocytozoon bennetti Valkiunas, 1993

Found in the blood of *Coracias garrulus*, caught in May 1997 at Nova Cherna. The intensity was very low, so we did not give a description. Dr. G. Valkiunas made species identification.

Plasmodium (*Haemamoeba*) *relictum* Grassi et Feletti, 1891 (Table 6)

Morphology: trophozoites obviously displace the host cell nucleus. The number of the merozoites in the fully-grown meronts is around 20. Gametocytes and meronts are round to ellipsoid. They change the shape of the infected erythrocyte and displace its nucleus. They can fill the entire host cell cytoplasm in the last stages of their growth. The gametocyte nucleus located centrally. Pigment granules small, dispersed and vary greatly in number.

Table 6. Measurements (in μm) of *Plasmodium relictum* (n=10).

Gametocytes	Range	Mean	Standard deviation
Length	5.0-7.5	6.7	0.3
Width	4.0-6.5	6.1	0.3

Hosts: *Lanius collurio, Passer montanus, Parus major, Panurus biarmicus.*

Intensity of infection: found in 7 birds. Except for 2 of them (in the blood of *Lanius collurio*), the intensity of infection was extremely low (below 1.0).

Localities: Nova Cherna in the blood of spring migrants and locally breeding birds, and at Durankulak in the blood of local breeding *Panurus biarmicus* (July 2001).

Notes: *Plasmodium relictum* is the most common *Plasmodium* species in birds (Valkiunas 1997).

Plasmodium (*Giovanolaia*) *polare* Manwell, 1934 (Table 7)

Morphology: meronts with 6-10 merozoites are situated most often in the polar zone of the infected erythrocyte. They are round or ellipsoid in shape. Gametocytes are with ameboid or entire margins and centrally located nucleus. Pigment granules are between 5 and 10, usually gathered in one of the ends of the parasite. Cases of double infection in 1 erythrocyte were observed.

Table 7. Measurements (in μm) of *Plasmodium polare* (n=10).

Gametocytes	Range	Mean	Standard deviation
Length	9.0-16.0	11.6	1.2
Width	2.0-3.0	2.4	0.3

Host: *Hirundo rustica.*

Intensity of infection: Found once with an intensity of infection of 21 parasites per 100 fields.

Locality: found at Nova Cherna in April 2001.

Notes: so far *P. polare* has been observed in the blood of some passerines of the swallow family - *Hirundo pyrrhonota* and *H. cucullata. H. rustica* was not included in the list of the hosts of *P. polare.* Found in all zoogeographical zones except the Australian (Valkiunas 1997).

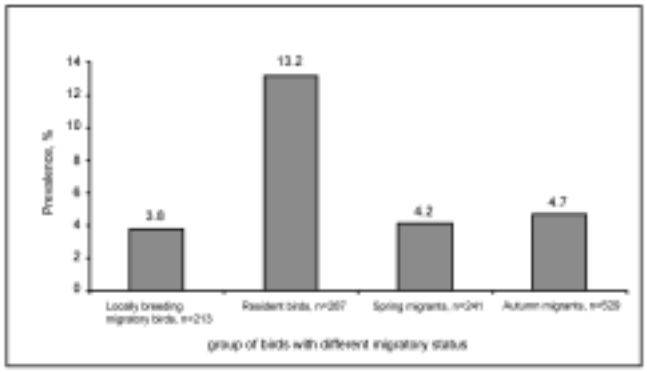

Fig. 1. Prevalence of *Plasmodium* infections of wild birds with different migratory status in Bulgaria.

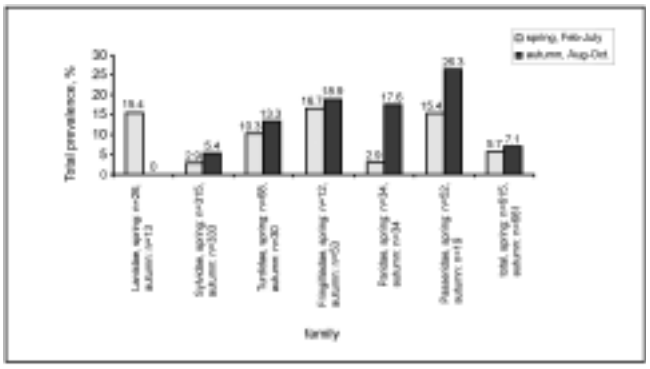

Fig. 3. Seasonal differences in the prevalence of the *Plasmodium* infections among the studied bird families.

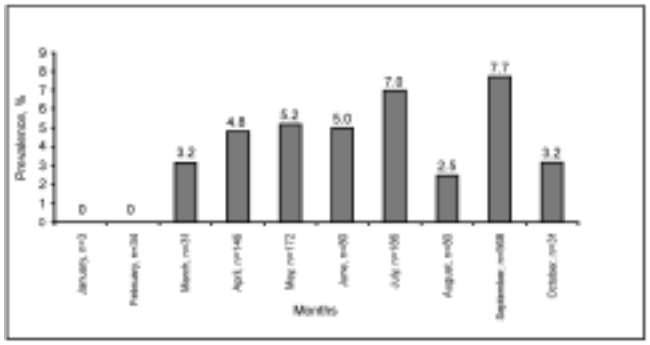

Fig. 2. Total prevalence of *Plasmodium* infections by month.

Plasmodium (*Novyella*) *vaughani* Novy et MacNeal, 1904 (Table 8)

Morphology: meronts are smaller than the erythrocyte nucleus. The number of the merozoites is between 4 and 8. Young gametocytes adhere only to the host cell membrane. Fully-grown gametocytes are elongated with centrally located nuclei. Pigment granules are between 3 and 12 in number, often gathered in a group. The fully-grown gametocytes and meronts do not displace the erythrocyte nucleus.

Table 8. Measurements (in μm) of *Plasmodium vaughani* (n=10).

	Range	Mean	Standard deviation
Length of gametocytes	10.0-12.0	10.8	0.6
Width of gametocytes	1.5-2.5	2.0	0.3
Length of meronts in erythrocyte	2.5-4.0	3.2	0.4
Width of meronts in erythrocyte	1.5-2.5	2.1	0.3

Hosts: *Turdus merula, Sylvia atricapilla, Acrocephalus schoenobaenus.*

Intensity of infection: found in 5 birds with intensity between 1.0 and 5.0 parasites per 100 fields.

Localities: the species was found at Rupite (April 2000) and Vrana (June 2000) in locally breeding birds (*Turdus merula, Sylvia atricapilla*) and at Nova Cherna in fall migrants (*Acrocephalus schoenobaenus*) (September 2000).

Notes: common species, found in all zoogeographical zones (Valkiunas 1997).

Infection prevalence and intensity

The total prevalence in the birds studied was 6.2% for *Plasmodium* and 1.3% for *Leucocytozoon* (n=1332). All *Leucocytozoon* infections were of low prevalence and intensity. *Leucocytozoon* was diagnosed in the blood of 9 bird species: *Asio otus, Coracias garrulus, Anthus trivialis, Turdus merula, Turdus philomelos, Sylvia atricapilla, Acrocephalus schoenobaenus, Phylloscopus trochilus* and *Oriolus oriolus.* Nine cases of infections were found in locally breeding birds (migratory and non-migratory), 6 in fall migrants and 2 in spring migrants.

In Table 9 the prevalence of *Plasmodium* infections in different bird species and families is shown. *Plasmodium* was found only in passerine birds in Bulgaria. It should be emphasized, that the number of passerines studied (1224 individuals), was considerably higher than the number of non-passeriform birds (108 individuals). The highest prevalence was found in Fringillidae (18.5%, n=65), Passeridae (18.3%, n=71) and Turdidae (11.2%, n=98). A low infection rate was found in Hirundinidae: 2.5% (n=81).

Table 9. Number of the examined birds, their migration status and blood parasite prevalence (in %) in different bird families and species. Migration status: Cm - close migrants (spends the winter mainly in the Mediterranean zone), Lm - long-distance migrants, Nm - non-migratory (resident) species.

Bird families/species	Migration status	Examined birds	*Plasmodium*			*Leucocytozoon*		
			Infected birds	Prevalence	Parasite species	Infected birds	Prevalence	Parasite species
Pelecanidae		14						
Pelecanus onocrotalus (Bruch)	Lm	14						
Ardeidae		10						
Ixobrychus minutus (L.)	Lm	10						
Anatidae		1						
Anas platyrhynchos (L.)	Cm	1						
Accipitridae		1						
Accipiter nisus (L.)	Cm	1						
Falconidae		1						
Falco subbuteo (L.)	Lm	1						
Charadriidae		1						
Charadrius dubius (Scop.)	Lm	1						
Scolopacidae		28						
Tringa nebularia (Gunn.)	Lm	1						
Tringa glareola (L.)	Lm	1						
Philomachus pugnax (L.)	Cm	1						
Limicola falcinellus (Pontop.)	Lm	1						
Calidris minuta (Leisler)	Lm	12						
Calidris alpina (L.)	Cm	2						
Calidris ferruginea (Pontop.)	Lm	6						
Arenaria interpres (L.)	Lm	3						
Phalaropus lobatus (L.)	Lm	1						
Laridae		3						
Larus ridibundus (L.)	Cm	2						
Sterna hirundo (L.)	Lm	1						
Columbidae		4						
Streptopelia turtur (L.)	Lm	1						
Columba livia(dom.)(Gm.)	Nm	3						
Cuculidae		5						
Cuculus canorus (L.)	Lm	5						
Strigidae		3				1		
Asio otus (L.)	Nm	1				1		*L. danilewskyi*
Otus scops (L.)	Lm	1						
Athene noctua (Scop.)	Nm	1						
Caprimulgidae		2						
Caprimulgus europaeus (L.)	Lm	2						
Alcedinidae		24						
Alcedo atthis (L.)	Nm	24						
Coraciidae		2				2		
Coracias garrulus (L.)	Lm	2				2		*L. eurystomi*, *L. benneti*
Picidae		12						
Dendrocopus major (L.)	Nm	3						
D. syriacus (Hemp. et Eher.)	Nm	3						
D. medius (L.)	Nm	2						
Picus canus (Gm.)	Nm	2						
P. viridis (L.)	Nm	1						
Jynx torquilla (L.)	Lm	1						
Hirundinidae		81	2	2.5				
Hirundo rustica (L.)	Lm	58	2	3.5	*P. polare*, *P. (Haemamoeba)* sp.			
Riparia riparia (L.)	Lm	18						

Table 9. (contd)

Bird families/species	Migration status	Examined birds	*Plasmodium*			*Leucocytozoon*		
			Infected birds	Prevalence	Parasite species	Infected birds	Prevalence	Parasite species
Delichon urbica (L.)	Lm	1						
Ptyonoprogne rupestis (Scop.)	Cm	4						
Motacillidae		31				2	6.5	
Anthus trivialis (L.)	Lm	25				2	8.0	*L. fringillinarum*
Motacilla flava (L.)	Lm	6						
Prunellidae		5						
Prunella modularis (L.)	Cm	5						
Turdidae		98	11	11.2		5	5.1	
Turdus merula (L.)	Nm	16	2	12.5	*P. vaughani*, *P.* (*Haemamoeba*) sp.	4	25.0	*L. fringillinarum*, *L. dubreuili*, *L. majoris*
T. philomelos (Brehm)	Cm	9	3		*P.* (*Haemamoeba*) sp. *Plasmodium* sp.	1		*L. dubreuili*
T. iliacus (L.)	Cm	1						
Phoenicurus phoenicurus (L.)	Lm	14						
Erithacus rubecula (L.)	Cm	16	1	6.3	*P.* (*Haemamoeba*) sp.			
Saxicola rubetra (L.)	Lm	4						
Luscinia luscinia (L.)	Lm	9	1		*P.* (*Haemamoeba*) sp.			
L. megarhynchos (Brehm)	Lm	29	3	10.3	*P.* (*Haemamoeba*) sp. *P.* (*Novyella*) sp.			
Sylviidae		648	27	4.2				
Sylvia nisoria (Bech.)	Lm	5						
S. borin (Bodd.)	Lm	7						
S. atricapilla (L.)	Lm	53	2	3.8	*P. vaughani* *P.* (*Haemamoeba*) sp.	1	1.9	*L. fringillinarum*
S. communis (Latham)	Lm	15	2	13.3	*P.* (*Haemamoeba*) sp.			
S. curruca (L.)	Lm	20	1	5.0	*Plasmodium* sp.			
Acrocephalus arundinaceus (L.)	Lm	162	8	4.9	*P.* (*Haemamoeba*) sp. *P.* (*Novyella*) sp. *Plasmodium* sp.			
A. palustris (Bech.)	Lm	52						
A. scirpaceus (Herm.)	Lm	65						
A. schoenobaenus (L.)	Lm	173	14	8.1	*P. vaughani*, *P.* (*Haemamoeba*) sp. *P*. (*Giovanolaia*) sp.	2	1.2	*L. majoris*
A. agricola (Jerdon)	Lm	5						
Hippolais icterina (Vieil.)	Lm	5						
Phylloscopus trochilus (L.)	Lm	36				3	8.3	*L. fringillinarum*
P. sibilatrix (Bech.)	Lm	11						
P. collybita (Vieil.)	Cm	5						
Locustella fluviatilis (Wolf)	Lm	9						
L. luscinioides (Savi)	Lm	25						
Muscicapidae		40	2	5.0				
Ficedula parva (Bech.)	Lm	6						
F. semitorquata (Hom.)	Lm	1						
F. albicollis (Temm.)	Lm	3						
F. hypoleuca (Pall.)	Lm	5						
Muscicapa striata (Pall.)	Lm	25	2	8.0	*P.* (*Haemamoeba*) sp.			
Aegithalidae		2						
Aegithalos caudatus (L.)	Nm	2						
Remizidae		7						
Remiz pendulinus (L.)	Cm	7						
Paradoxornithidae		8	1					
Panurus biarmicus (L.)	Cm	8	1		*P. relictum*			

Table 9. (contd)

Bird families/species	Migration status	Examined birds	*Plasmodium*			*Leucocytozoon*		
			Infected birds	Prevalence	Parasite species	Infected birds	Prevalence	Parasite species
Paridae		68	7	10.3				
Parus major (L.)	Nm	47	7	14.9	*P. relictum*, *P.* (*Haemamoeba*) sp. *P.* (*Giovanolaia*) sp.			
P. caeruleus (L.)	Nm	17						
P. palustris (L.)	Nm	4						
Sittidae		5						
Sitta europaea (L.)	Nm	5						
Certhiidae		6						
Certhia brachydactyla (Brehm)	Nm	1						
C. familiaris (L.)	Nm	5						
Troglodytidae		8						
Troglodytes troglodytes (L.)	Nm	8						
Laniidae		42	4	9.5				
Lanius collurio (L.)	Lm	39	3	7.7	*P. relictum*, *P.* (*Haemamoeba*) sp.			
L. minor (Gm.)	Lm	3	1		*Plasmodium* sp.			
Corvidae		3	1					
Pica pica (L.)	Nm	2						
Garrulus glandarius (L.)	Nm	1	1		*P.* (*Giovanolaia*) sp.			
Oriolidae		3	1			1		
Oroilus oriolus (L.)	Lm	3	1		*P.* (*Novyella*) sp.	1		*L. majoris*
Sturnidae		3						
Sturnus vulgaris (L.)	Cm	3						
Passeridae		71	13	18.3				
Passer domesticus (L.)	Nm	29	9	31.0	*P.* (*Haemamoeba*) sp. *Plasmodium* sp.			
P. hispaniolensis (Temm.)	Lm	22	2	9.1	*P.* (*Haemamoeba*) sp.			
P. montanus (L.)	Nm	20	2	10.0	*P. relictum*			
Fringillidae		65	12	18.5				
Coccotrhaustes coccothraustes (L.)	Nm	2						
Fringilla coelebs (L.)	Cm	14	3	21.4	*P.* (*Haemamoeba*) sp.			
Carduelis chloris (L.)	Nm	30	7	23.3	*P.* (*Haemamoeba*) sp.			
C. carduelis (L.)	Cm	19	1	5.3	*P.* (*Haemamoeba*) sp.			
Emberizidae		22	1	4.6				
Emberiza hortulana (L.)	Lm	1						
E. schoeniclus (L.)	Cm	17						
E. citrinella (L.)	Nm	1						
Miliaria calandra (L.)	Cm	3	1		*P.* (*Haemamoeba*) sp.			
Total		1332	82	6.2		17	1.3	

Plasmodium infections were most common in local non-migratory birds (Fig. 1). The total prevalence for these birds was 13.2% (n=287), which is higher than the rate established for the local migratory birds: 3.8% (n=213) (F-test, $p<0.001$). There was no significant difference in the prevalence between fall migrants: 4.7% (n=529) and spring migrants: 4.2% (n=241) (F-test, $p>0.05$). The prevalence in local migratory birds and transitory migrants (both fall and spring) did not differ significantly. The prevalence for the close migrants was 5.8% (n=65) and for the long-distance migrants - 4.1% (n=975). The difference was not significant (F-test, $p>0.05$). The migration status of each bird species is shown in Table 9.

Plasmodium parasites of the subgenus *Haemamoeba* were most commonly found (61.9% of all infections). Other subgenera were rarely found: *Novyella* (9.5%) and *Giovannolaia* (4.8%). About 23.8% of the *Plasmodium* infections were not determined to subgenus level.

The seasonal distribution of the *Plasmodium* infections showed small monthly variations (Fig. 2). Nevertheless a trend of increasing the rates from March to September could be marked. The highest prevalence was found in September (7.7%).

The comparison between the average prevalence for spring and summer (from March until the end of July) and the autumn prevalence (August-October) (Fig. 3) showed that in all studied families, except Laniidae, the autumn rates were higher. Differences were not significant (F-test, $p>0.05$), except for the family Paridae, where a significant difference was found (F-test, $p<0.05$).

Plasmodium infection rates did not differ significantly between males (10.2%, n=124) and females (11.2%, n=130) (F-test, $p>0.05$).

Adults and young birds (<1- year old) in summer and autumn periods (June - October) showed almost the same prevalence: 7.8% (n=293) for the adults and 7.4% (n=402) for the young birds (F-test, $p>0.05$). In this comparison the birds with difficult age determination are excluded.

The infection intensity of *Plasmodium* was usually low or very low (less than 1 parasite in 100 microscope fields at magnification 2000x). Part of the infections of the lowest intensity was probably missed. So the total *Plasmodium* prevalence given, 6.2%, is lower than the real one. Intensive infections with more than 10 parasites in 100 fields were found in 5 birds (4 of them in spring and summer).

Four cases (in 4 bird species) of mixed infections with 2 subgenera of *Plasmodium* were found: in the blood of *Parus major, Haemamoeba* and *Giovannolaia*; in *Fringilla coelebs, Haemamoeba* and *Giovannolaia* or *Novyella*; in *Acrocephalus schoenobaenus, Haemamoeba* and *Giovannolaia* or *Novyella*; and in *Turdus merula - Haemamoeba* and *Novyella*. In the last case *Leucocytozoon dubreuili* was also found. Similar cases have been reported frequently by other authors (Valkiunas 1997).

Mixed invasions of *Plasmodium* and other haemosporidians were also found: 9 cases of *Plasmodium/Haemoproteus* (one or 2 species) type and one case of *Plasmodium/Leucocytozoon* type. One adult *Oriolus oriolus* infected with 4 parasites was caught: *Plasmodium* (*Novyella*) sp., *Haemoproteus orioli, Leucocytozoon majoris* and *Trypanosoma* sp. (29.05.1999, Nova Cherna). A similar case was found in *Fringilla coelebs* infected with *Plasmodium* (*Haemamoeba*) sp., *Haemoproteus fringillae, H. dolniki* and *Trypanosoma* sp. (14.06.2000, Vrana campsite). Three cases of mixed infections of *Plasmodium/ Hepatozoon* type were also found (2 of them in *Parus major* and 1 in *Acrocephalus schoenobaenus*). A total number of 18 (22%) mixed infections of *Plasmodium* (n=82) were diagnosed.

DISCUSSION

The number of *Plasmodium* and *Leucocytozoon* species known in Bulgaria (4 and 6 respectively) is considerably lower than expected until now. For *Plasmodium* this is due to the difficulties in the species identification, especially in the cases with low rates of intensity of infection. At Curonian Spit in the Baltic Sea 11 species of *Plasmodium* and 15 of *Leucocytozoon* were described (Valkiunas 1997).

The total prevalence of *Leucocytozoon* in Bulgaria (1.3%) is lower than in Northern and Central Europe. At Curonian Spit the prevalence was 12.2% (Valkiunas 1985); in Poland, 12.4 - 13.0% (Dymowska and Żukowski 1965, 1968; Ramisz 1965); in Central Europe, 6% (Kučera 1981a). In Southeastern Kazakhstan (a region on a similar geographic latitude as Bulgaria), a total prevalence of 2.2% was found (1.13% only for the passerines) (Jakunin 1972). These data are similar to ours.

In contrast to the *Leucocytozoon* the *Plasmodium* infection rates were on average higher in Bulgaria than in most of the other regions in Eurasia (Manwell 1956, Jakunin 1972, Pierce and Mead 1976, Valkiunas 1985). In Central Europe Kučera (1981b) found a higher total prevalence of *Plasmodium* in bird groups that were not included in our investigation (orders Galliformes and Columbiformes). Higher infection rates than these in Bulgaria were found in Africa: 13.6% in Egypt (Helmy Mohammed 1958) and 13.0% in Morocco (Gaud and Petitot 1945).

A comparison between *Plasmodium/Leucocytozoon* prevalence in birds of different migratory status was made by many authors (Ramisz 1965; Kučera 1981a, b; Valkiunas 1987, 1997). Likewise, in the present study, a high total prevalence of *Plasmodium* infections for the local non-migratory birds was also found in Poland

where up to 81% of *Plasmodium* infected birds were locally breeding non-migratory birds (Ramisz 1965). Kučera (1981b) also found in Central Europe a higher prevalence of *Plasmodium* in the non-migratory birds compared with the long distance migrants. It is our opinion, that the main reason for the lower *Plasmodium* prevalence found for the long-distance migrants in the present study is the high number of non-infected autumn migrants (mainly young birds) in our sample. These birds belong to the populations from Northern parts of Europe and Asia, where there is no local transmission of malarial parasites (Valkiunas 1997).

Ramisz (1965), Jakunin (1972) and Kučera (1981b) mentioned that the levels of parasitemia for *Plasmodium* were higher in spring and summer than in autumn. Not only the high number of the vectors could cause this spring peak in this period but also by spring relapses of parasitemia (Valkiunas 1997, Krone *et al.* 2001). According to Valkiunas (1997) the best weather conditions for the transmission of the malarial parasites in Holarctic could be reached only in the end of the summer period. Probably this could explain the higher *Plasmodium* prevalence found by us for family Paridae, including mostly locally breeding, resident birds. The higher *Plasmodium* prevalence in September, compared with August, found in the present study, could be connected with more favourable (more humid) weather conditions for the vectors (dipterans of family Culicidae) during September. In Bulgaria August is usually very dry month, which is not the case in September.

In some publications (Kučera 1981b, Valkiunas 1987, Hatchwell *et al.* 2000), it was shown that adult birds are more often infected with *Plasmodium* than are young birds (<1-year old). But in other studies just the opposite data were gathered (Bennet and Fallis 1960, Jakunin 1972). Our data showed almost equal rates of parasitemia for adults and young birds. This proportion probably varies in different groups of birds and in different local populations.

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ACTA
PROTOZOOLOGICA

Redescription of a Poorly-known Marine Cyrtophorid Ciliate, *Dysteria pusilla* (Claparède et Lachmann, 1859) (Protozoa: Ciliophora: Cyrtophorida) from Qingdao, China

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Summary. The living morphology, infraciliature and silverline system of a poorly known marine cyrtophorous ciliate *Dysteria pusilla* (Claparède et Lachmann, 1859), isolated from a fish-culturing tank off Qingdao, China, have been investigated. An improved diagnosis for *Dysteria pusilla* is supplied: small marine *Dysteria*, body rectangular in outline when viewed from side, 15-30 x 10-20 µm *in vivo*; with one right ventral and 2 frontoventral kineties in right field; 7 short fragments of kineties in left equatorial field; oral structure simplified; three contractile vacuoles, two ventrally located, and one positioned dorso-frontally; macronucleus sausage-like.

Key words: Cyrtophorida, *Dysteria pusilla*, marine ciliate, morphology and infraciliature.

INTRODUCTION

The species-rich cyrtophorid ciliates *Dysteria* have been found worldwide in marine and freshwater benthic biotopes and frequently described (Dujardin 1841; Huxley 1857; Claparède and Lachmann 1859; Kent 1882; Möbius 1888; Calkins 1902; Schouteden 1906; Hamburger and von Buddenbrock 1911; Lepsi 1927; Kahl 1931, 1935; Wang and Nie 1932; Wang 1934; Wailes 1943; Tucolesco 1962; Biernacka 1963; Dragesco 1966; Jankowski 1967; Wilbert 1971; Borror 1972; Agamaliev 1974, 1983; Aladro-Lubel *et al*. 1990; Carey 1992; AL-Rasheid 1997). These organisms are bilaterally compressed and crawling over the substrate on their ventral side, mostly feed on bacteria, diatoms and small flagellates. Although over 30 nominal species have been reported over the last century, only within last few decades have silver impregnation techniques been employed for their study (Fauré-Fremiet 1965; Deroux 1965, 1976; Dragesco and Dragesco-Kernéis 1986; Gong *et al*. 2002). These studies have demonstrated that the ventral ciliary structure is highly species-specific and is therefore a reliable character for species separation, whereas body size, shape and other morphological characters relating to the organism *in vivo* are either variable or observer-dependent rendering identification difficult.

Recently, during an ecological as well as taxonomical survey on ciliates in biofilm (microbiotecton) of mariculture environments, we isolated a poorly-described form,

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Dysteria pusilla, of which the infraciliature and statistical data remained unknown and are supplied here.

MATERIALS AND METHODS

Dysteria pusilla (Claparède et Lachmann, 1859) was collected from the water tanks for culturing abalone (*Haliotis discus*) in the Research Laboratory of Mariculture, Ocean University of Qingdao (Tsingtao), China (36°08'N, 120°43'E). Ten glass slides, fixed in a slide frame as artificial substrate, were immersed in the water till biofilm was formed. After being exposed for about 15 days, the slides were carefully taken out and transferred to Petri dishes with marine water from the sampling site. Isolated specimens were maintained in the laboratory for about 1 week as raw cultures (water temperature 17 °C, salinity *ca* 30‰) in Petri dishes for observation and further studies.

Living cells were observed by differential interference microscopy. The infraciliature was revealed using the protargol impregnation method according to Wilbert (1975). The Chatton-Lwoff silver nitrate method was used to demonstrate the silverline system (Song and Wilbert 1995). Living individuals were examined and measured at 1000x magnification; drawings of stained specimens were performed at 1250x with the aid of a camera lucida. Terminology is mainly according to Corliss (1979) and Petz *et al.* (1995).

A few terms concerning the infraciliature of *Dysteria* are here defined.

Frontoventral kineties (FvK): somatic kineties, that extend over almost the entire length of the body; the anterior ends extend beyond the level of the cytostome and almost reach the apical groove; positioned between RvK and Re.

Right ventral kineties (RvK): ventral kineties of variable length, the anterior ends of which terminate at or below the level of cytostome; positioned between LK and FvK.

Left equatorial kineties (LK): short, densely packed ventral kineties, positioned equatorially, to the left of the RvK.

Right equatorial kinety (Re): the rightmost ventral kinety, positioned equatorially and to the right of the FvK; length depends on individuals, but usually short.

Fragment of external kinety (Fe): short kinety formed by several basal bodies, antero-dorsally positioned at the end of the FvK.

RESULTS

Morphology and infraciliature of *Dysteria pusilla* (Claparède et Lachmann, 1859) (Figs 1-5, 7-18; Tables 1-3)

Improved diagnosis of *Dysteria pusilla*: small marine *Dysteria*, body rectangular in outline when viewed from side, 15-30 x 10-20 µm *in vivo*; with one right ventral and 2 frontoventral kineties in right field; 7 short fragments of kineties in left equatorial field; oral structure simplified; three contractile vacuoles, two ventrally located, and one positioned dorso-frontally; macronucleus sausage-like.

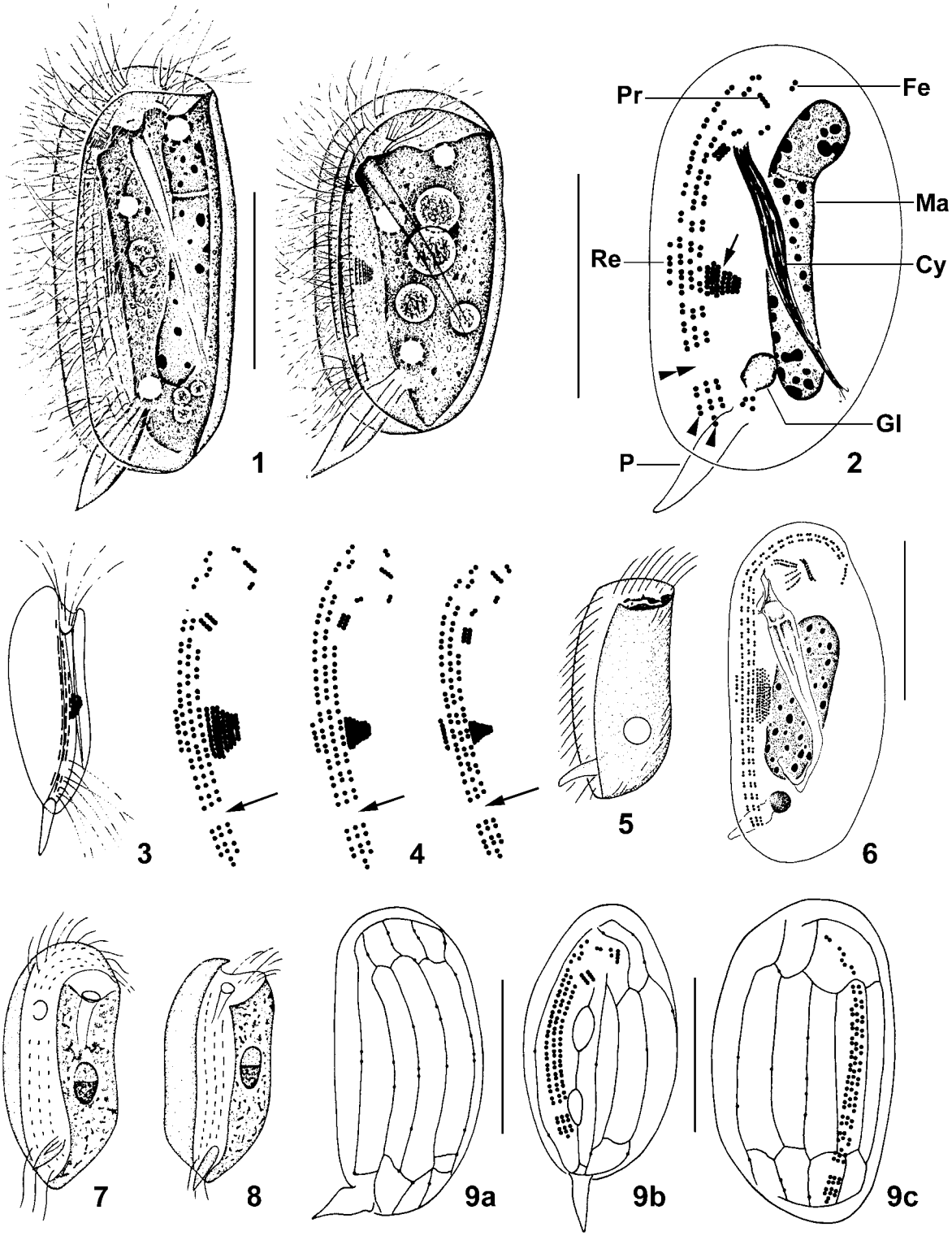
Neotype specimens: one neotype slide of protargol impregnated specimens is deposited in the Natural History Museum, UK with registration number 2003.3.26.1. Two paraneotypes are deposited in the Laboratory of Protozoology, Ocean University of China, P. R. China (number LF2001-12-02).

Description: size 15-30 x 10-20 µm *in vivo*, slender form length: width about 2:1, while in plump specimens only 3:2 (Figs 1, 10). Body shape rectangular in outline when viewed from side: ventrally straight, dorsal side slightly convex. Both anterior and posterior margin blunt rounded (Figs 1, 10). Cells bilaterally flattened (*ca* 2:3); right plate more arched and slightly larger than left (about 24 x 14 *vs.* 21 x 11 µm) (Figs 1, 3). No conspicuous groves or ridges on lateral sides. Cilia about 8 µm long *in vivo*. As in its congeners, ciliary rows mostly restricted to the ventral groove between two plates (Fig. 3). Podite (P) about 8 µm in length, distal end pointed, emerges subcaudally from left posterior ventral side. Cytoplasm colourless, usually containing several small food vacuoles, 2-4 µm in diameter. Cytostome in anterior 1/5-1/6 of cell length and ventrally located. Cytopharynx typical, diagonally oriented (Figs 2, 12, 13, 15).

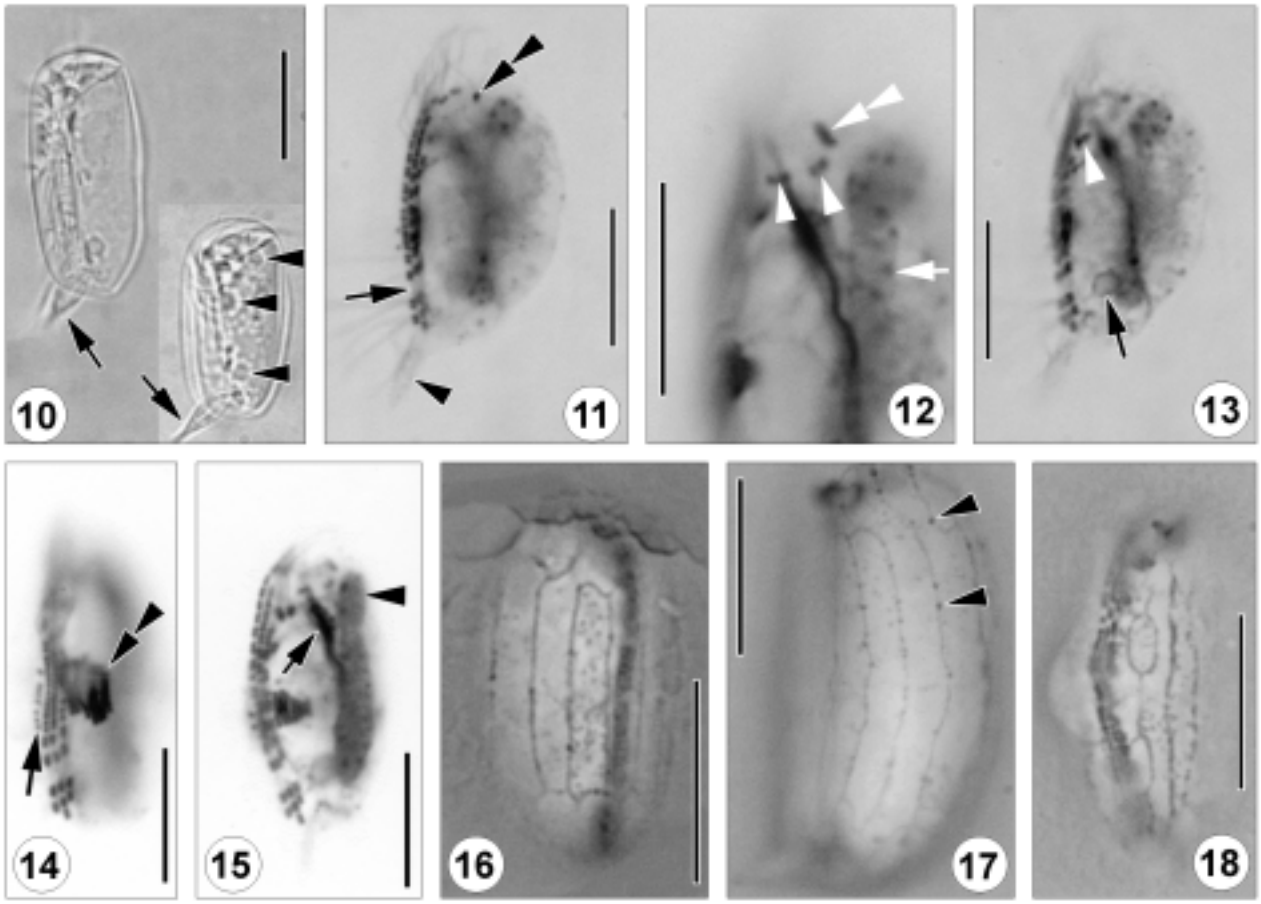
Three contractile vacuoles, 2-4 µm in diameter, of which one is located antero-dorsally in anterior 1/5-1/6 of cell length, and the other two are ventrally positioned respectively (Figs 1, 10).

Movement typical by crawling over substrate or (occasionally) swimming very slowly in water for a short while.

Infraciliature as shown in Figs 2, 4, 11. Right field occupied by 0-1 (see Table 1) short right equatorial kinety (Re) comprising about 5 basal bodies and three long ventral kineties (two frontoventral and one right ventral kinety). All 3 ventral kineties composed of loosely arranged kinetosomes, which are, unlike in most other congeners, basically not fragmented except in the posterior part, where there is a conspicuous gap present (about 1.5 µm wide; double-arrowheads in Fig. 2; arrows in Fig. 4). Two frontoventral kineties (see Fig. 2, arrows) equal in length, each row consisting of 30-40 basal bodies (Table 1). One right ventral kinety (RvK) terminating subapically posterior to the level of cytostome, with about 20 basal bodies. The fragment of external kinety consisting of only one pair of basal bodies (Fig. 2,



Figs 1-9. *Dysteria pusilla* (1-4, 9, original; 5, from Claparède and Lachmann 1859; 7, 8 from Kahl 1935. 1, 3, 5, 7, 8, from life; 2, 4, protargol impregnation; 9, silver nitrate impregnation) and *D. cristata* (6, from Gong *et al.* 2002, protargol impregnation). **1 -** left side views of two slender and wider individuals; **2 -** left view of infraciliature, arrow indicates left equatorial kineties, arrowheads mark the posterior ends of two frontoventral kineties, double-arrowheads indicate the posterior gap of ventral kineties; **3** - ventral view; **4** - showing arrangement of the oral and somatic kineties from three individuals, arrows point to posterior gaps; note that that most oral structure is highly reduced into pairs of kinetosomes; **5 -** left side view; **6** - left side view of infraciliature; **7, 8** - left side (**7**) and ventral (**8**) view; **9a-c** - left (**a**) and right side (**c**) as well as left-ventral view (**b**) of silverline system. Cy - cytopharynx, Fe - fragment of external kinety, Gl - glandule, Ma - macronucleus, P - podite; Pr - preoral kinety; Re - right equatorial kinety. Scale bars 12 µm (1, 2, 9); 20 µm (6).



Figs 10-18. Photomicrographs of *Dysteria pusilla* from life (10), after protargol (11-15) and silver nitrate impregnation (16-18). **10 -** left view of two typical individuals; arrows indicate the podite, arrowheads mark the three contractile vacuoles; **11** - left view of infraciliature, arrow indicate the posterior gap in ventral kineties, arrowhead points to the podite, double-arrowheads mark the fragment of external kinety consisting of two kinetosomes; **12** - anterior portion, note the two pairs of kinetosomes (arrowheads) and the preoral kinety (double-arrowheads), arrow indicates the sausage-like macronucleus; **13** - left view, showing the argentophilic glandule (arrow) and preoral kinety (arrowhead); **14** - focusing on kinety level, to demonstrate the right equatorial kinety (arrow) and the densely-arranged left equatorial kineties (double-arrowheads); **15** - general view, note the cytopharynx (arrow) and the macronucleus (arrowhead); **16-18** - silverline system from right (**16)** and left side view **(17**), and left-ventral view (**18)**, arrowheads indicate the tiny argentophilic granules in silverlines. Scale bars 10 µm.

Table 1. Morphometric characteristics of *Dysteria pusilla* (from protargol impregnated specimens). Abbreviations: Max - maximum, Mean - arithmetic mean, Min - minimum, n - number of individuals examined, SD - standard deviation.

Characters	Min	Max	Mean	SD	n
Body length (µm)	17	24	21.3	2.0	36
Body width (µm)	8	16	12.1	2.1	36
Frontoventral kineties, number	2	2	2	0	36
Right ventral kineties, number	1	1	1	0	36
Left equatorial kineties, number	7	7	7	0	36
Basal bodies in one frontoventral kinety, number	30	41	32.8	7.7	15
Basal bodies in fragment of external kinety, number	2	2	2	0	16
Basal bodies in right equatorial kinety, number	0	7	3.8	2.1	16
Macronucleus, length (µm)	10	19	15.8	2.1	36
Macronucleus, width (µm)	2.5	4	2.8	0.4	36
Glandule, diameter (µm)	1	2.4	1.9	0.4	32

Table 2. Comparison of *Dysteria pusilla* with three closely-related marine *Dysteria* species.

Characters	*D. pusilla* (Claparède et Lachmann, 1859)	*D. cristata* (Gourret et Roeser, 1888) Kahl, 1931	*D. calkinsi* (Calkins, 1902) Kahl, 1931	*D. ovalis* (Gourret et Roeser, 1886) Kahl, 1931
Body size *in vivo* (μm)	15-30 x 10-20	40-50 x 25 -30	30-50 x 20-25	-
Body shape from side view	rectangular	wide oval	rectangular	oval
Contractile vacuoles, number	3	2	-	1*
Macronucleus, size (μm)	16 x 3	13 x 6	25 x 15	-
Right ventral kineties, number	1	1	3	2
Left equatorial kineties, number	7	5-7	*ca* 6	-
Frontoventral kineties, number	2	2	2	2
Basal bodies in one frontoventral kinety, number	30-41	72-90	-	-
Caudal spine	absent	absent	absent	absent
Argentophilic glandule, diameter (μm)	1- 2.4	2.5 -3	-	-
Podite, length (μm)	*ca* 8	*ca* 8	*ca* 15	-
Data source	Original	Gong *et al.* (2002)	Song and Wilbert (2002)	Fauré-Fremiet (1965)

- Data not available; * Data from Kahl (1931)

Table 3. Comparisons between the seven species of *Dysteria* for which the infraciliature is known.

Species	Body size *in vivo* (μm)	Right ventral kineties, No.	Left equatorial kineties, No.	Frontoventral kineties, No.	Data source
D. pusilla (Claparède et Lachmann, 1859)	15-30 x 10-20	1	7	2	Original
D. cristata (Gourret et Roeser, 1888) Kahl, 1931	40-50 x 25-30	1	5-7	2	Gong *et al.* (2002)
D. armata Huxley, 1857	-	2	-	4	Fauré-Fremiet (1965)
D. monostyla (Ehrenberg, 1838) Kahl, 1931	80-110 x 30-40	3	6-9	2	Gong *et al.* (2002)
D. brasiliensis Faria, Cunha *et* Pinto, 1922	100-130 x 30-34	3	7-8	2	Song and Packroff (1997)
D. ovalis (Gourret et Roeser, 1886) Kahl, 1931	-	2	-	2	Fauré-Fremiet (1965)
D. calkinsi (Calkins, 1902) Kahl, 1931	30-50 x 20-25	3	*ca* 6	2	Song and Wilbert (2002)
D. antarctica Gong *et al.* 2002	145 x 65	2	*ca* 6	3	Petz *et al.* (1995)

- Data not available.

Fe). Seven left kineties as short rows of densely packed kinetosomes in mid-body area (Fig. 2, arrow; Fig. 12, double-arrowhead). Macronucleus long and sausage-like, about 16 x 3 μm after protargol impregnation longitudinally positioned in mid-body, characteristically heteromerous; micronucleus not observed. One argentophilic glandule (Fig. 2, Gl; Fig. 13, arrow) subcaudally positioned, about 2 μm in diameter, often together with 3-4 unciliated basal bodies, always present near the base of podite.

Oral kineties simplified and difficult to define, but basically consisting of one short single-rowed and one double-rowed fragments, as well as 2 pairs of basal bodies (seldom one).

Silverline system on both left and right plate generally similar, composed of *ca* 10 longitudinal silverlines. Two traverse silverlines positioned subapically and subcaudally, respectively; silverline pattern of ventral field comprising two loops connected by a single line (Figs 9b, 18). Tiny argentophilic granules sparsely distributed on silverlines (Figs 9a-c, 16-18).

DISCUSSION

Comparison with related species

Dysteria pusilla was originally described by Claparède and Lachmann (1859) under the name *Aegyria pusilla*, which was very briefly characterized by "very small and without ribs", no further information was given about the size, the number of contractile vacuoles and the shape of macronucleus in both original and the subsequent redescription (Claparède and Lachmann 1859, Kent 1882), Hamburger and von Buddenbrock (1911) reported a population isolated from the North Sea, Germany, with body size *ca* 32 x 24 μm. Later, this organism was simply mentioned again by Kahl (1931), and was illustrated possessing an elliptical macronucleus with body size of 25 μm in his subsequent document (Kahl 1935). Since then, no redescriptions have been made. In the absence of data concerning the infraciliature, we identified our organism mainly on the basis of its size, body shape, and the habitat. The only difference is the shape of macronucleus: according to Kahl's (1935) redescription, *D. pusilla* has an oval shaped macronucleus, while, in Qingdao population, it is consistently sausage-like. Since the shape of macronucleus, as to the authors' knowledge, is - in most cases - a population-dependent feature, hence, we consider this difference as an intra-species variation.

As much as we know, 7 species have been described using modern methods (Table 3): *D. armata*, *D. monostyla*, *D. brasiliensis*, *D. antarctica*, *D. calkinsi*, *D. ovalis* and *D. cristata* (Deroux 1965, Fauré-Fremiet 1965, Dragesco and Dragesco-Kernéis 1986, Petz *et al.* 1995, Song and Packroff 1997, Gong *et al.* 2002, Song and Wilbert 2002). The former four species have considerable larger cell size (over 60 μm), and more ventral kineties (4 or more *vs.* 3), and thus can be clearly separated from *D. pusilla*.

Dysteria pusilla differs from *D. calkinsi* (Calkins, 1902) Kahl, 1931 in terms of: (1) the general appearance *in vivo* (rectangular *vs.* elongate with 2 conspicuous, longitudinally positioned ridges); (2) the infraciliature (3 *vs.* 5 ventral kineties) and (3) macronuclear size (16 x 3 *vs.* 25 x 15 μm) (Song and Wilbert 2002).

Although the cell size of *D. ovalis* (Gourret et Roeser, 1886) Kahl, 1931 remains unknown, it can be separated from *D. pusilla* by the body shape *in vivo* (oval *vs.* rectangular in *D. pusilla*) and the infraciliature (consistently 4 *vs.* 3 ventral kineties) (Fauré-Fremiet 1965).

Dysteria cristata (Gourret et Roeser, 1888) is perhaps the most similar organism to the Qingdao population of *D. pusilla*, i.e. both species have 3 ventral kineties (Figs 2, 6; Tables 2, 3). However, *D. pusilla* is identified by: (1) ventral kinety sparsely ciliated (30-40 basal bodies in each row *vs.* 72-90 in *D. cristata*); (2) the ventral kineties (FvK and RvK) in *D. pusilla* consistently exhibit a cilia-free gap at the posterior portion (*vs.* continuous in *D. cristata*); (3) highly simplified oral infraciliature in the former (see Figs 2, 6), and (4) the number of contractile vacuoles (3 *vs.* 2) (Gong *et al.* 2002).

Considering the small size, living body shape and habitat, at least two other incompletely described species (infraciliature unknown), *D. compressa* (Gourret et Roeser, 1888) and *D. navicula* Kahl, 1928, should be compared as well with *D. pusilla*.

Dysteria compressa differs from *D. pusilla* in the caudal spine (present *vs.* absent) and possibly also in the number of contractile vacuoles (2 *vs.* 3) (Table 2).

Dysteria navicula can be distinguished from *D. pusilla* by its larger body size (*in vivo* 35- 45 x 14-18 *vs.* 15-30 x 10-20 μm), the body shape (slender and spindle-shaped *vs.* rectangular) and the number of contractile vacuoles (2 *vs.* 3) (Table 2) (Kahl 1931).

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Pseudotrypanosoma elphinstonae sp. n., a Trichomonad Symbiotic in *Schedorhinotermes* (Isoptera: Rhinotermitidae)

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Summary. A new species of *Pseudotrypanosoma*, *P. elphinstonae* sp. n., is described which is symbiotic within the hindguts of the rhinotermitid termites *Schedorhinotermes secundus* and *S. intermedius*. *P. elphinstonae* possesses most of the features of the genus: 4 anterior flagella, prominent costa and recurrent flagellum forming an undulating membrane and simple bean-shaped parabasal body. The mastigont complex is of similar composition and arrangement to other trichomonads but the pelto-axostylar complex is greatly simplified being composed of a single layer of microtubules which do not over lap and cannot be distinguished into separate structures. The undulating membrane is much smaller than in related species; the costa is smaller and simpler and there are no microtubular bundles connecting it to the recurrent flagellum. Comparison of the ultrastructure of *P. elphinstonae* sp. n. with that of *P. giganteum* demonstrated that *P. elphinstonae* sp. n. in addition to being much smaller in size had a correspondingly simper ultrastructural organisation lacking several organelles which characterise the latter species.

Key words: Isoptera, Parabasalida, *Pseudotrypanosoma elphinstonae* sp. n., Rhinotermitidae, *Schedorhinotermes*, Trichomonadida, ultrastructure.

INTRODUCTION

Five genera of trichomonads have been reported to possess 4 anterior flagella plus 1 recurrent flagellum forming part of the undulating membrane (4+R arrangement): *Pseudotrypanosoma* Grassi, 1917, *Trichomitopsis* Kofoid et Swezy, 1919, *Pentatrichomonas* Mesnil, 1914 (4+R+I, I being an independent anterior flagellum), *Tetratrichomonas* Parisi, 1910, and *Trichomonas* Donné, 1836. Additionally 1 species of *Tritrichomonas* Kofoid, 1920 has the 4+R arrangement rather than the usual arrangement of 3+R for this genus. These genera are considered to be phylogenetically unrelated (Gerbod *et al.* 2001), representing multiple independent additions of another flagellum to the basic or privileged basal bodies (3+R) (Brugerolle 1991). Of these, *Pseudotrypanosoma* has one of the narrowest distributions being confined to Australian termites of the genus *Porotermes*.

Pseudotrypanosoma was initially described by Grassi (1917), who assigned one species to the genus: *P. giganteum* which was endosymbiotic in the Austra-

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lian termite, *Porotermes adamsoni*. Descriptions of both the genus and type species was subsequently emended by Kirby (1931), who also made mention of "smaller, more typical trichomonads" in a second termite host, *Po. grandis*, but did not formally describe these forms. *P. giganteum* was described a third time by Sutherland (1933) who also examined the "small trichomonads" from *Po. grandis* and assigned them to a second *Pseudotrypanosoma* species: *P. minimum*. Cleveland (1961) re-examined *P. giganteum* from *Po. adamsoni* describing the cell division cycle of this species in great detail. Cleveland (1961) was of the opinion that *Pseudotrypanosoma* was synonymous with *Trichomonas* as both genera exhibited similar morphology despite the great disparity in size between *P. giganteum* and most species of *Trichomonas*. Despite this, Cleveland (1961) did not make a clear taxonomic statement synonymising *Pseudotrypanosoma* with *Trichomonas* and it has been universally ignored by subsequent treatments of the trichomonads (e.g. Honigberg 1963, Yamin 1979). This has been partly vindicated by recent molecular phylogenetic studies which have suggested that *Pseudotrypanosoma* is distinct from *Trichomonas* and if they were to be synonymised, then *Pentatrichomonoides* and *Trichomitus* must also be synonymised with *Trichomonas* (Keeling *et al*. 1998). Recently, the ultrastructural characterisation of *Pseudotrypanosoma giganteum* has reinforced its distinctiveness from *Trichomonas* particularly in the structure of the costa (Brugerolle 1999). Here we report a third species in the genus *Pseudotrypanosoma* which is the first to be described from a termite other than *Porotermes*.

MATERIALS AND METHODS

Schedorhinotermes intermedius and *S. secundus* colonies were collected by hand from under fallen timber, within dead fallen branches and from within tubular galleries within the bark of living trees. Individual termites representing the worker, major soldier and minor soldier castes were collected from each colony. Voucher collections were made of each colony by preserving 5 of each caste in 70% ethanol and they were used to identify the termite species collected. Nest material was collected along with termites and each colony was provided with tissue paper soaked in water as a moisture and food source to maintain the colony in the laboratory. Workers were examined shortly after collection by dissecting the hindgut into a small drop of Locke's fluid. Some workers were removed directly from the nest and examined but they generally yielded "dirty" preparations with abundant coarse wood fibres and siliceous particles. "Clean" preparations were generated by isolating individual workers from nest material and rearing them on water-soaked tissue paper for at least 4 days to purge them of dirt and coarse wood fibre in their guts.

Light microscopic observations were performed on Giemsa-stained smears made by spreading gut content diluted with a small volume of Locke's fluid on slides. Partially air dried smears were fixed with methanol and stained with Giemsa's stain. Giemsa slides were examined without cover slips by bright-field microscopy under immersion oil. Both "clean" and "dirty" preparations were examined by light microscopy to determine if cleaning cause artefactual changes to the cells. Cells were drawn using a camera lucida, and measurements were made using a calibrated eye piece micrometer for each cell dimension except for cell length, anterior flagellum length and undulating membrane length which was measured from drawings by planometer. Measurements are presented as a range of values followed by the average in parentheses.

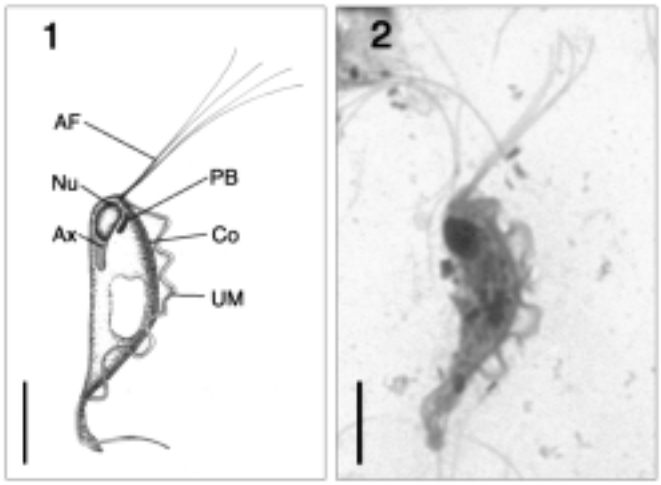
Specimens for transmission electron microscopy were collected exclusively from "clean" termites dissected into Locke's fluid and fixed in at least 10 volumes of 3% glutaraldehyde in 0.066 M cacodylate buffer (pH 7.2) for 30 min. Fixed samples from several termites were pooled and washed three times in Sorenson's phosphate buffer (pH 6.8) for 30 min each. Cells were post-fixed in 4% osmium tetroxide for 1 h and washed 3 times in distilled water (10 min, 10 min and overnight). Specimens were then dehydrated in a graded series of acetone solutions (5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 95, 100 and 100%) for 10 min. each. Cells were gradually infiltrated with Epon resin (25, 50, 75% Epon in 100% acetone for 1 h each, 100% Epon overnight) and embedded in fresh 100% Epon, pelleted by gentle centrifugation and cured for 1 day at 60°C. Semithin survey sections were cut with glass knives, stained with 1% toluidine blue and used to orientate sections. Ultra-thin sections (70 nm and 90 nm) were cut with diamond knives, mounted on formvar-coated copper slot grids, stained with 5% uranyl acetate in 50% methanol for 2 min, washed in distilled water for 30 s and dried. The sections were then counter-stained with Reynold's 2% lead citrate for 1 min, washed in distilled water for 30 s and dried prior to examination. Sections were examined in a JEOL 1010 transmission electron microscope.

RESULTS

Colonies of *S. secundus* were collected from the following localities Brisbane, QLD (8 colonies), Herberton, QLD (1 colony), Nanango, QLD (1 colony) and New Brighton, NSW (1 colony); 1 colony of *S. intermedius* was collected from Brisbane (QLD). All soldiers and workers from every colony examined were host to a small trichomonad species whose characteristics were consistent with the description of the genus *Pseudotrypanosoma* Grassi, 1917. The species appeared to be novel and its morphology and ultrastructure are described below.

Pseudotrypanosoma elphinstonae sp. n.

Type host: *Schedorhinotermes secundus*
Other host: *Schedorhinotermes intermedius*



Figs 1-2. Morphology of *Pseudotrypanosoma elphinstonae* sp. n. **1** - line diagram, **2** - light micrograph. Scale bars 10 µm.

Habitat: termite hindgut.

Type locality: Pinjarra Hills, Brisbane, QLD.

Type material: holotype deposited with the Queensland Museum (Brisbane, Australia), accession number: G463726.

Description: cells long, narrow and sinuous, 10.2-21.6 (16.3) µm long by 2.4-7.8 (4.1) µm wide (Figs 1, 2). Live cells highly flexible, capable of coiling upon themselves but rarely becoming spherical (characteristic of other *Pseudotrypanosoma* spp). Four anterior flagella, 8.7-19.2 (11.9) µm long, emerge just ventral of the cell apex, flagella often grouped into 2 pairs proximally, splitting distally. Nucleus ovoid, 1.8-3.6 (2.8) µm long by 1.2-2.4 (1.9) µm wide, located at anterior of cell immediately beneath the anterior flagella. Axostyle short and rod-like, 1.2-3.6 (2.4) µm long by 0.6-1.8 (1.1) µm wide, projects from nucleus posteriorly through middle of the cell, axostyle is non-emergent. Undulating membrane sinusoidal, 7.5-20.4 (13.7) µm long reaching almost to posterior end of the cell, recurrent flagellum extends freely posterior of the undulating membrane short distance.

Differential diagnosis: *P. elphinstonae* differs from *P. giganteum* Grassi, 1917 on the basis of body size (it is much smaller) and costa morphology (it is narrow and not greatly contractile in the former species). *P. elphinstonae* differs from *P. minimum* Sutherland, 1933 in having a very short, non-projecting axostyle whereas the latter species has a strongly projecting axostyle.

Etymology: *P. elphinstonae* is named after a good friend and great dancer Miss Kara Elphinstone.

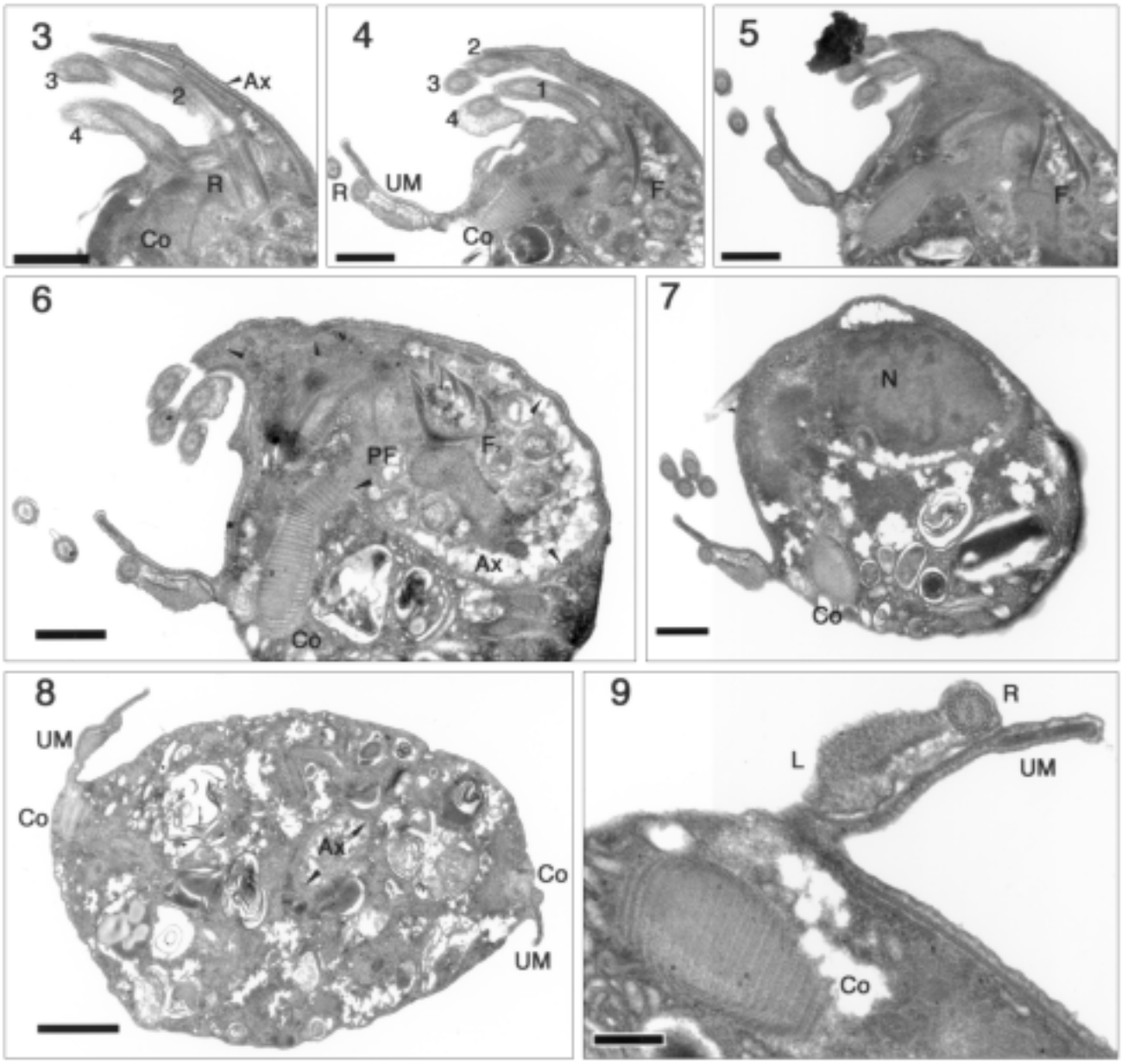
Ultrastructural study

Mastigont system

There are 4 anterior flagella and 1 recurrent flagellum which forms part of the undulating membrane (Figs 3, 4, 10). The anterior flagella are deeply recessed within a ventral pocket; the anterior end of the cell thus forms the periflagellar canal from which the flagella emerge (Figs 3, 10). Flagellum 2 is adherent to the roof of the periflagellar canal and there is a thickening of the cell membrane in this area (Fig. 4). The basal bodies of the anterior flagella are associated to form two pairs - 1,4 and 2,3 (Fig. 6). The basal body of the recurrent flagellum (R) is perpendicular to the other basal bodies and emerges from the centre of the diamond which they form (Fig. 10). There is a small pocket around the base of the recurrent flagellum as it emerges from the cell (Fig. 10). Basal bodies 1 and 3 bear a hooked lamina emerging from the top of the basal body and curving around it clockwise before running posteriorly for a short distance (Fig. 13). The sigmoid fibres (F_2) are located posterio-dorsal to the basal bodies and are attached to the basal body 2 (Fig. 14). F_2 is composed of a dorsal set of 5 fibres which are unbranching and a single ventral fibre which splits into 4-5 fibres before curving dorsally towards the end of the fifth dorsal fibre (Figs 4-6). The whole structure thus has a claw-like appearance in profile. The reticular bodies found in *P. giganteum* by Brugerolle (1999) were absent; a superficially similar structure, the scroll-form body was located dorsal to the basal bodies but was composed of convoluted membranes not microtubules (Fig. 15).

Nucleus and axostyle

The pelto-axostylar complex is simple composed of a single layer of microtubules throughout. There is no pelta distinct from the axostyle. The capitulum of the axostyle instead opens out to form a broad hood overlap the mastigont structures (Figs 6, 12). There is no over or doubling of the microtubules as has been observed in other trichomonads where the pelta overlap the axostyle. The axostyle is a thin sheath wrapped tightly around the nucleus and projecting posteriorly into the main body of the cell (Figs 8, 12). The nucleus is located within the anterior end of the axostyle immediately posterior to the Golgi stack (Figs 11, 12). Nuclear chromatin is densely

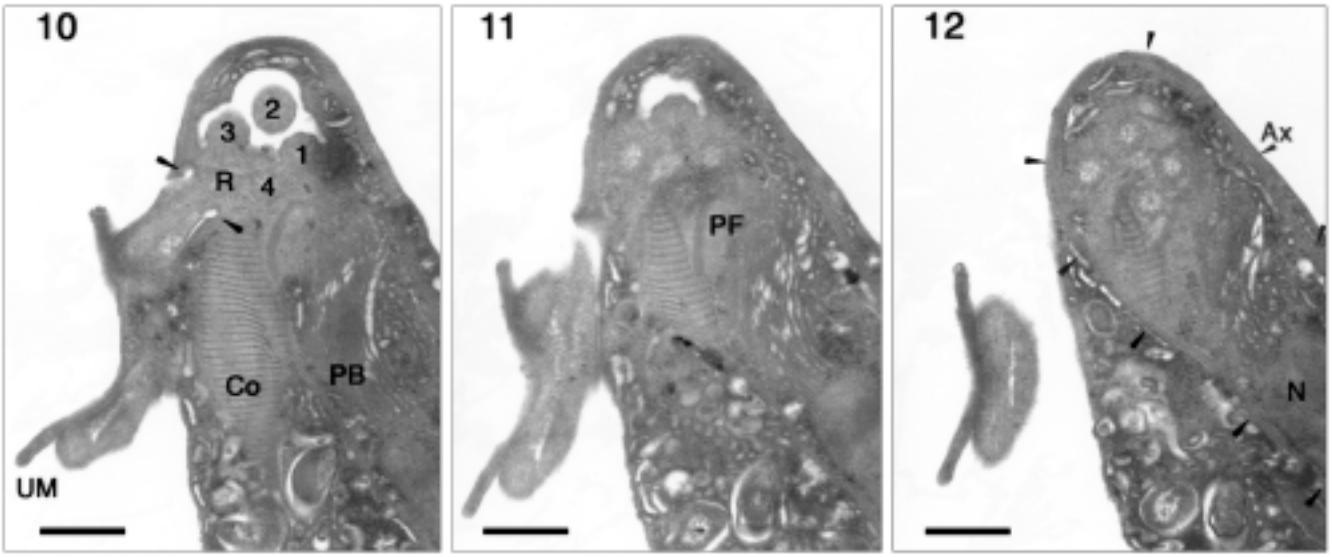


Figs 3-9. Transmission electron micrographs of *P. elphinstonae* sp. n., transverse sections. **3-8** - serial sections through the same cell, anterior to posterior; **9** - costa and undulating membrane. Arrow heads in 6 and 8 show course of axostyle. 1 - flagellum 1, 2 - flagellum 2, 3 - flagellum 3, 4 - flagellum 4, Ax - axostyle, Co - costa, F_2 - sigmoid fibre, L - lamella, N - nucleus, PF - parabasal fibre, R - recurrent flagellum, UM - undulating membrane. Scale bars 10 µm (3-8); 1 µm (9).

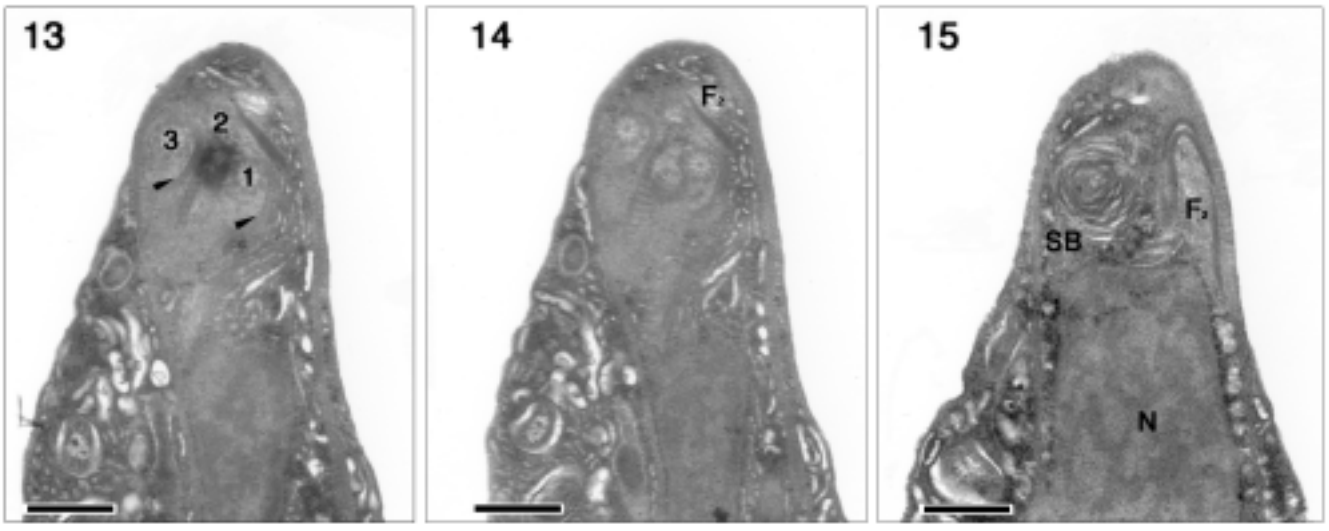
packed but has not condensed into discrete bodies (Figs 7, 15).

Parabasal apparatus

The parabasal apparatus is simple composed of a single parabasal fibre and a single Golgi stack of cisternae (Figs 10, 11). It is not branched as in many other trichomonads and is entirely contained within the pelto-axostylar complex. The parabasal fibre is a short, thick, electron opaque fibre attached anteriorly to basal body 4, runs posteriorly parallel to the costa and joins the Golgi ventrally at the point where the cisternae approach the costa (Fig. 11). The parabasal fibre is attached to the Golgi superficially; it does not penetrate the stack as in *P. giganteum*. The whole parabasal apparatus is small, squeezed between the nucleus and the costa (Figs 10-12).



Figs 10-12. Transmission electron micrographs of *P. elphinstonae* sp. n., sagittal sections I. **10-12 -** serial sections through the same cell, ventral to dorsal. Arrowheads in 10 highlight the sac-like opening at the base of the recurrent flagellum. Arrowheads in 12 show the course of the axostyle. 1 - flagellum 1, 2 - flagellum 2, 3 - flagellum 3, 4 - flagellum 4, Ax - axostyle, Co - costa, N - nucleus, PB - parabasal body, PF - parabasal fibre, R - recurrent flagellum, UM - undulating membrane. Scale bars 1 µm.



Figs 13-15. Transmission electron micrographs of *P. elphinstonae* sp. n., sagittal sections II. **13-15 -** serial sections through the same cell, ventral to dorsal, arrowheads in 13 denote the hooked lamella. 1 - flagellum 1, 2 - flagellum 2, 3 - flagellum 3, F_2 - sigmoid fibre, N - nucleus, SB - scroll-form body. Scale bars 1 µm.

Undulating membrane and costa

The costa is a thick, striated cord wrapped in a loose spiral around the cell in association with the undulating membrane (Figs 8, 9). Anteriorly, it is a narrow cord attached to the posterior of basal body 2 and directed posteriorly between basal bodies 3 and 4 (Fig. 12). It thickens significantly immediately posterior to basal body 4 giving the appearance of also being attached to this basal body (Fig. 11). For much of its length, the costa is of constant width, is roughly circular in cross-section and heavily striated. The undulating membrane is composed

Table 1. Morphometrics of *Pseudotrypanosoma elphinstonae* sp. n., measurements in micrometers.

Character	x	SD	Min	Max	n
Body length (L)	16.31	3.002	10.2	21.6	40
Body width (W)	4.11	1.172	2.4	7.8	40
Shape index (L/W)	4.16	1.010	2.04	6.0	40
Anterior flagellum length	11.94	2.688	8.7	19.2	40
Nucleus length	2.85	0.430	1.8	3.6	40
Nucleus width	1.95	0.330	1.2	2.4	40
Axostyle length	2.4	0.775	1.2	3.6	26
Axostyle width	1.11	0.333	0.6	1.8	26
Undulating membrane length (UM)	13.68	2.922	7.5	20.4	40
Undulating membrane proportion (L/UM)	1.2	0.135	0.95	1.53	40

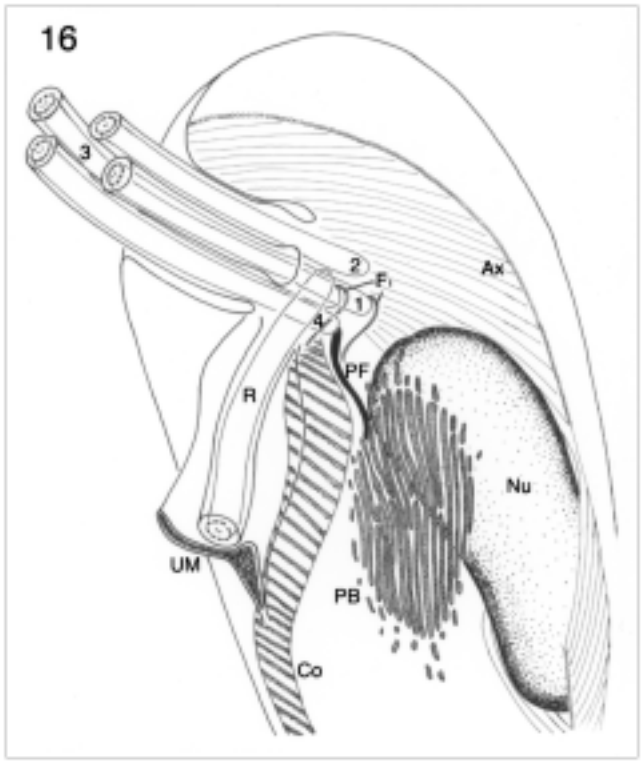


Fig. 16. Cut away view of the mastigont system of *Pseudotrypanosoma elphinstonae* sp. n., sigmoid fibre and scroll-form body omitted in the interests of clarity. 1 - basal body 1, 2 - basal body 2, 3 - basal body 3, 4 - basal body 4, Ax - axostyle, Co - costa, F_1 - hooked lamella, Nu - Nucleus, PB - parabasal body/golgi apparatus, PF - parabasal fibre, R - recurrent flagellum, UM - undulating membrane.

of the recurrent flagellum and lamella (Fig. 9). The recurrent flagellum is dilated in a long thin band which projects back towards the costa, this dilated portion lacks internal structure (Fig. 9). No microtubular bundles were found to connect to the undulating membrane to the costa as has been observed in *P. giganteum*.

DISCUSSION

Whilst superficially similar the trichomonads which possess 4 anterior flagella are readily distinguishable from each other by their distinctive ultrastructural characteristics. *P. elphinstonae* differs from *Trichomonas gallinae* in the arrangement of the fibres associated with the flagellar bases: (1) absence of a shallow periflagellar canal; (2) lack of a pelta which conspicuously overlaps the anterior end of the axostyle; (3) very different structure to the sigmoidal fibre (2 sheets composed of 5 and 1 microtubules in the former species versus a single sheet of 9 microtubules in the latter) (Mattern *et al*. 1967). In addition the structure of the parabasal body of *T. gallinae* is much more complex than that of *P. elphinstonae*. In *Trichomonas* there are 2 parabasal fibres, each the diameter of a kinetosome, PF1 derives from basal body 2 and curves around the basal body 3 to the right and external to the hooked lamina and PF2 has a common origin with the costa, between basal bodies 3 and 4 before running dorsally along the length of the costa; the parabasal body itself is composed of an irregular stack of few discs (Mattern *et al*. 1967). In contrast in *P. elphinstonae* there is a single parabasal fibre derived from basal body 4 which attaches superficially to the dense, regularly stacked Golgi apparatus. The undulating membrane and costa is much less developed in *Trichomonas* than in *Pseudotrypanosoma*. The costa itself is much smaller, about the diameter of a kinetosome whereas in *P. elphinstonae* it is about 3 times the diameter. There is also no contact between the recurrent flagellum and the undulating membrane along its length in *Trichomonas* whereas they are intimately associated in *P. elphinstonae* and *P. giganteum* (Mattern *et al*. 1967,

Brugerolle 1999). These differences challenge the proposal of Cleveland (1961) that *Pseudotrypanosoma* and *Trichomonas* are synonymous and is consistent with molecular phylogenetic results suggesting the distinctiveness of *Pseudotrypanosoma* (Keeling *et al.* 1998, Gerbod *et al.* 2001).

Pentatrichomonas is a second genus with 4 anterior flagella (plus an independent flagellum which gives the cell the appearance of 5 anterior flagella), which has been suggested, is closely related to *Pseudotrypanosoma* on the basis of molecular sequence analysis (Gerbod *et al.* 2001) despite their widely separated hosts (mammals *vs*. termites). Some features of the kinety ultrastructure supports this hypothesis (Honigberg *et al.* 1968); in *Pentatrichomonas* the 4 anterior flagella are grouped in pairs and the sigmoidal fibre splits into several subfibres of similar appearance to that seen in *Pseudotrypanosoma* (Honigberg *et al.* 1968). Most of the other features of the cell are however identical to those found in *Trichomonas* so the significance of the ultrastructural similarities between *Pentatrichomonas* and *Pseudotrypanosoma* are difficult to gauge.

Pseudotrypanosoma elphinstonae is very similar to *P. giganteum* but differs in several features (Brugerolle 1999). The arrangement of the basal bodies and their associated fibres is very similar, basal bodies 1-4, R and the hooked lamina fibres are all arranged identically in the two species. The sigmoid fibre (F_2) has a similar structure (5 dorsal and 1 ventral) and origin (basal body 2) in the two species but is much smaller and divides into fewer secondary fibres in *P. elphinstonae* (4-5 secondary fibres *vs*. 30 in *P. giganteum*). The reticulate bodies found in *P. giganteum* were not present in *P. elphinstonae*. The costa was a much less conspicuous organelle in *P. elphinstonae* than in *P. giganteum*. Even considering the smaller size of the cell, the costa of *P. elphinstonae* was proportionately smaller, less conspicuously contractile in living cells and was never observed independent of lysed cells. The undulating membrane of *P. elphinstonae* is also simpler than that of *P. giganteum*. The dilated recurrent flagellum of the former species is smaller and lacks the internal structure of para-axonemal fibres found in the latter. The most striking difference between *P. elphinstonae* and *P. giganteum* is the absence of a pelta in the former species and its replacement by an elaboration of the axostyle's capitulum. *P. giganteum* has a large pelta which, similar to other trichomonads, is composed of a ribbon of 5 microtubules at it's base where it overlaps the axostyle capitulum (Mattern *et al.* 1967, Honigberg *et al.* 1968, Brugerolle 1999). There is no such overlapping area in *P. elphinstonae* and the hood-like structure similar to the pelta appears to be continuous with the axostyle. For this reason we have chosen to refer to this structure as an expanded axostyle rather than a simplified pelta. Considering the differences in size and structure of the organelles between the two species it is apparent that *P. elphinstonae* is much smaller and simpler in structure than *P. giganteum*. There are few similar examples comparing the ultrastructure of congeneric trichomonads which vary significantly in their cell size. Further investigation of such pairs may greatly improve our understanding of the probable functions of the various components of the mastigont system.

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PROTOZOOLOGICA

Nosema chaetocnemae sp. n. (Microspora: Nosematidae), a Microsporidian Parasite of *Chaetocnema tibialis* (Coleoptera: Chrysomelidae)

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Summary. A microsporidian parasite infecting *Chaetocnema tibialis* is reported for the first time and described by light and electron microscopy in the present study. The infection rate in the population at Samsun (Turkey) reaches up to 42%. Spores are oval, measure 3.52 ± 0.41µm (2.85-4.27) in length and 2.09 ± 0.26 µm (1.90-2.85) in width, possess a diplokaryon and have 13 coils of the polar filament. The morphological and ultrastructural features indicate that the described microsporidian is a member of the genus *Nosema.* It is described as *Nosema chaetocnemae* sp. n. after the name of the genus of its host.

Key words: biological control, *Chaetocnema tibialis,* Microsporidia, *Nosema chaetocnemae* sp. n.

INTRODUCTION

Chaetocnema tibialis (Chrysomelidae: Coleoptera) is one of the most important pests of sugar beets in Turkey. Chemical pesticides utilized to control this pest have hazardous effects on the environment. In contrast, biological control agents have certain advantages over chemicals as control agents. They are non-polluting and thus environmentally safe and acceptable. It is believed that entomopathogenic microorganisms can decrease insect population densities and reduce the duration of outbreaks (Myers 1988). Consequently, any natural enemies of this pest are of great interest as potentially valuable biological control agents. As a group, Microsporidia are important pathogens of insects and are considered to be important regulators of the population dynamics (Linde *et al*. 2000). They form the majority of the protista pathogenic to insects and cause economically serious diseases in pest insects (Tanada and Kaya 1993).

Although chrysomelids are frequently infected by protista, no parasitic protist has been recorded up to now from *C. tibialis*. The first microsporidian described from the Chrysomelidae was *Nosema phyllotretae*, observed in *Phyllotreta atra* and *Phyllotreta undulata* (Weiser 1961). Toguebaye and Bouix (1989) presented a list of *Nosema* parasites described in the family Chrysomelidae. However, there is not a single microsporidian record from *C. tibialis*. In the present paper, we report on

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Nosema chaetocnemae sp. n., a microsporidian parasite of *C. tibialis* in Turkey.

MATERIAL AND METHODS

The adults of *Chaetocnema tibialis* were collected from March to October in 2000 in Çarşamba (Samsun) in Turkey.

Light Microscopy

Each beetle was dissected and wet smears were examined under a microscope for parasites. When infection was observed, a fragment of infected host tissue was lacerated and spread on a slide. The smear was air-dried and then fixed with methanol for 10 min. Afterwards the slides were washed with distilled water and stained for approximately 10 h in a freshly prepared 5% solution of Giemsa stain. They were then washed in running tap water, air-dried and examined under a microscope (Toguebaye *et al*. 1988). Detected spores were measured and photographed.

Electron microscopy

Different portions of infected beetles were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1-2 h, rinsed in cacodylate buffer, postfixed in reduced OsO_4 according to Karnovsky (1971) (a fresh 1:1 mixture of 2% OsO_4 and 3% $K_4[Fe(CN_6)]$ for 1.5 h, rinsed in cacodylate buffer and dehydrated in ethanol prior to embedding in Spurr's resin (Spurr 1969). Thin sections were mounted on Pioloform-coated copper grids, which were stained with saturated uranyl acetate and Reynolds' lead citrate (Reynolds 1963). They were examined in a Philips 208 electron microscope (TEM).

Host specificity of the parasite

We also carried out tests for host specificity of this parasite with two other chrysomelids, *Phyllotreta undulata* and *P. atra.* For this, a semi-purified spore suspension with a concentration of 2 x 10^6 spores/ml was experimentally tested. A film of the spore suspension was applied to the surface of fresh cabbage leaves and 30 beetles in each group were allowed to feed on these leaves for 25 days. Infected insects were dissected, and spores were released on the microscopic slide.

RESULTS

The infection was found in the adults of *C. tibialis* in Çarşamba (Samsun), Turkey. When the infected insects were dissected, a large quantity of spores was released on the microscopic slide. The spores of the parasite have a characteristic appearance and are easily distinguished (Figs 1-3). Fresh spores are oval and measure 3.52 ± 0.41µm (2.85-4.27) in length and 2.09 ± 0.26 µm (1.90-2.85) in width (n = 50). Microscopic examination of parasitized individuals revealed the presence of the parasite in the gut, muscles, tracheal cells and Malpighian tubules. Frequently, the tissues were completely filled with large quantities of spores. Plasmodia with 4 nuclei are formed (Fig. 4). Sporonts are diplokaryotic (Fig. 5).

Ultrastructural studies show that the polar filament of the described species has 13 coils (Fig. 6). The polar filament was measured 115 nm in diameter in the anterior 9 spirals and 75 nm in the posterior 4 spirals. The filament contains a central core surrounded by 4 concentric layers. The core is surrounded by a relatively electron-lucent layer with a substructure consisting of about 20 small granules (Fig. 7). The diplokaryotic spore has two closely approached nuclei that are slightly elongate in the direction of the major axis of the spore. They are found in the central part of the spore. The spore wall is 176.5 to 213 nm thick and made up of a clear endospore (127.5 to 158 nm) and an exospore (50 to 55 nm) (Fig. 7). Exospore is uniform. A polar sac was well developed (Fig. 8). The polaroplast seems to be relatively vesicular (Fig. 8).

***Nosema chaetocnemae* sp. n.**

Spores: oval, 3.52 x 2.09 µm (fresh spores), 13 coils, relatively vesicular polaroplast.

Location in host: gut, tracheae, muscles and Malpighian tubules.

Host: *Chaetocnema tibialis* (Coleoptera: Chrysomelidae).

Location: Çarşamba (Samsun), Turkey.

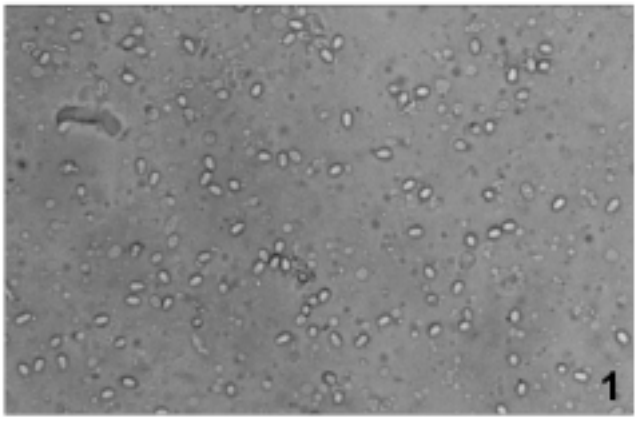
Prevalence: 112 of 431 examined beetles were infected by the parasite. Infection rate reached 42%.

Type material: the preparations for light (Preperat No: MY.P-01) and electron microscopy are stored at Department of Biology, Karadeniz Technical University, Turkey.

Etymology: the name of the species refers of its host.

We observed that last coils of the polar filament are narrow. Larsson (1986) recorded that as the most posterior coils are the most immature, an isofilar polar filament can be mistaken for anisofilar in immature spores. It has been interpreted that the polar filament of *Nosema* is isofilar (Larsson 1999, Canning and Vávra 2000). Hovewer, Toguebaye and Bouix (1986) observed anisofilar polar filament in *Nosema galerucellae* from *Galerucella luteola* (Chrysomelidae: Coleoptera).

Experimental infections showed that the parasite did not infect the other two chrysomelids, *P. undulata* and *P. atra*. An unpublished study by us confirms this result. In that study we searched for protozoan parasites of



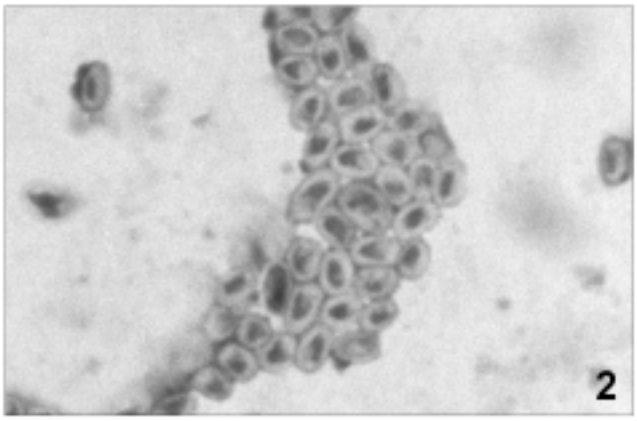
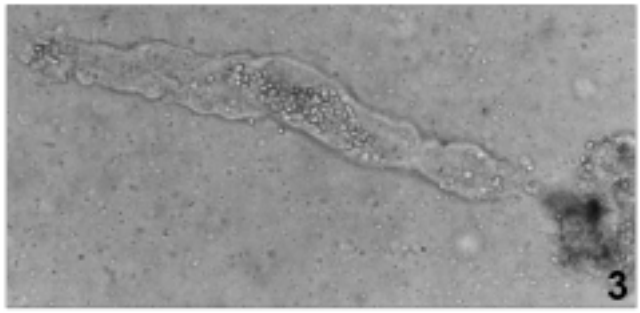


Fig. 1. Fresh spores of *N. chaetocnemae* sp. n., (x 600).
Fig. 2. *N. chaetocnemae* sp. n. spores stained with Giemsa, (x 2000).



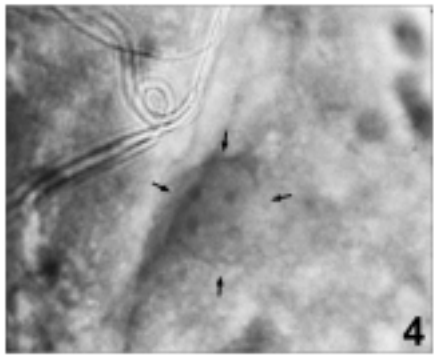


Fig. 3. *N. chaetocnemae* sp. n. spores in Malpighian tubules, (x 400).
Fig. 4. A plasmodium with 4 nuclei, (x 1500).

P. undulata and *P. atra*. We did not observe any microsporidian parasite in *P. undulata* and *P. atra* samples (more than 100 beetles of each species), although they were collected in the same plantation in which we observed the *Nosema* infection in the *C. tibialis* population.

DISCUSSION

Light and electron microscopic studies of the microsporidian parasite of *Chaetocnema tibialis* indicate that it belongs to the genus *Nosema* Naegeli, 1857. The recorded parasite has typical characters of the genus *Nosema* such as shape of the fresh (Fig. 1) and stained spores (Fig. 2), spore size, plasmodia with 4 nuclei (Fig. 4), diplokaryotic stages (Figs 5, 6), uniform exospore (Figs 6, 7) and the thickness of the spore wall (Larsson 1986, 1999). The exospore of the parasite is uniform and 50 to 55 nm. It is usually 40-60 nm in the genus *Nosema* (Larsson 1986). The polar filament was measured 115 nm in diameter in the mature coils. It is 100 nm in *N. herpobdellae* (see Spelling and Young 1983), and 117 nm in *N. tractabile* (see Larsson 1981).

Certain features observed using light and electron microscopy have led to the definition of 3 genera of microsporidians, *Nosema* Naegeli, 1857, *Unikaryon* Canning, Lai and Lie, 1974 and *Pleistophora* Gurley, 1893 in the Chrysomelidae (Toguebaye *et al*. 1988). Microsporidians of the genus *Nosema* are diplokaryotic at all developmental stages and currently recognized as being disporous, while the genera *Unikaryon* and *Pleistophora* are monokaryotic at all stages (Toguebaye *et al*. 1988).

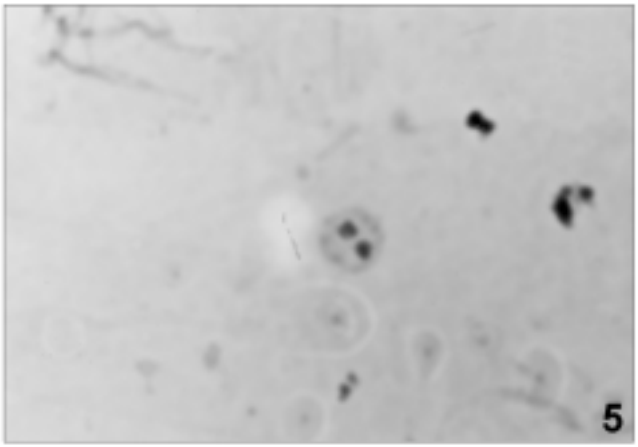
At least 200 of the 800 or so microsporidian (Microspora) species described (Canning 1990), belong to the genus *Nosema* (Sprague 1981). This seemingly disproportionate number of *Nosema* species may be due partly to incorrect identifications. Early descriptions, based mainly on spore morphology and lacking ultrastructural details, have sometimes resulted in the unnecessary creation of new species (Malone and McIvor 1995). In the most recent identification keys to microsporidian genera it was necessary to use at least a minimum of ultrastructural characters (Larsson 1983, 1988, 1999). The spore is the most important life cycle stage for the identification of microsporidia above the

Table 1. *Nosema* species described in the family Chrysomelidae (Coleoptera)

Nosema species	Spore mesurations	Infected organ	Host	Locality
N. phyllotretae Weiser, 1961	4.2 x 2 to 3 µm	Adipose body	*Phyllotreta atra* *Phyllotreta undulata*	England
N. leptinotarsae Lipa, 1968	2 to 5 x 1.9 to 3.3 µm	Haemolymph	*Leptinotarsa decemlineata*	U.S.S.R.
N. gastroideae Hostounský et Weiser, 1973	3 to 4.8 x 2.5 to 3 µm	Overall infestation	*Gastrophysa polygoni* and several experimental hosts	Czechoslovakia
N. polygrammae Hostounský et Weiser, 1975	4.8 x 2.05 µm	Gut	*Polygramma undecimlineata*	Cuba
N. equestris Hostounský et Weiser, 1980	4 to 5 x 3 µm	General infestation	*Gastrophysa viridula* *Leptinotarsa decemlineata*	Czechoslovakia
N. couilloudi Toguebaye et Marchand, 1984	3.4 to 4 x 1 to 1.5 µm	Gut	*Nisotra* sp.	Senegal
N. birgii Toguebaye et Marchand, 1986	6.2 x 3.5 µm	Eggs and general	*Mesoplatys cincta* infestation, larvae and imago	Senegal
N. nisotrae Toguebaye et Marchand, 1989	5.8 x 3.1 µm	General infestation	*Nisotra* sp.	Senegal
N. galerucellae Toguebaye et Bouix, 1989	4.95 x 2.89 µm	Gut principally, adipose body, muscles, tracheae and Malpighian tubules	*Galerucella luteola*	France
N. chaetocnemae present paper	3.52 x 2.09 µm	Gut, tracheae, muscles and Malpighian tubules	*Chaetocnema tibialis*	Turkey

species level by ultrastructural studies. The spore is always present and provides abundant characters to evaluate (Larsson 1999). The ultrastructure of many *Nosema* spp. has been described (Sato *et al*. 1982, Avery and Anthony 1983, Toguebaye and Marchand 1984, Toguebaye and Bouix 1989, Hsu *et al*. 1991). These studies provide useful information for the identification of *Nosema*. The ultrastructural characteristics of spores of the genus *Nosema* was given by Sato *et al*. (1982) and Canning and Vávra (2000). Therefore, we compared the spore ultrastructure of this *Nosema* species with other *Nosema* species infecting the family Chrysomelidae (Coleoptera) in order to create new species.

In the literature there is no microsporidian record from *Chaetocnema tibialis*. The parasite presented in this paper is the first microsporidian described from *C. tibialis*. Sprague *et al*. (1992) listed the host as the first of the taxonomic characters because a host is prerequisite to the parasitism. The host affinity is generally recognized as a valid taxonomic character, at least in microsporidia infecting insects. According to Toguebaye and Bouix (1989), up to now ten species belonging to the genus *Nosema* have been described from the family Chrysomelidae; their distinctive characteristics are shown in Table 1. The spore dimension is a good feature for comparison of the ten *Nosema* species from chrysomelids. As seen in Table 1, our microsporidian differs from all *Nosema* species in spore size. On the other hand, the number of polar coils provides a further useful taxonomic criterion for differentiating species (Cheung and Wang 1995). The number of polar coils of the described parasite (13) is different from the number of coils of the 4 chrysomelid parasites, *Nosema galerucellae*



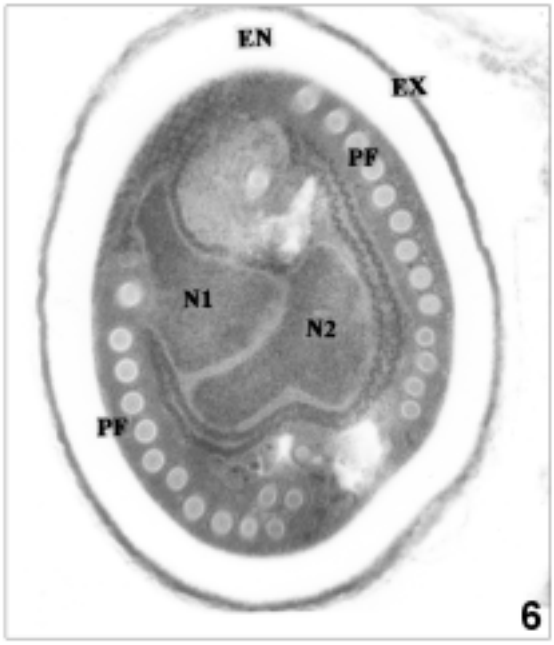
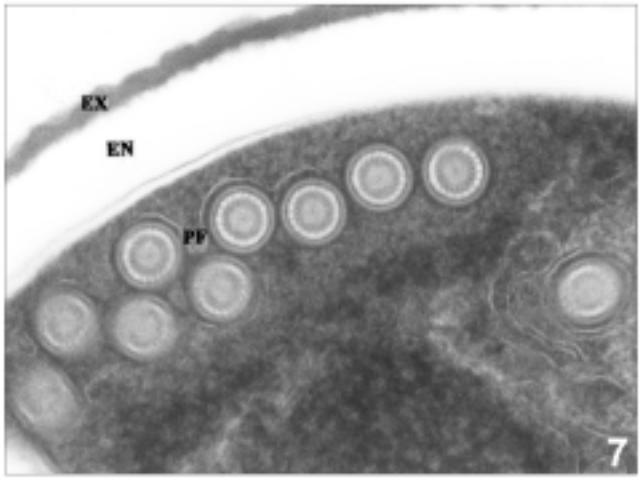


Fig. 5. Diplokaryotic sporont, (x 1000).
Fig. 6. Section of a *N. chaetocnemae* sp. n. spore showing 13 coils of the polar filament (PF) and a clearly visible diplokaryon (N1 and N2). EN - endospore, EX - exospore, (x 25,000).



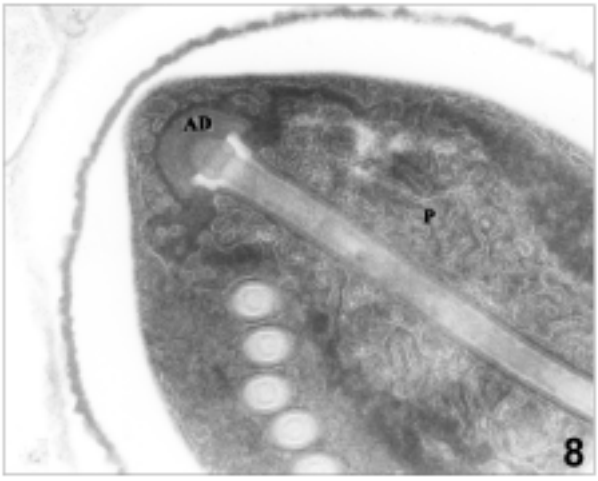


Fig. 7. Cross section of the polar filament (PF) of a *N. chaetocnemae* sp. n. spore. EN - endospore, EX - exospore, PF - polar filament, (x 80,000).
Fig. 8. Section of the anterior portion of a *N. chaetocnemae* sp. n. spore showing anchoring disc (AD) and vesicular polaroplast (P), (x 50,000).

(7-9 coils) (Toguebaye and Bouix 1989), *Nosema couilloudi* (8-10 coils) (Toguebaye and Marchand 1984), *Nosema birgii* (12-14 coils) (Toguebaye and Marchand 1986) and *Nosema nisotrae* (15-18 coils) (Toguebaye and Marchand 1989).

As seen in Table 1, in all cases, at least one character of the known species is different from this new chrysomelid parasite, either spore dimensions, number of polar filament coils, thickness of the spore wall, host and geographic location of the host, host spectrum, and infected organs. Therefore, the described characters of the *Nosema* from *C. tibialis* seem to be sufficiently distinct in order to create a new species.

Nosema chaetocnemae sp. n. infects the gut, muscles, tracheal cells and Malpighian tubules. Similar tissue infection was observed for *N. galerucellae* in *Galerucella luteola* (Toguebaye and Bouix 1989) in France. Hostounský and Weiser (1973) observed overall infestation including muscles with *Nosema gastroideae* in *Gastroidea polygoni* (Chrysomelidae: Coleoptera). They found oval or tubular masses of spores in the centre of the muscle. However, *N. couilloudi* (Toguebaye and Marchand 1984) and *N. polygrammae* (Hostounský and Weiser 1975) infect only the gut of *Nisotra* sp. and *Polygramma undecimlineata* (Chrysomelidae: Coleoptera) respectively.

When the infected insects were dissected, a large quantity of spores was released into the water on the

microscopic slide. Under natural conditions this great amount of spores is released after the death of infected beetles and this favours a quick spread of infection among the insect population. Thus, *N. chaetocnemae* sp. n. might have some importance in regulating the abundance of *C. tibialis*.

As a group, the microsporidia were thought to be the most important protozoan pathogens of insects. The systematic position of the microsporidia has been contested recently. Several molecular phylogenies suggest that microsporidia are closely related to fungi (Hirt *et al*. 1999, Keeling *et al*. 2000). However, Weiser *et al*. (2002) included this group to Protista.

In this extensive survey conducted during 2000 in Turkey, we confirmed the presence of this pathogen in *C. tibialis* populations in Çarşamba (Samsun), Turkey. The infection rate in Samsun reached 42%. Turkey represents a bridge, which joins Asia to Europe. Therefore, the results of the study are of great importance for the geographical distribution of *Nosema* species from chrysomelids (Table 1).

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Description of Two New Myxosporean Species Parasitic in Freshwater Fishes from the Yangtze River in China

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Summary. Two new species of myxosporeans (Myxosporea: Myxidiidae), *Myxidium tuanfengensis* sp. n. and *Zschokkella saurogobionis* sp. n., parasitic in freshwater fishes collected from the Yangtze River of China are described in this paper. *M. tuanfengensis* was found in the liver parenchyma and intestine lumen of *Leptobotia taeniops* Sauvage, 1878, while *Z. saurogobionis* was found in the gall bladder of *Saurogobio dumerili* Bleeker, 1871. The diagnostic characters of *M. tuanfengensis* are: round or elliptical polysporous plasmodia averaging 118 µm in size; spore oval in frontal view with smooth surface and nearly spindle-shape in sutural view with slightly sinuous sutural ridge, averaging 19.5 x 9.75 x 8.9 µm in size; two large spherical polar capsules 6.8 µm in diameter, with polar filament wound in 4 to 5 coils. The diagnostic characters of *Z. saurogobionis* are: spore elliptical in both frontal and sutural view measuring 18.3 x 9.8 x 10.8 µm in size; fine sutural ridge in S-form, spore shell marked with 10 to 12 distinct lines paralleled with the sutural line; two spherical polar capsules, 6.7 µm in diameter, with polar filament in 5 coils.

Key words: fish parasite, myxosporean, Myxidiidae, *Myxidium tuanfengensis* sp. n., *Zschokkella saurogobionis* sp. n.

INTRODUCTION

Although the parasite fauna of the fresh water fishes in lower and middle branches of the Yangtze River of China have been described by many authors (Nie *et al*. 1999, 2000; Yao 2001), the protozoan parasites in fishes of these river stretches are still little known. Nevertheless, it is necessary to obtain data on protozoan infection in fish in order to recognize pathogenicity inflicted by many of them on commercial and aquarium fish as described by Zhao and Song (2001). While re-examining samples of myxosporean parasitic in freshwater fishes collected at the town of Tuanfeng in Huanggang City, Hubei Province, China, two of them have been found to be new members of the family Myxidiidae. This paper describes the two new myxosporean species of the genera *Myxidium* and *Zschokkella*.

MATERIALS AND METHODS

The host fishes, *Leptobotia taeniops* Sauvage, 1878 and *Saurogobio dumerili* Bleeker, 1871, which kept in 10% buffered formalin, were collected from the branches water of Yangtze River at the town of Tuanfeng in Huanggang City, Hubei Province, China, during the investigations on the fauna of parasites from freshwater fishes of the

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Yangtze River in 1981-1985 (Hsieh 1988, Yu and Wu 1989). All body organs were examined and twenty spores of each myxosporean were measured with an eyepiece micrometer. Permanent preparations were fixed with absolute methanol and stained with Giemsa. Illustrations were drawn with the aid of camera Nikon FDX-35 and all the measurement are in micrometers (μm).

RESULTS AND DISCUSSION

Myxidium tuanfengensis sp. n. (Figs 1-5)

Diagnosis: round or elliptical polysporous plasmodia 118 (100-130) ± 8.7 in diameter.

Trophozoites: polysporous plasmodia (Fig. 1).

Spore: spore oval in frontal view with smooth surface, both anterior and posterior ends rounded, nearly spindle in sutural view with the sutural ridge slightly sinuous; spores measure 19.5 (18.0-20.8) ± 0.8 x 9.75 (9.1-10.4) ± 0.6 x 8.9 (8.8-9.2) ± 0.1 in size; two large spherical polar capsules with polar filament wounded in 4 to 5 coils; no iodinophilous vacuole.

Host: *Leptobotia taeniops* Sauvage, 1878.

Prevalence: 6 of 12 fish examined was infected (50%).

Site of infection: liver parenchyma and intestine lumen.

Locality: Tuanfeng Town (30° 30' N, 111° 05' E) of the Huanggang City in Hubei province, China.

Date of sampling: June 1984.

Type specimens: syntypes on slide No. B018, deposited in the Laboratory of Fish Diseases, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China.

Etymology: the name of this species has been derived from the sampling site, Tuanfeng Town.

Taxonomic affinities

Bütschli (1882) was the first to establish the genus *Myxidium*. Up to now, 69 species of the genus have been reported from freshwater fish in China (Chen and Ma 1998). Species of this genus share the following characters: spore spindle-shaped or close to spindle-shaped with polar capsules in both ends in front view; most species are coelozic and usually have straight sutural line.

In the light of the morphologically similar forms, the following species should be compared with our species: *Myxidium songtaoensis* Xiao et Feng, 1997; *M. macrocapsulare* Auerbach, 1910 and *M. tongrenensis* Feng et Xiao, 1996. *M. songtaoensis* differs from our form in having elongated elliptical spores, smaller in length (12.0-16.0) and in width (6.9-7.5) as well as in having 6-7 striations parallel to the sutural ridge (Xiao and Feng 1997). *M. macrocapsulare* can be distinguished by possessing striations on shell surface and smaller spores (10.0-12.0 x 6.0) (Auerbach 1910a). *M. tongrenensis* appears distinctly different from our species in bearing 8-10 striations parallel to the sutural ridge and smaller spores (10.5-12.0 x 5.5-6.5) (Xiao and Feng 1997). Our new species is distinguished by the following features: (A) larger dimension of spores and (B) smooth shell surface. Furthermore, *Leptobotia taeniops* Sauvage, 1878 is a new host for myxosporeans in China according to the published articles (Table 1).

Zschokkella saurogobionis sp. n. (Figs 6-9)

Diagnosis: larger dimension of spores.

Trophozoites: not observed.

Spore: spore elliptical in both frontal and sutural view with 18.3 (17.2-19.2) ± 1.0 x 9.8 (9.0-10.8) ± 0.8 x 10.8 (10.2-11.5) ± 0.6 in size; fine sutural ridge in S-form, stretching from one end to the other end, spore shells marked with 10 to 12 distinct striations parallel to the sutural line; two spherical polar capsules, 6.7 ± 0.5 (6.2-7.2) in diameter, with polar filament in 5 coils; no iodinophilous vacuole.

Host: *Saurogobio dumerili* Bleeker, 1871.

Prevalence: 9 of 10 fish examined was infected (90%).

Site of infection: gall bladder.

Locality: Tuanfeng Town (30° 30' N, 111° 05' E) of the Huanggang City in Hubei province, China.

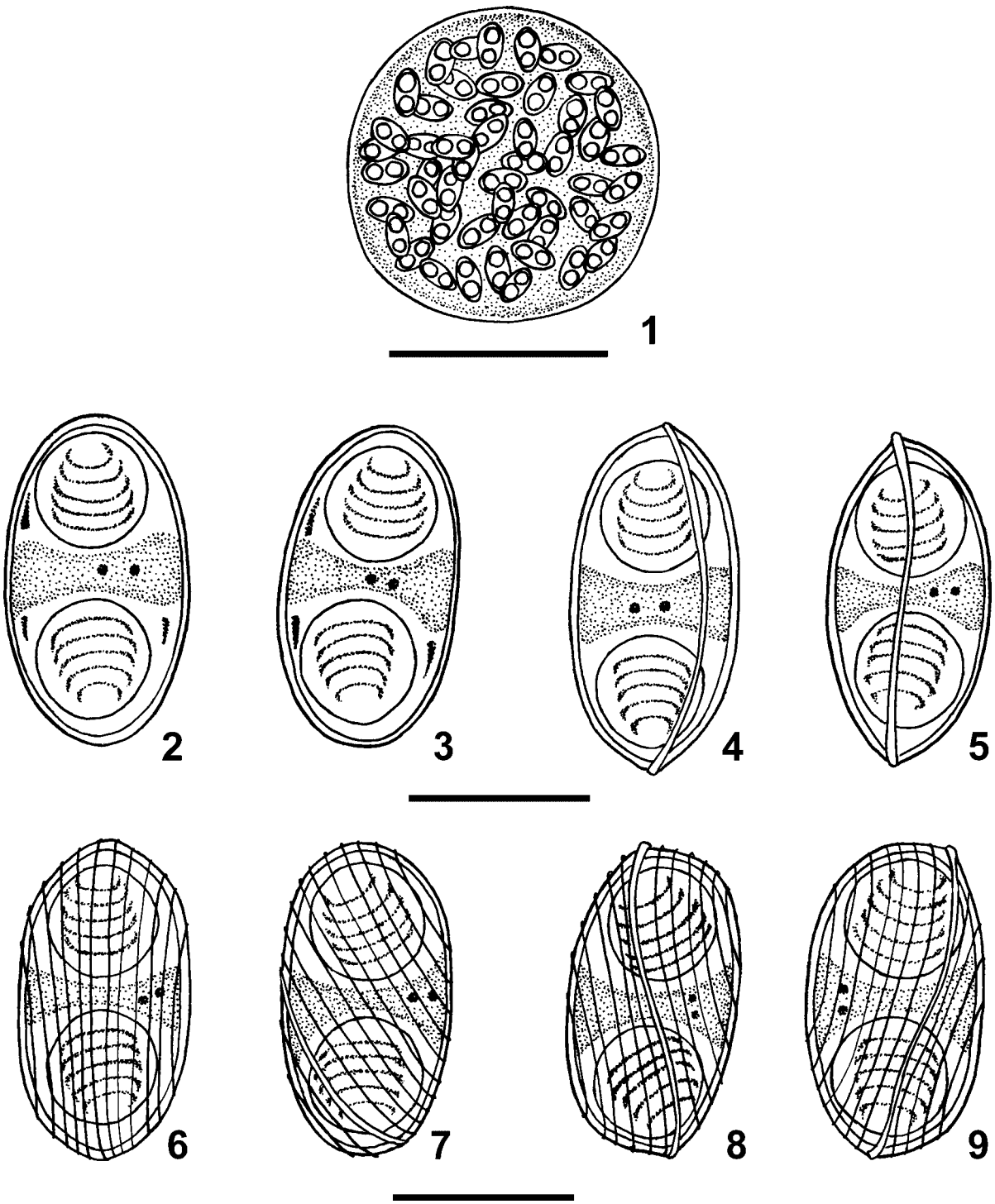
Date of sampling: June 1984.

Type specimens: syntypes on slide No. C009, deposited in the Laboratory of Fish Diseases, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China.

Etymology: this species has been named after the fish host, *Saurogobio dumerili* Bleeker, 1871.

Taxonomic affinities

Auerbach (1910b) established the genus *Zschokkella* in which the spores were elliptical (or close to elliptical) or oval (or close to oval) with two round polar capsules and curved sutural line. Since then, 24 species were reported in China (Chen and Ma 1998). According to the spore shape, three species, *Zschokkella striata* Schulman, 1962; *Z. parasiluri* Fujita, 1927 and *Z. nova*



Figs 1-5. *Myxidium tuanfengensis* sp. n.: **1** - polysporous plasmodia; **2, 3** - mature spore in front view; **4, 5** - mature spore in sutural view. **Figs 6-9.** *Zschokkella saurogobionis* sp. n.; **6, 7** - mature spore in front view; **8, 9** - mature spore in sutural view. Scale bars 100 µm (1); 10 µm (2-9).

Table 1. Comparison of the related species of *Myxidium* Bütschli, 1882 with *M. tuanfengensis* sp. n. D - diameter, L - length, T - thickness, W - width (all measurements in µm).

Species	Host	Infection locus	Shape of spore	Size of spore	Size of PC[b]	Striations on SS[c]	Data on source
M. tuanfengensis sp. n.	*Leptobotia taeniops*	Liver parenchyma and intestine lumen	Oval	L: 19.5 (18.0-20.8) ± 0.8[a] W: 9.75 (9.1-10.4) ± 0.6 T: 8.9 (8.8-9.2) ± 0.1	D: 6.8 (5.7-7.8) ± 0.5	None	Present paper
M. songtaoensis Xiao et Feng, 1997	*Pseudorasbora parva*	Gall bladder	Elongated elliptical	L: 12.0-16.0 W: 6.9-7.5	L: 4.5-5.1 W: 4.5-5.0	6-7, parallel to the sutural ridge	Xiao and Feng 1997
M. macrocapsulare Auerbach, 1910	*Scardinius erythrophthalmus*	Gall bladder	Elongated elliptical	L: 10.0-12.0 W: 6.0	D: 3.0-4.0	Possessing	Auerbach 1910a
M. tongrenensis Feng et Xiao, 1996	*Gnathopogon argentatus*	Gall bladder	Close to spindle	L: 10.5-12.0 W: 5.5-6.5	L: 3.4-4.0 D: 3.2-4.0	8-10, parallel to the sutural ridge	Xiao and Feng 1997

[a] Mean ± SD (Min-Max); [b] polar capsule; [c] shell surface

Table 2. Comparison of the related species of *Zschokkella* Auerbach, 1910 with *Z. saurogobionis* sp. n. D - diameter, L - length, T - thickness, W - width (all measurements in µm).

Species	Host	Infection locus	Shape of spore	Size of spore	Size of PC[b]	Striations on SS[c]	Data source
Z. saurogobionis sp. n.	*Saurogobio dumerili*	Gall bladder	Elliptical	L: 18.3 (17.2-19.2) ± 1.0[a] W: 9.8 (9.0-10.8) ± 0.8 T: 10.8 (10.2-11.5) ± 0.6	D: 6.7 (6.2-7.2) ± 0.5	10-12, parallel to the sutural line	Present paper
Z. striata Schulman, 1962	*Pseudogobio rivularis*	Gall bladder	Elliptical	L: 12.9-14.0 W: 6.3-7.0	L: 4.2-5.6 W: 3.8-4.2	Possessing	Chen and Ma 1998
Z. parasiluri Fujita, 1927	*Parasilurus asotus*	Gall bladder	Elliptical	L: 11.94-14.0 W: 4.0-6.0	L: 3.7-5.0 W: 3.5-4.5	5-6, parallel to the sutural line	Chen and Ma 1998
Z. nova Klokacewa, 1914	*Carassius vulgaris*	Gall bladder	Pointed elliptical	L: 9.5-11.5 W: 6.5-7.0	D: 3.0-3.5	8-11, parallel to the sutural line	Klokacewa 1914

[a] Mean ± SD (Min-Max); [b] polar capsule; [c] shell surface

Klokachewa, 1914 should be compared with our new species. *Z. parasiluri* differs our species with distinctly fewer striations on the shell surface and smaller spores (11.94-14.0 x 4.0-6.0) while *Z. striata* differed our species from elliptical-shaped and smaller size of spores (12.9-14.0 x 6.3-7.0) (Chen and Ma 1998). *Z. nova* has pointed elliptical, smaller spores (9.5-11.5 x 6.5-7.0) and polar capsules (diameter: 3.0-3.5) when compared with our new species (Klokacewa 1914). In addition, the species mentioned above were obtained from different hosts and *Saurogobio dumerili* Bleeker, 1871 is a new host for *Zschokkella* in China (Table 2).

In view of the differences described in this paper, the two myxosporeans species are considered as new species when we consulted related published papers.

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Three New Species of *Myxobolus* Bütschli, 1882 from Different Food Fishes of West Bengal, India

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Summary. Investigations on the incidence of myxozoan parasites (Myxozoa: Bivalvulida) in fishes have assumed immense importance in view of severe pathogenicity and mortality caused by these organisms on their hosts. The present communication records three new species of myxosporeans, *Myxobolus calcariferum* sp. n.; *Myxobolus chinsurahensis* sp. n. and *Myxobolus mrigalhitae* sp. n. from different food fishes viz., *Lates calcarifer* (Bloch), *Anabas testudineus* (Bloch) and Hybrid between Mrigal [*Cirrhinus mrigala* (Hamilton - Buchanan)] and Rohu [*Labeo rohita* (Hamilton - Buchanan)] of West Bengal, India respectively.

Key words: Bivalvulida, India, *Myxobolus calcariferum* sp. n., *Myxobolus chinsurahensis* sp. n., *Myxobolus mrigalhitae* sp. n., Myxozoa.

INTRODUCTION

Bütschli (1882) established the genus *Myxobolus* with the type species *Myxobolus mulleri.* When the genus was first established, it included myxozoans having spores with or without an iodinophilous vacuole and with one or two polar capsules. Thélohan in the year 1892 proposed a new genus *Myxosoma* for those species lacking an iodinophilous vacuole. Since the establishment of the genus in 1882 different workers from various parts of the world have described several species from freshwater and marine (mostly estuarine) fishes under the genus *Myxobolus.* Landsberg and Lom (1991) listed 453 species of *Myxobolus* in fishes.

Three new species of *Myxobolus* viz., *Myxobolus calcariferum* sp. n.; *Myxobolus chinsurahensis* sp. n. and *Myxobolus mrigalhitae* sp. n. respectively from *Lates calcarifer* (Bloch), *Anabas testudineus* (Bloch) and Hybrid between Mrigal [*Cirrhinus mrigala* (Hamilton - Buchanan)] and Rohu [*Labeo rohita* (Hamilton - Buchanan)] carp came out from our routine examinations during 1998-99. The description of these three myxozoans has been made in this communication in accordance with the guideline of Lom and Arthur (1989) and Lom and Dyková (1992).

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MATERIALS AND METHODS

The host fishes were collected alive from the local fish markets from September, 1998 to December, 1999 and were brought to the

laboratory and were immediately examined thoroughly for their myxozoan parasites. Sporogonic plasmodia, when found, were carefully removed with the help of a sterile forceps, smeared on clean grease-free slides with drops of 0.5% NaCl solution, covered with thin cover-glasses and properly sealed for examination under the oil immersion lens of Olympus CH-2 phase contrast microscope. Some of the fresh smears were treated with various concentrations (2-10%) of KOH solution for the extrusion of polar filaments. The India ink method of Lom and Vavrá (1963) was employed for observing the mucus envelope of spores. For permanent preparations, air-dried smears were stained with Giemsa after fixation in acetone-free absolute methanol. Measurements (based on twenty fresh spores treated with Lugol's iodine) were taken with the aid of a calibrated ocular micrometer. All measurements are presented in micrometers as mean ± SD followed in parentheses by the range. Drawings were made on fresh/stained materials with the aid of a Camera Lucida (Mirror type) and computer programme Corel Draw 9.0.

RESULTS

Myxobolus calcariferum sp. n. (Figs 1-3)

Plasmodia: round (400 in diameter), creamy white coloured plasmodia are developed within the gill lamellae of infested host fishes. They contain a large number of mature spores only.

Spores: the mature spores, in fresh condition, are light yellowish in colour. Spores are 6.6 ± 0.32 (6.1-7.1) x 6.2 ± 0.21 (5.7-6.5), almost spherical in front or valvular view (Figs 1, 3) and broadly lenticular in sutural view (Fig. 2) with symmetrical shell valves of uniform thickness. Both the ends of the spores are rounded, but the anterior part of the spore is bulged out to accommodate the polar capsules. Sutural ridge is median and straight (Fig. 2), although sutural line is not observed in fresh as well as in stained preparations.

Two polar capsules are equal [4.2 ± 0.22 (3.8-4.5) x 2.3 ± 0.21 (2.0-2.7)] in size and pyriform in shape with rounded posterior ends and cover anterior 2/3rd of the spore cavity. Anterior tip of the capsule is pointed in most of the forms. Polar filament is thick and makes 4-5 loose spiral coils inside each capsule (Fig. 1). The polar filament is, however, not extruded although all the conventional methods for the extrusion of polar filament (viz., treatments with various concentrations of KOH, H_2O_2 and urea solution) have been performed.

The intercapsular ridge or appendix is absent.

The posterior 1/3rd of the spore cavity is filled with finely granular, homogenous mass of sporoplasm, which extends slightly into the intercapsular space (Figs 1, 3). Two very small dot-like compact sporoplasmic nuclei of 0.8 ± 0.11(0.6-0.9) diameter are situated centrally (Figs 1, 3). These are transversely parallel or oblique in position. Iodinophilous vacuole and mucus envelope around spores are absent.

Spore index:

LS: WS = 1: 0.939

LP: WP = 1: 0.548

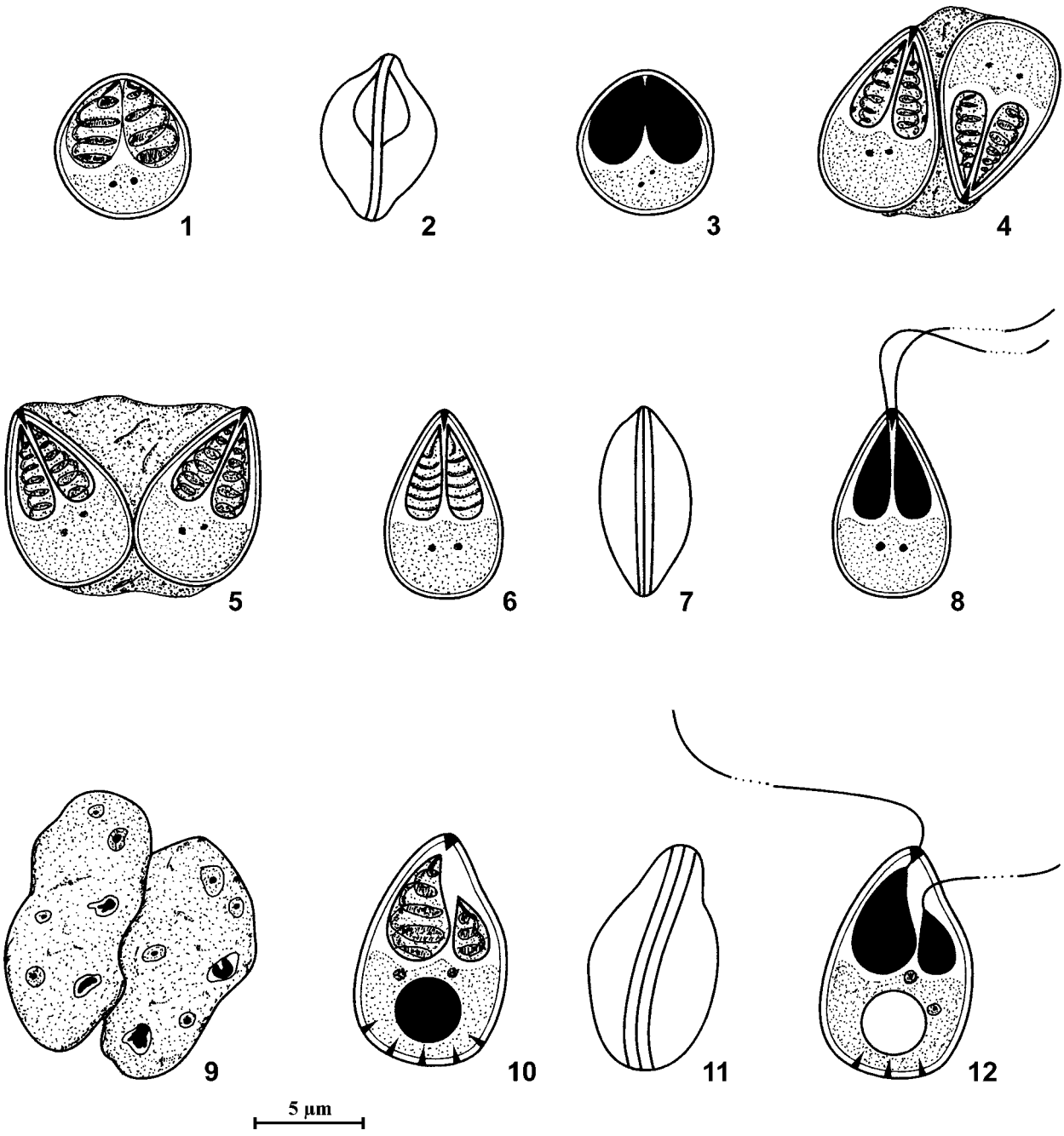
LS: LP = 1: 0.636

WS: WP = 1: 0.371

Intraspecific variability: the present myxozoan obtained from the gill lamellae of *Lates calcarifer* (Bloch) shows little variations among the specimens. The anterior end in a few spores is slightly pointed. Anterior extremity of two polar capsules is almost fused in some forms. A few spores with coarse sporoplasmic granules are also common in some subpopulation.

Taxonomic affinities: the present myxozoan species is assigned to the genus *Myxobolus* Bütschli, 1882 in spite of the absence of the iodinophilous vacuole in the sporoplasm of the spore since several authors have been considering the genus *Myxosoma* Thélohan, 1892 a junior synonym of the genus *Myxobolus* (Walliker 1968, Mitchell 1977, Lom and Noble 1984, Landsberg and Lom 1991, Lom and Dyková 1992) as mere presence of an iodinophilous vacuole in the sporoplasm of the spore can not be accepted as a distinguishing character for the separation of the genus *Myxosoma* from the genus *Myxobolus*.

The present myxozoan shows similarity with *Myxobolus* (= *Myxosoma*) *funduli* Kudo, 1918; *M. indicum* Tripathi, 1952; *M. barbi* Tripathi, 1952; *M. conspicuus* Kudo, 1966 and *M. ampullaceus,* Lalitha Kumari, 1969 reported from *Fundulus* sp., *Cirrhinus mrigala, Barbus ticto, Notropis gilberti* and *Barbus kolus* respectively in having close morphometric data of the spores. However, the spherical spores of the present species are apart from the pyriform spores of *M. funduli*. The present myxozoan differs from *M. barbi, M. conspicuus* and *M. ampullaceus* by the absence of an iodinophilous vacuole in the sporoplasm of the spore while the iodinophilous vacuole is present in last three myxozoans. Moreover, the spores of *M. conspicuus* and *M. ampullaceus* are broader than the present myxozoan (breadth of *M. conspicuus* and *M. ampullaceus* are 6.5-8.0 and 6.4-7.9 respectively). The similarity of the present species with *M. indicum* is untenable since the spores of the former species have two equal polar capsules while the two polar capsules in



Figs 1-3. Camera lucida drawings of spores of *Myxobolus calcariferum* sp. n.; **1** - fresh spore in valvular view - Lugol's iodine; **2** - fresh spore in sutural view - Lugol's iodine; **3** - fixed spore in valvular view - Giemsa.
Figs 4-8. Camera lucida drawings of plasmodia and spores of *Myxobolus chinsurahensis* sp. n.; **4, 5** - Disporous pansporoblasts - Lugol's iodine; **6** - fresh spore in valvular view - Lugol's iodine; **7** - fresh spore in sutural view - Lugol's iodine; **8** - fixed spore in valvular view with extruded polar filaments - Giemsa.
Figs 9-12. Camera lucida drawings of plasmodia and spores of *Myxobolus mrigalhitae* sp. n.; **9** - multinucleate pansporoblasts - Lugol's iodine; **10** - fresh spore in valvular view - Lugol's iodine; **11** - fresh spore in sutural view - Lugol's iodine; **12** - fixed spore in valvular view with extruded polar filaments - Giemsa.

the latter species are unequal. It also disagrees with *M. barbi* in the absence of an intercapsular ridge (intercapsular ridge is distinct in *M. barbi*).

Further, the present myxozoan closely resembles in shape with *M. carnaticus* Seenappa et Manohar, 1980, *M. curmucae* Seenappa et Manohar, 1980 and *M.vanivilasae* Seenappa et Manohar, 1980. But the dimensions of spores of *M. carnaticus* (8.6 x 6.8) are larger than in the present species. Moreover, presence of intercapsular ridge and unequal polar capsules distinctly clarify their dissimilarity. The present species differs from *M. curmucae* and *M. vanivilasae* as spores of *M. curmucae* and *M. vanivilasae* with intercapsular ridge (which is absent in the present form) are larger (dimensions of spores of *M. curmucae* and *M. vanivilasae* are 9.8 x 7.6 and 8-10 x 7-9 respectively) and possess both equal and unequal polar capsules. Furthermore, the present species under study resembles *M. anili* Sarkar, 1989 in shape, presence of equal polar capsules and absence of iodinophilous vacuole. But the spores of latter species are broadly ellipsoid with slightly acuminate anterior end (in the former, spores are spherical with rounded anterior end); polar capsules have short neck like structure in *M. anili,* which is not encountered in the present species under consideration too.

In view of such differences with the closely related species, the present myxozoan has been considered as a new species for which the name *Myxobolus calcariferum* sp. n. is given.

Type host: *Lates calcarifer* (Bloch).

Type locality: Naihati, 24 Parganas (North), West Bengal, India.

Type specimens: paratypes are spores stained in Giemsa, in the collection of H.W.M. Laboratory of Parasitology, U. S. A., No. HWML 16707.

Prevalence and intensity of infection: 67/385 (17.40 %).

Etymology: the species is named after its type host *Lates calcarifer* (Bloch).

***Myxobolus chinsurahensis* sp. n. (Figs 4-8)**

Plasmodia: numerous yellowish kidney shaped plasmodia are attached with the scales of infested host fishes - *A. testudineus*. These are 59-73 long and 29-41 wide and contain some late developmental stages (Figs 4, 5) as well as mature spores (Figs 6-8).

Spores: mature histozoic spores are 8.4 ± 0.43 (8.0-9.7) x 5.4 ± 0.29 (5.1-6.1), creamy white to yellowish in colour, tear shaped in valvular view with rounded posterior and bluntly pointed anterior ends (Figs 6,8), although a few spores show a slightly pyriform shape in valvular view. In sutural view the spores are lenticular with prominent sutural ridge and line in fresh as well as in Lugal's iodine preparations (Fig. 7). The shell valves are moderately thick, uniform, without any parietal fold or markings. There lies a prominent but very small intercapsular ridge (Figs 6,8).

Polar capsules are two in number, equal in size and pyriform with greatly rounded posterior and sharply pointed anterior ends converging closely but open side by side. These are 4.4 ± 0.74 (3.9-6.6) long and 2.1 ± 0.16 (1.8-2.5) wide. Five to six tight coils are formed inside each polar capsule (Fig. 6). Polar filaments are thin and extrude through the anterior bluntly pointed end of the spores in extremely rare cases (Fig. 8).

Granular homogenous mass of sporoplasm fills the extracapsular space of the spore cavity and contains two sporoplasmic nuclei of 0.6 ± 0.13 (0.5-0.9) diameters (Figs 6, 8). Spores lack any mucus envelope around as well as the iodinophilous vacuole within their sporoplasm.

Spore index:

LS: WS = 1: 0.643

LP: WP = 1: 0.477

LS: LP = 1: 0.524

WS: WP = 1: 0.389

Intraspecific variability: general structures of fresh and stained specimens of *Myxobolus chinsurahensis* sp. n. obtained from *Anabas testudineus* (Bloch) are quite identical. However, some minor variations can be noticed in the spore morphology. Some spores have a pyriform shape with slightly elongated anterior necks. The polar capsules are, accordingly, have a rather elongated pyriform shape. In a few instances sporoplasmic distribution is limited just to the posterior part of the polar capsules and not throughout the extracapsular space of the spore cavity.

Taxonomic affinities: according to Tripathi's (1952) grouping of the genus *Myxobolus*, the present species is placed in the first group, as it has polar capsules of equal size and an intercapsular ridge. It resembles *M. mrigalae* Chakravarty, 1939 reported from scales of *Cirrhinus mrigala*; *M. bengalensis* Chakravarty et Basu, 1948 reported from branchiae of *Catla catla*; *M. sphericum* Tripathi, 1952 reported from inner sides of the scales of *Cirrhinus mrigala*; *M. potaili* Lalitha Kumari, 1969 reported from the branchiae of *Labeo potail*; *M.* (=*Myxosoma*) *magauddi* Bajpai, Kundu et Haldar, 1981 from brain of *Trichogaster fasciatus*; *M. rohitae*

Haldar, Das et Sharma, 1983 reported from the scale of *Labeo rohita* and *M. molae* Sarkar, 1993 reported from the Kidney of *Amblypharyngodon mola* in shape as well as in morphometry of the spores. However, the spores of *M. mrigalae* and *M.* (=*M.*) *magauddi* possess a pair of distinctly unequal polar capsules and hence are different from the myxozoan species under study. The spores of *M. potaili* have an anterior knob and striated shell valves, which are not seen in the present species. *M. bengalensis* although possesses spores having a similar shape and length but the slightly narrower spores (breadth of spore of *M. bengalensis* is 6.4-6.8), a pair of larger and narrower polar capsules (dimensions of polar capsule in *M. bengalensis* is 4.2-5.4 x 2.5-3.2) and prominent small intercapsular ridge (which is absent in *M. bengalensis*) make the present species very distinct from the former one. Moreover, the oval spores of *M. molae* with larger dimension [SP: 8.5-9.5 (9.0) x 7.0-8.5 (7.4); PC: 5.0-6.5 (6.0) x 3.8-4.5 (4.0)] possess a small spherical iodinophilous vacuole, which is not observed in the much smaller, and tear shaped forms of the present species under study. The spores of *M. sphericum* and *M. rohitae* resemble the present myxozoan by their site of infection. But shape of the spores in former two species are different (spores of *M. sphericum* is spherical and that of *M. rohitae* are ovoid to rounded in vulvular view) from the present one under consideration as it was tear shaped in structure.

This species also resembles, either in size or in shape, some myxobolid species reported from other countries. These are *M. dispar* Thélohan, 1895; *M. exiguus* Thélohan, 1895; *M. musculi* Keysselit, 1908; *M. lobatus* Dogel, 1932; *M. amurensis* Akhmerov, 1960 and *M.* (=*Myxosoma*) *acuta* Fujita, 1912. Of these, the spores of *M. dispar, M. musculi* and *M.* (=*M.*) *acuta* have a pair of highly unequal polar capsules and hence differ from the present species. *M. exiguus* has larger spores with a few thickenings in the shell valves and its anterior ends are narrow and round which are not found in the spores of the present species. *M. lobatus* shows very close resemblance in the morphometry of the spore and polar capsules. However, the nearly parallel polar capsules and very indistinct intercapsular spine are different from the strongly convergent polar capsules and prominent intercapsular ridge of the myxozoan under consideration. Finally, *M. amurensis* has larger spores than those of the present form. Moreover, the pair of the polar capsules of *M. amurensis* are broadly pyriform and their openings are distantly placed at the perfectly round anterior end of the spore while the spores of the present myxozoan have bluntly pointed anterior ends and a pair of strongly convergent pyriform polar capsules having very closely set openings.

Considering these differences with the other related species, the present myxosporidan species is proposed as a new species and named *Myxobolus chinsurahensis* sp. n.

Type host: *Anabas testudineus* (Bloch).

Type locality: Chinsurah, Hooghly, West Bengal, India.

Type specimens: paratypes are spores stained in Giemsa, in the collection of H.W.M. Laboratory of Parasitology, U. S. A., No. HWML 16708.

Prevalence and intensity of infection: 43/279 (15.41 %).

Etymology: the species is named after its type locality Chinsurah, in West Bengal.

***Myxobolus mrigalhitae* sp. n. (Figs 9-12)**

Plasmodia: fully developed plasmodia are round in shape and attached with the gill filaments of infested host fishes. The earlier stages, uni- and binucleate pansporoblasts are not present but a few multinucleate pansporoblasts are observed (Fig. 9). The multinucleate pansporoblasts are irregular in shape. These have 12 nuclei, which segregate into two equal groups and form 2 sporoblasts. So the pansporoblast is disporous. The nuclei are of different shapes and nature. The capsulogenous nuclei are elongate, valvular nuclei are large and irregular and sporoplasmic nuclei appear oval.

Spores: immature spores are round to spherical in shape. Two polar capsules occupy most part of the spore cavity. Fully developed or mature spores are pyriform in front view (Figs 10, 12) and are 10.8 ± 0.32 (10.2-11.3) x 7.9 ± 0.18 (7.6-8.1) in dimensions. The posterior end is broad and rounded whereas the anterior end is narrow and blunt. The spore valves are thick at the posterior end, but thin at the anterior one. The sutural ridge is broad with straight median sutural line (Fig. 11). In the posterior extremity of the spore there lie 2-5 parietal folds (Figs 10, 12). The inner wall of the spore valve forms a thickening of intercapsular appendix (Figs 10, 12).

Two polar capsules are unequal. The larger one is 4.8 ±0.30 (4.3-5.2) x 2.9 ± 0.16 (2.7-3.2), pyriform with broader posterior and bluntly pointed anterior ends. The smaller polar capsule is tear-shaped and 3.0 ± 0.09 (2.9-3.2) x 2.1 ± 0.06 (2.0-3.2) in measurement with slightly curved anterior end. The polar filament makes

5-6 and 3-4 spiral coils inside larger and smaller polar capsules respectively (Fig. 10). Polar filament from the larger polar capsule extrudes through the anterior end of the spore but that from the smaller one extrudes through the anterolateral end, i.e., there lie two separate openings for the extrusion of two polar filaments (Fig. 12).

The sporoplasm is granular, homogenous and fills the extracapsular space. Two karyosomatic sporoplasmic nuclei of 1.1 ± 0.08 (1.0-1.2) diameter and a spherical glycogen containing iodinophilous vacuole having a diameter of 3.0 ± 0.09 (2.9-3.3) are present in the posterior part of the sporoplasm (Fig. 10).

Spore index:

LS: WS = 1: 0.731

LLPC: WLPC = 1: 0.604

LSPC: WSPC = 1: 0.7

LLPC: LSPC = 1: 0.625

WLPC: WSPC = 1: 0.724

Intraspecific variability: the variability in the spore and polar capsule morphology among the individuals of *Myxobolus mrigalhitae* sp. n. obtained from the gill filaments of hybrid between Mrigal and Rohu carp during the study period was not very significant. However, spore with slightly acuminate anterior end and larger polar capsule with 7-8 turns of polar filament were noticed in very few forms.

Taxonomic affinities: the present species with a pair of unequal polar capsule and an intercapsular ridge well satisfies the generic character of *Myxobolus* Bütschli, 1882 and can well be placed in the last fourth group of Tripathi's (1952) grouping of the genus *Myxobolus.*

The present species shows similarities either in shape or in morphometry with *M. toyami* Kudo, 1915 reported from the connective tissue of gill lamellae of *Cyprinus carpio haematopterus; M. mrigalae* Chakravarty, 1939 reported from the scales of *Cirrhinus mrigala; M. indicum* Tripathi, 1952 reported from the scales, liver and intestinal wall of *Cirrhinus mrigala; M. anisocapularis* Shulman, 1962 reported from the gill lamellae of *Hemibarbus labeo* and *H. maculatus; M. chondrostomi* Donec, 1962 reported from the muscles of *Chondrostoma nasus; M. koli* Lalitha Kumari, 1969 reported from the liver and intestine of *Barbus punjaubensis; M. bhadrensis* Seenappa et Manohar, 1981 reported from the muscle of *Labeo rohita* and *M. vedavatiensis* Seenappa et Manohar, 1981 reported from the gills of *Cirrhinus mrigala*.

Of these above mentioned species, the spores of *M. toyami* (LS: 14.0-15.0); *M. anisocapsularis* (LS: 15.0-15.5); *M. chondrostomi* (LS: 13.5-17.0) are larger than that in the present species. The spores of *M. mrigalae* (SP: 7.2 x 8.2, LPC: 5.1 x 3.0, SPC: 3.0 x 2.0); *M. koli* (SP: 8.4 x 6.0, LPC: 4.3 x 2.8, SPC: 2.0 x 1.2) and *M. osmaniae* (SP: 12.0-15.0 x 7.1-10.0, LPC: 5.0-7.1 x 2.1-3.6, SPC: 2.9-3.9 x 1.4-2.9) show much similarity with the present species. However, spherical to oval spore of *M. mrigalae,* oval spore of *M. koli* and somewhat pyriform spore of *M. osmaniae* are distinctly different from the pyriform spore of the species under study. Similarly beside the shape, the spores of *M. bhadrensis* (SP: 9.5 x 7.1, LPC: 3.5 x 3.2, SPC: 2.5 x 1.7) and *M. vedavatiensis* (SP: 13.8 x 9.2, LPC: 6.0-7.0 x 3.0-4.0, SPC: 3.0-5.0 x 2.0-3.0) are morphometrically different from the spores of present myxozoan. The present species also differs from the oval (SP: 9.5-10.8 x 7.5-8.2) and much smaller polar capsules (LPC: 2.7-3.6 x 1.8, SPC: 1.8 x 1.0) of *M. indicum.*

Besides, the present myxozoan is also compared with a very recent species of the genus *Myxobolus*-*M. ophthalmusculata* Basu et Haldar, 2002 reported from the eye muscles of *Cirrhinus mrigala.* But the pyriform spore with smooth shell valves and slightly undulating sutural ridge of the latter species is larger (SP: 13.1 x 8.0) in dimensions from the pyriform spore with 2-5 parietal folds and straight median suture of the present species under consideration. Furthermore, the host of the present species is a hybrid between Mrigal (*Cirrhinus mrigala*) and Rohu (*Labeo rohita*) carp from which no such species are described earlier.

Considering the above facts, it is evident that the present species is a new myxozoan and hence it has been proposed as *Myxobolus mrigalhitae* sp. n. in this dissertation.

Type host: hybrid between Mrigal (*Cirrhinus mrigala*) and Rohu (*Labeo rohita*) carp.

Type locality: Kalna, Burdwan, West Bengal, India.

Type specimens: paratypes are spores stained in Giemsa, in the collection of H.W.M. Laboratory of Parasitology, U. S. A., No. HWML 16706.

Prevalence and intensity of infection: 241/372 (64.72%).

Etymology: the species is named after its type host hybrid between Mrigal (*Cirrhinus mrigala*) and Rohu (*Labeo rohita*) carp.

DISCUSSION

In this communication three new species of *Myxobolus* Bütschli, 1882 have been reported among which two viz. *Myxobolus calcariferum* sp. n., *Myxobolus chinsurahensis* sp. n., although have no iodinophilous vacuole are placed in the genus *Myxobolus* Bütschli, 1882 based on the spore characters given by Kudo (1920, 1933).

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Short Communication

Response of *Amoeba proteus* to Microinjection with Active Rac1 and RhoA Proteins

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Summary. Molecular mechanisms underlying the unique locomotion of highly motile free-living *Amoeba proteus*, a widely used model of amoeboid movement, still remain unknown. Recently, we have shown that blocking amoeba endogenous Rac- and Rho-like proteins led to the distinct and irreversible changes in the behaviour of these large locomoting cells and to a significant inhibition of their locomotion. To further elucidate the mechanism of Rho pathway, we tested the effect of introduction of active recombinant human RhoA and Rac1 proteins on amoebae cells. While the degree of the inhibition of migration of cells treated with both proteins seems to be similar to cells microinjected with anti-RhoA and anti-Rac1 antibodies, there are distinct differences in cell behaviour and morphology when compared with the blocked phenotypes. The results indicate the important and complex role of Rho-family proteins in amoebae migration.

Key words: *Amoeba proteus*, cell behaviour, cytoskeleton, locomotion, Rac, Rho.

INTRODUCTION

Rho family low-molecular GTP-binding proteins have been found in all eukaryotic organisms - from amoebae (Rędowicz and Korn 2000) and plants (Valster *et al*. 2000) to humans (Hall 1998). It has been known from more than a decade now that its members, Rho, Rac and Cdc42, govern many cellular functions associated with the actin-based cytoskeleton such as cell motility, endocytosis and exocytosis, muscle contraction, neurite outgrowth or cytokinesis (Takai *et al*. 2001). These proteins are active in GTP-bound form and inactive in GDP-bound form. Since their intrinsic GTPase activity is very low, the hydrolysis is enhanced by several regulatory proteins. GDP to GTP exchange is stimulated by guanine nucleotide exchange factor (GEF), GTPase-activating proteins (GAPs) increase the intrinsic rate of GTP hydrolysis and, in addition, guanine nucleotide dissociation inhibitors (GDIs) inhibit both the exchange of GTP and the hydrolysis of the bound GTP (Takai *et al*. 2001). It has been found that microinjection of active RhoA or its constitutively active mutant (Val14RhoA) into quiescent fibroblasts promotes stress fibers and focal adhesion assembly, active Rac1 stimulates

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lamellipodia and membrane ruffles formation, and activation of Cdc42 results in producing filopodia and associated adhesion complexes (Ridley 1995, Hall 1998). Rnd1, also a member of Rho family, promotes disassembly of actin filament structures and loss of cell adhesion (Nobes *et al*. 1998). Rho family proteins exert their functions through the activation of target proteins that upon binding to the active form of Rho family member are activated and affect their specific substrate proteins. RhoA binds, for example, to Rho-associated kinase (ROCK or ROK) that, in turn, phosphorylates and affects several cytoskeletal proteins such as myosin light chain phosphatase, myosin regulatory light chains, vimentin, cofilin or ezrin/moesin/radixin (Rędowicz 1999). Rac1 and Cdc42 activate, among others, PAK kinase (p21-activated kinase) that phosphorylates myosin regulatory light chains, myosin light chain kinase or amoeboid myosin I heavy chain (Brzeska *et al*. 1999, Daniels and Bokoch 1999).

The role of Rho family proteins in amoebae remains practically an unexplored area despite the distinct organization of actin-based cytoskeleton in amoeba cells (Stockem and Kłopocka 1988). Godbold and Mann (2000) have shown that expression in *Entamoeba histolytica* of C3 transferase, the specific RhoA inhibitor, resulted in the inhibition of its cytolytic activity thus indicating the involvement of Rho-dependent signal transduction in amoeba pathogenicity. Our previous observations that inhibition of endogenous Rac1-like and RhoA-like proteins leads to the significant and irreversible changes in morphology and locomotion of *Amoeba proteus* also confirm the crucial role of these small GTPases in protozoans, and suggest a possibility of a different mechanism of the Rho-mediated pathway (Kłopocka and Rędowicz 2003).

Here, by observing amoebae microinjected with active recombinant human Rac1 and RhoA, we attempted to further elucidate the role of Rho family proteins in *Amoeba proteus.*

MATERIALS AND METHODS

Cell culture

Amoeba proteus (strain Princeton) was cultured at room temperature in the standard Pringsheim medium [0.848 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.081 mM $MgSO_4 \cdot 7H_2O$, 0.112 mM $Na_2HPO_4 \cdot 2H_2O$, 0.349 mM KCl, 0.007 mM $FeSO_4 \cdot 7H_2O$; pH 6.8-7.2]. Amoebae were fed on *Tetrahymena pyriformis* twice a week and always used for experiments on the third day after feeding.

Protein samples

The recombinant human fusion GST-RhoA and GST-Rac1 proteins (GST - glutathione S-transferase) were expressed in the standard *E. coli* expression system using pGEX-2T vector as it has been described by Self and Hall (1995). The plazmids carrying the wild type RhoA and Rac1 proteins were from Dr. S. Gutkind laboratory (NIH, Bethesda). The expressed proteins were purified to homogeneity using glutathione-Sepharose 4B beads (Amersham, Pharmacia) and stored at -20°C, after an overnight dialysis at 4°C, in a buffer containing 25 mM Tris-HCl, 50 mM KCl, 4 mM EGTA, 20% glycerol, 0.2 mM DTT, 0.1 mM PMSF, 1μM GDP, and a set of protein inhibitors ("Complete" tablets from Roche). The exchange of GDP into GTP bound into the active site was essentially based on the method described by Yamamoto *et al*. (1990). Briefly, protein aliquots were taken directly before the experiment, adjusted to 12.5 mM Tris HCl, 5 mM EDTA and 0.5 mM DTT, supplemented with 2 mM GTP (from Sigma, USA) and incubated for 10 min at 30°C following the addition of $MgCl_2$ up to 10 mM final concentration. The GTP-bound RhoA and Rac1 were used for microinjection within 30 min after treatment.

Locomotion studies

The effect on *Amoeba proteus* was examined at room temperature by observation of living amoebae after microinjecting them with GTP-bound GST-Rac1 and GST-RhoA. The final concentrations of microinjected proteins after 1:10 dilution with Pringsheim medium were 45 μg/ml for GST-RhoA, and 45 and 110 μg/ml for GST-Rac1, which are within the range used for studies on fibroblasts (Ridley 1995). Control cells were microinjected with either Pringsheim medium or a buffer in which the proteins were prepared (see the Protein samples section). In each experiment, not less than seven amoebae were examined. Microinjections were carried out directly on standard microscopic slides with glass micropipettes held in an Eppendorf micromanipulator. After each microinjection the sequence of up to 500 DIC Nomarski images has been acquired. The time-lapse (5-s intervals) images have been registered using cooled CCD camera Retiga 1300 (QImaging Co., Canada) connected by firewire interface to PC computer running AQM advance 6-image acquisition software (Kinetic Imaging Ltd., U. K.). The same computer program was used to assess the velocity of locomotion of the frontal edge(s) and retraction of the uroid. The rate of migration of both uroidal and frontal parts were calculated separately.

RESULTS AND DISCUSSION

Cells microinjected either with the control buffers (Figs 1a and 1b) or with the GTP-bound forms of Rac1 (Fig. 2a) and RhoA (Fig. 2b) were observed and recorded for about 30 min after treatment, and the results are demonstrated by selected images.

Amoebae microinjected with Pringsheim medium (Fig. 1a) or the protein buffer (Fig. 1b) resumed the normal migration within two to five minutes after the treatment. Amoebae microinjected with GTP-binding

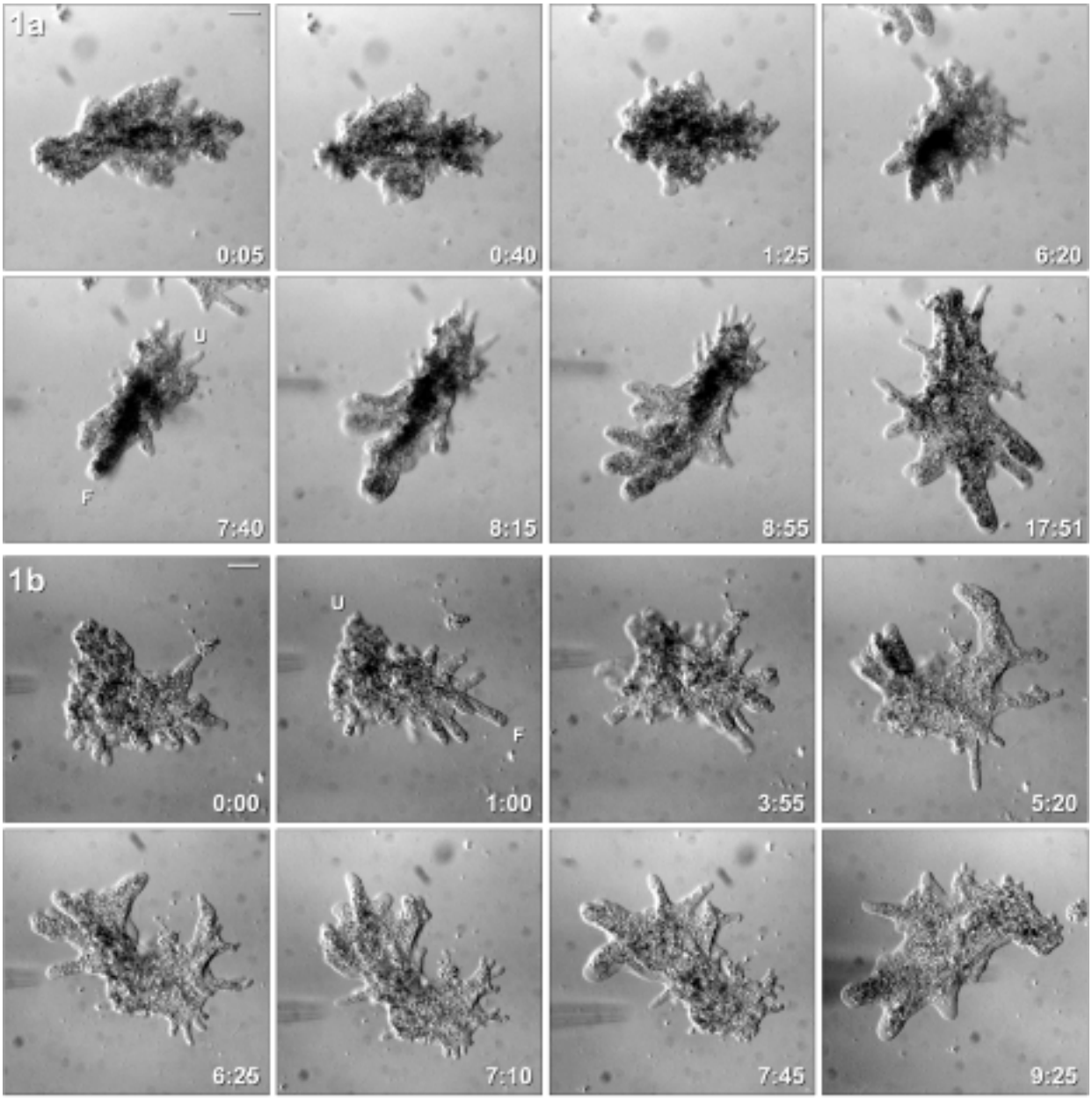

Fig. 1. Behaviour of *Amoeba proteus* after microinjection with either Pringsheim medium (a) or the buffer in which proteins were prepared (b). Numbers in the left bottom corner of each panel in this figure and in Fig. 2 reflect time (in min) after the treatment. Scale bar - 50 µm.

proteins remained irreversibly changed during the course of the experiment (Fig. 2a and b).

Cells introduced with 45 µg/ml Rac-GTP (Fig. 2a) attempted to migrate within three to four minutes after injection but their locomotive shape as well as the rate of migration were changed in comparison to control cells. They also did not appear to be so flat as the cells treated with anti-Rac antibodies (Kłopocka and Rędowicz 2003), they extended few wide pseudopodia that were able to advance in various directions. Adding about three times more of protein (110 µg/ml) caused even more pronounced changes (not shown).

Cells treated with Rho-GTP (Fig. 2b), contrary to cells treated with anti-RhoA antibodies or C3 trans-

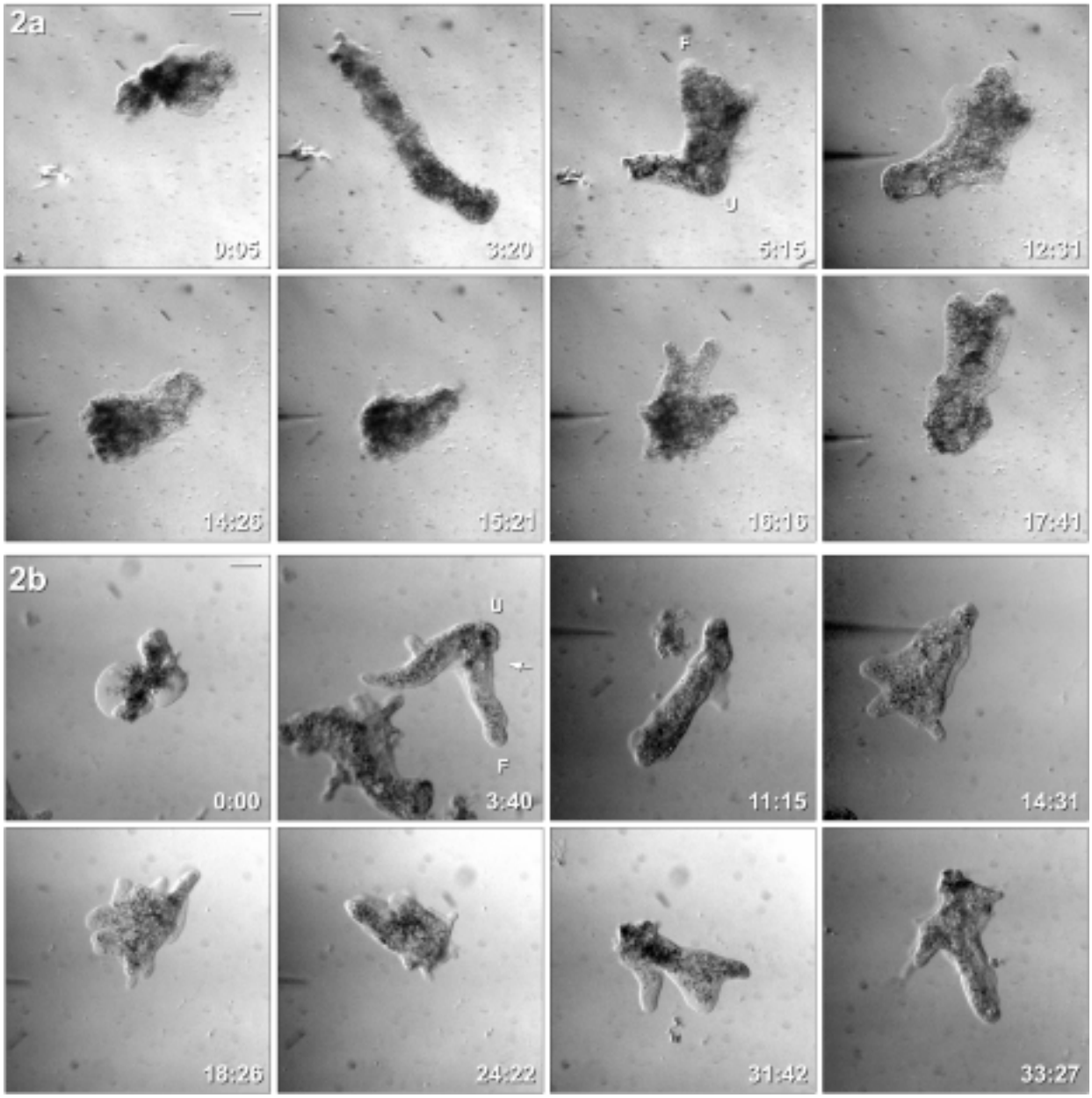

Fig. 2. Behaviour of *Amoeba proteus* after microinjection with the GTP-bound GST-Rac1 (a) and GTP-GST-RhoA (b) proteins at the 45 µg/ml concentration. Scale bar - 50 µm.

ferase (Kłopocka and Rędowicz 2003) were able to dislocate, did not round up and formed up to few, sometimes very long, protrusions.

Quantification of the migration rates of the cells microinjected either with control buffers or with active RhoA and Rac1 proteins, calculated separately for front and uroid, is presented in Fig. 3. Advancing of the frontal edges was inhibited about 41% and 48% when cells were treated with 45 µg/ml of RhoA and Rac1, respectively. The rate of the uroidal retraction was also lower than that of control cells, with the observed inhibition of about 50% for RhoA and 45% for Rac1 (Fig. 3a).

It is noteworthy that amoebae microinjected with Rac or Rho exhibited a kind of sequential pattern of migration in which, after several minutes of impaired locomotion, there were several periods lasting up to ten seconds

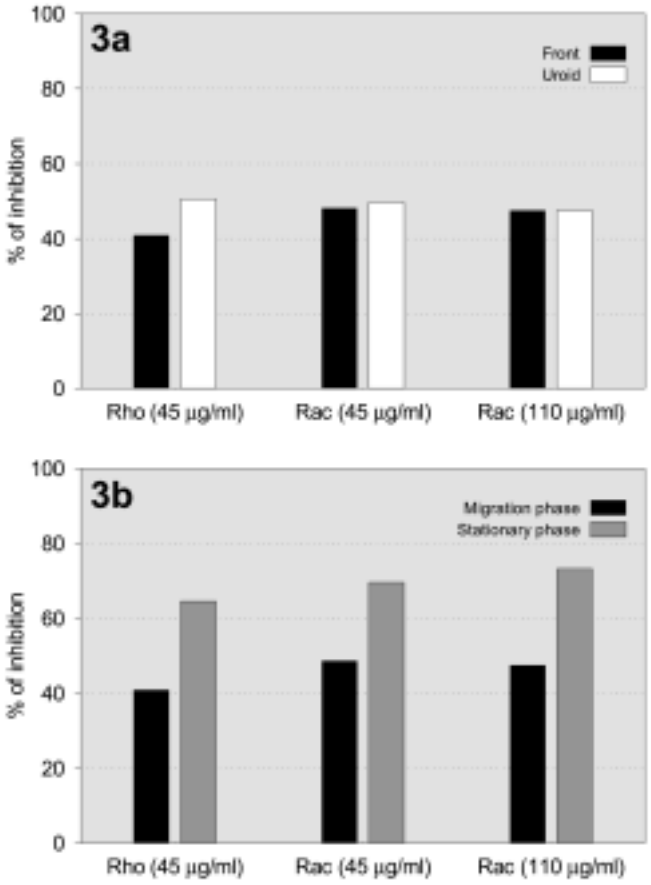

Fig. 3. Effect of introducing of active RhoA (45 µg/ml) and Rac1 (45 µg/ml and 110 µg/ml) on the rate of frontal progression and uroidal locomotion during the migration phase (a) and front advancing during the stationary phase (b). Results are expressed as % of the control cells migration rate.

when cells contracted, rounded up, strongly adhered to the glass surface and practically stopped locomotion (Figs 2a, 15:21; 2b, 24:22). Afterwards they resumed the migration and after several minutes they stopped again. During these periods (termed here as stationary phases) amoebae were yet able to extend protrusions but the rate of pseudopodial migration has significantly decreased when compared to cells treated with the buffer alone or during their migration phase. The inhibition, compared to control cells, was about 65% for cells treated with RhoA, about 70% for cells microinjected with 45 µg/ml of Rac1, and about 73% for cells treated with 110 µg/ml of Rac1 (Fig. 3b). Phenotypes observed during stationary phases resembled the phenotypes of amoebae in which endogenous Rac- and Rho-like proteins had been blocked with anti-RhoA and anti-Rac1 antibodies (Kłopocka and Rędowicz 2003). Interestingly, shortly after the resumption of the migration the inhibition of formation of the protrusion reached the overall frontal migration of treated amoebae (about 41% for RhoA and about 48% for both Rac1 concentrations). It is plausible to think that the stationary phase reflects the state in which GTP bound in the active sites of the introduced proteins has been *in vivo* exchanged to GDP (Takai *et al*. 2001).

It has been earlier observed that constitutively active Rac1 protein (Val12Rac) did not affect fibroblasts migration in the wound closure test (Nobes and Hall 1999). However, introducing constitutively active Rho A protein (Val14Rho) caused about 95% inhibition of wound closure (Nobes and Hall 1999). Constitutively active Cdc42 protein (Val12Cdc42), the member of Rho family that has not been found in different amoebae (Lohia and Samuelson 1996, Rędowicz and Korn 2000, Kłopocka and Rędowicz 2003) and in slime mold *Dictyostelium discoideum* (Wilkins and Insall 2001), had practically no effect on fibroblasts migration (Nobes and Hall 1999). The authors explain these results in terms of the cooperation and coordination in time and space of distinct pathways controlled by these Rho family proteins.

Our results, different from the ones reported for fibroblasts, reflect the distinct and complex nature of the Rho-family pathways in *Amoeba proteus*. Because there is very little known about amoebae Rho-family proteins, their structure and functions, it is impossible to speculate on the possible mechanisms underlying the observed phenotypes. Certainly, one should keep in mind the gross differences in the complexity and dynamics of amoebae actin-based cytoskeleton, and the fact that these cells can migrate even about 500 faster that the fibroblasts in the wound closure test. These free-living cells are always ready to act and they probably do not need for their survival the signal transduction machinery as it has evolved in metazoan cells. It is also quite possible that amoebae have more, unknown yet, members of Rho family as it has been found in *E. histolytica* (Lohia and Samuelson 1993, 1996) and *D. discoideum* (Wilkins and Insall 2001). Introducing the excess of one of them may shift their delicate balance and activate the other protein(s) pathway(s) that under normal conditions are not functioning during migration. The role of the amoebae Rho-family proteins certainly needs to be further investigated since, besides the contribution to the general knowledge, the results might have more practical aspect, as it has been shown by Godbold and Mann (2000) inactivation of Rho-dependent signal transduction

pathway in *Entamoeba histolytica* leads to decrease of its invasiveness.

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