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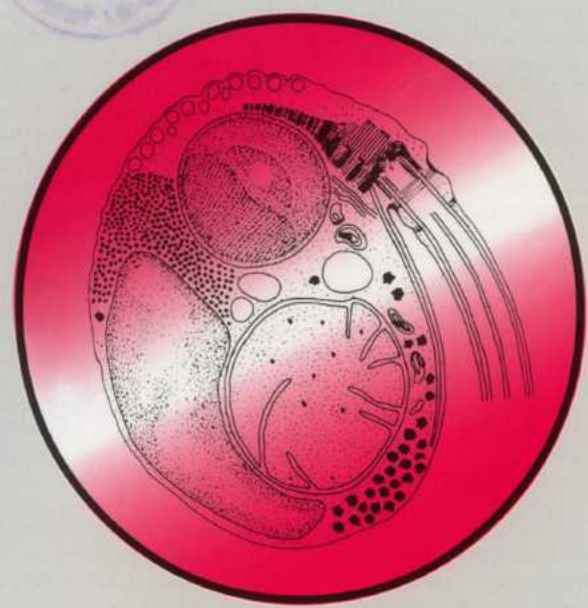
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A Cladistic Approach for the Classification of Oligotrichid Ciliates (Ciliophora: Spirotricha)

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Summary. Currently, gene sequence genealogies of the Oligotrichea Bütschli, 1889 comprise only few species. Therefore, a cladistic approach, especially to the Oligotrichida, was made, applying Hennig's method and computer programs. Twenty-three characters were selected and discussed, i.e., the morphology of the oral apparatus (five characters), the somatic ciliature (eight characters), special organelles (four characters), and ontogenetic particulars (six characters). Nine of these characters developed convergently twice. Although several new features were included into the analyses, the cladograms match other morphological trees in the monophyly of the Oligotrichea, Halteriia, Oligotrichia, Oligotrichida, and Choreotrichida. The main synapomorphies of the Oligotrichea are the enantiotropic division mode and the *de novo*-origin of the undulating membranes. Although the sister group relationship of the Halteriia and the Oligotrichia contradicts results obtained by gene sequence analyses, no morphologic, ontogenetic or ultrastructural features were found, which support a branching of *Halteria grandinella* within the Stichotrichida. The cladistic approaches suggest paraphyly of the family Strombidiidae probably due to the scarce knowledge. A revised classification of the Oligotrichea is suggested, including all sufficiently known families and genera.

Key words: classification, computer programs, *Halteria* problem, Hennig's cladistic method, taxonomy.

INTRODUCTION

Since the Oligotrichea have not, except for the tintinnids, left fossil records, their phylogeny can only be reconstructed from the known features of extant species. In 1992, Petz and Foissner proposed the first cladistic system for the Oligotrichea on suprafamilial level, using morphologic and ontogenetic features. Ac-

cording to their genealogy and revised classification, the Halteriia are an adelphotaxon to the subclass Oligotrichia, which contains two orders, the Strombidiida and the Oligotrichida with the suborders Tintinnina and Strobilidiina (Fig. 1a). In earlier and even some recent classifications, however, the halteriids are a sister taxon to the strombidiids (Fig. 1b; Kahl 1932, Fauré-Fremiet 1970, Corliss 1979, Small and Lynn 1985, Maeda 1986, Montagnes and Lynn 1991, Laval-Peuto *et al.* 1994, Song *et al.* 1999, Lynn and Small 2002). Likewise, gene sequence analyses do not reflect the results of Petz and Foissner (1992) and of the other authors mentioned because *Halteria grandinella* clusters with the

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stichotrich *Oxytricha granulifera* (Fig. 1c; Baroin-Tourancheau *et al.* 1992, Hoffman and Prescott 1997, Shin *et al.* 2000, Bernhard *et al.* 2001, Snoeyenbos-West *et al.* 2002, Croft *et al.* 2003, Hewitt *et al.* 2003, Modeo *et al.* 2003, Strüder-Kypke and Lynn 2003, Agatha *et al.* 2004). On the other hand, the separation of the tintinnids and strobilidiids from the Oligotrichida, based on the shape of the membranellar zone (closed *vs.* C-shaped), is widely accepted and supported by gene sequence data (Small and Lynn 1985, Petz and Foissner 1992, Laval-Peuto *et al.* 1994, Lynn and Small 2002); only Song *et al.* (1999) followed Kahl's (1932) classification in assigning the aloricate Strobilidiidae, Strombidiidae, and Halteriidae to the same suborder Oligotrichina separated from the loricate tintinnids.

Although molecular methods are frequently regarded as superior to traditional ones, using the subjective evaluation of morphologic characters, different molecules and methods often provide conflicting conclusions (Chen and Song 2002, Mayr and Bock 2002). Furthermore, currently too few gene sequences of Oligotrichea are available to elucidate their phylogenetic relationships at familial and generic level. Thus, a cladistic approach was made based on morphologic, ontogenetic, and ultrastructural data, and especially on the evolution of the ciliary patterns suggested by Agatha (2004).

MATERIALS AND METHODS

Cladistic analyses. The phylogenetic relationships within the Oligotrichea, with emphasis on the Oligotrichida, were elucidated by applying Hennig's argumentation method (Hennig 1982, Ax 1984, Sudhaus and Rehfeld 1992) and the computer programs PAUP 4.0b10 (Swofford 2002), HENNIG86, and FreeTree (http://www.natur.cuni.cz/~flegr/programs/freetree) with the Hypotrichea, i.e., the hypotrichs and stichotrichs, as out-group. The parsimony tree generated by the PAUP-program was founded on differently weighted features (for details, see Table 2), while equivalent weighting was used for the parsimony calculations with HENNIG86 and in the distance matrix cladogram produced with FreeTree (Jaccard index, UPGMA average linkage method, bootstrap re-sampled 1,000 times). The cladograms were printed by TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Morphologic, ontogenetic, and ultrastructural data from the original literature were the basis for the analyses (Grim 1974, Mirabdullaev 1985, Foissner *et al.* 1988, Song 1993, Agatha and Riedel-Lorjé 1998, Agatha 2003b, as well as the papers cited in Maeda 1986 and Agatha 2004). However, only sufficiently known genera were considered, i.e., *Cyrtostrombidium* Lynn and Gilron, 1993; *Laboea* Lohmann, 1908; *Limnostrombidium* Krainer, 1995; *Novistrombidium* Song and Bradbury, 1998; *Parallelostrombidium* Agatha, 2004; *Paratontonia* Jankowski, 1978; *Pelagostrombidium* Krainer, 1991;

Omegastrombidium Agatha, 2004; *Pseudotontonia* Agatha, 2004; *Spirotontonia* Agatha, 2004; *Spirostrombidium* Jankowski, 1978; *Strombidium* Claparède and Lachmann, 1859; and *Tontonia* Fauré-Fremiet, 1914. Other genera, such as *Echinostrombidium* Jankowski, 1978, *Lissostrombidium* Jankowski, 1978, *Metastrombidium* Fauré-Fremiet, 1924, *Peristrombidium* Jankowski, 1978, and *Seravinella* Alekperov and Mamajeva, 1992, were not taken into account because their type species are insufficiently known. Twenty-three characters were selected.

Terminology. Halteriids have two undulating membranes (Figs 2a, b): the inner membrane is named endoral, while the outer membrane is called paroral; both are assumed to correspond to the endoral and paroral of the Stichotrichida (Szabó 1935). In long-term cultures of halteriids, the paroral is occasionally reduced (Foissner, pers. commun.), a process which probably happened also in the evolution of the Oligotrichia (see below). The homology of the inner membrane of halteriids and oligotrichids is indicated not only by the same position, but also by the *de novo*-origin, monostichomonad structure, and perilemma cover (Petz and Foissner 1992, Petz 1994, Song and Wang 1996, Agatha 2003a, Agatha *et al.* 2004). Thus, the inner membrane of the Oligotrichia should likewise be called endoral (Figs 2c, d). The direction of the spiral of the girdle kinety is determined in top view according to Montagnes and Taylor (1994).

The taxonomic ranks used in the present paper follow the revised classification shown in Table 3.

RESULTS AND DISCUSSION

Characters, character states, and convergences considered

The Oligotrichea share several features with the Hypotrichea: a macronuclear replication band (Salvano 1975, Raikov 1982); an apokinetal development of the oral primordium (Foissner 1996); a conspicuous membranellar zone; and stichomonad undulating membranes on the right side of the buccal cavity (Grain 1972, Laval 1972, Grim 1987, Agatha 2003a). According to Corliss (1979), a stichomonad undulating membrane consists of a single row of identically orientated basal bodies. This is also shown in transmission electron micrographs of *Strombidium* and *Novistrombidium* provided by Modeo *et al.* (2003), although a dikinetidal structure of the endoral is described in the text.

In the Hypotrichea, and at least in dividing cells of Halteria, the somatic kineties are composed of dikinetids, bearing a distinct cilium only at each anterior basal body (Szabó 1935, Grain 1972, Grim 1974, Ruffolo 1976, Petz and Foissner 1992); even the cirri of some stichotrichs show a dikinetidal composition (Wirnsberger-Aescht *et al.* 1989). In contrast to the Hypotrichea and Halteriia, most Oligotrichida have only a single longitudinal kinety,

Table 1. Character states and coding used for the construction of the traditional cladogram shown in Figure 4.

	Character states	
	Apomorphy	Plesiomorphy
1	Paroral lacks (coded 1), paroral and endoral lack (coded 2)	Endoral and paroral (coded 0)
2	Membranellar zone apical (coded 1)	Membranellar zone ventral (coded 0)
3	Membranellar zone closed (coded 1)	Membranellar zone C-shaped (coded 0)
4	Without ventral membranelles (coded 1)	Ventral membranelles (coded 0)
5	Cyrtos-like pharyngeal fibres (coded 1)	Common pharyngeal fibres (coded 0)
6	Reduction of somatic ciliature to ≤ 2 kineties (coded 1)	Comprehensive somatic ciliature (coded 0)
7	Dextral spiral of somatic kineties (coded 1)	Longitudinal somatic kineties (coded 0)
8	Ventral kinety longitudinal (coded 1)	Ventral kinety dextrally spiralled (coded 0)
9	Ends of girdle kinety near posterior end of ventral side (coded 1), posterior portion of girdle kinety inversely orientated and parallel to ventral kinety (coded 2), girdle kinety horizontal (coded 3), girdle kinety sinistrally spiralled (coded 4)	Girdle kinety dextrally spiralled (coded 0)
10	Somatic cilia clavate (coded 1)	Somatic cilia rod-shaped or fusiform (coded 0)
11	Somatic kinetids bare (coded 1)	Somatic kinetids ciliated (coded 0)
12	Somatic cilia arranged in bristle complexes (coded 1)	Somatic cilia arranged in ordinary rows (coded 0)
13	Kinetodesmal fibre of somatic kinetids lacking or transient (coded 1)	Kinetodesmal fibres of somatic kinetids permanent (coded 0)
14	Polysaccharidic cortical platelets (coded 1)	No or other cortical platelets (coded 0)
15	Perilemma (coded 1)	Without perilemma (coded 0)
16	Oligotrichid extrusomes (trichites; coded 1)	No or other extrusomes (coded 0)
17	Tail (coded 1)	Without tail (coded 0)
18	Enantiotropy (coded 1)	Homeotropy (coded 0)
19	Stomatogenesis hypoapokinetal in temporary tube (coded 1) or pouch (coded 2) or permanent neoformation organelle (coded 3)	Stomatogenesis epiapokinetal (coded 0)
20	Posterior end of oral primordium performs clockwise rotation (coded 1)	Anterior end of oral primordium performs anticlockwise rotation (coded 0)
21	Undulating membranes originate *de novo* (coded 1)	Undulating membranes originate from oral primordium or cirral anlagen (coded 0)
22	Entire somatic ciliature originates *de novo* (coded 1)	At least parts of somatic ciliature originate by intrakinetal proliferation (coded 0)
23	Extensive reorganization of somatic ciliature (coded 1)	No or indistinct (intrakinetal) reorganization of somatic ciliature (coded 0)

i.e., the ventral kinety. Nevertheless, its structure is identical to that of the hypotrich and halteriid kineties, and even the girdle dikinetids bear only a single distinct cilium at each left basal body (Fauré-Fremiet and Ganier 1970, Agatha 2003a, Modeo *et al.* 2003, Agatha *et al.* 2004). This peculiarity led to the evolution of the ciliary patterns discussed by Agatha (2004). In the Choreotrichida,

however, the somatic kinetids are probably subject to several secondary modifications (Hedin 1976, Grim 1987, Lynn and Montagnes 1988, Montagnes and Lynn 1991, Agatha 2003b).

The cladistic analyses are founded on four groups of characters: the morphology of the oral apparatus (characters 1-5), the somatic ciliature (characters 6-13),
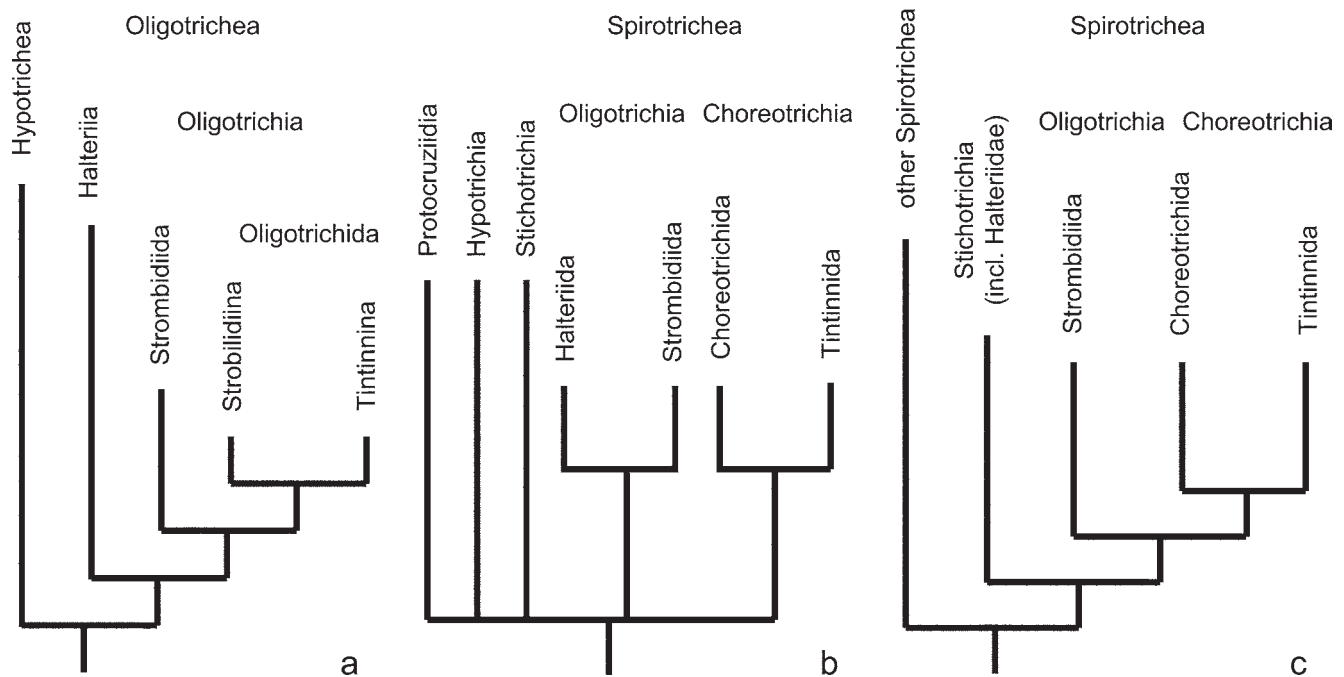
Figs 1a-c. Cladograms showing different models of the phylogenetic relationships within the Spirotricha. **a -** according to Petz and Foissner (1992); **b -** according to Lynn and Small (2002); **c -** according to Strüder-Kypke and Lynn (2003).

special organelles (characters 14-17), and ontogenetic particulars (characters 18-23). The characters and their states are summarized in Table 1 and their distribution over the taxa is summarized in Table 2.

Character 1: Number of undulating membranes. Stichotrichs and some hypotrichs have two undulating membranes. Likewise, halteriids have an endoral and a minute paroral (Figs 2a, b; Szabó 1935, Grain 1972, Petz and Foissner 1992); the latter may be reduced in long-term cultures (Foissner, pers. commun.). Thus, it is assumed that the ancestor of the Hypotrichea (hypotrichs and stichotrichs) and Oligotrichea had two undulating membranes, of which the outer was lost in the Choreotrichida, the Oligotrichida, and convergently in some Hypotrichida, e.g., *Euplotes* (Grain 1972; Ruffolo 1976; Grim 1987; Petz and Foissner 1992; Agatha 2003a, b; Agatha *et al.* 2004). The Cyrtostrombidiidae lack any undulating membrane (own observ.; Lynn and Gilron 1993).

Character 2: Arrangement of membranellar zone. The adoral zone of membranelles is C-shaped and extends on the ventral side of the Hypotrichea. In the Halteriia and Oligotrichida, it is also C-shaped, but occupies the apical cell end. This arrangement is regarded as apomorphy.

Character 3: Shape of membranellar zone. In contrast to the Hypotrichea, Halteriia, and Oligotrichida, the adoral zone of membranelles of the Choreotrichida is circular and thus probably represents a derived state (Fig. 2d).

Character 4: Ventral membranelles. In the Oligotrichea and some stichotrichs, the adoral zone of membranelles is bipartited into large distal and small proximal membranelles. In three oligotrich genera, however, the ventral (proximal) portion is absent: *Cyrtostrombidium* Lynn and Gilron, 1993; *Metastrombidium* Fauré-Fremiet, 1924; and *Seravinella* Alekperov and Mamajeva, 1992. This is likely an apomorphy.

Character 5: Cyrtos. The pharyngeal fibres of *Cyrtostrombidium* Lynn and Gilron, 1993 are thick in protargol preparations, resembling the cyrtos (cytopharyngeal basket) of the Nassophorea, Phyllopharyngea, and Prostomatea (Lynn and Small 2002). Since the fibres of the other Spirotricha are distinctly finer, this feature is probably derived, especially, as it is accompanied by the lack of an endoral and ventral membranelles.

Character 6: Reduction of somatic ciliature. The ancestor of the Hypotrichea and Oligotrichea is sup-
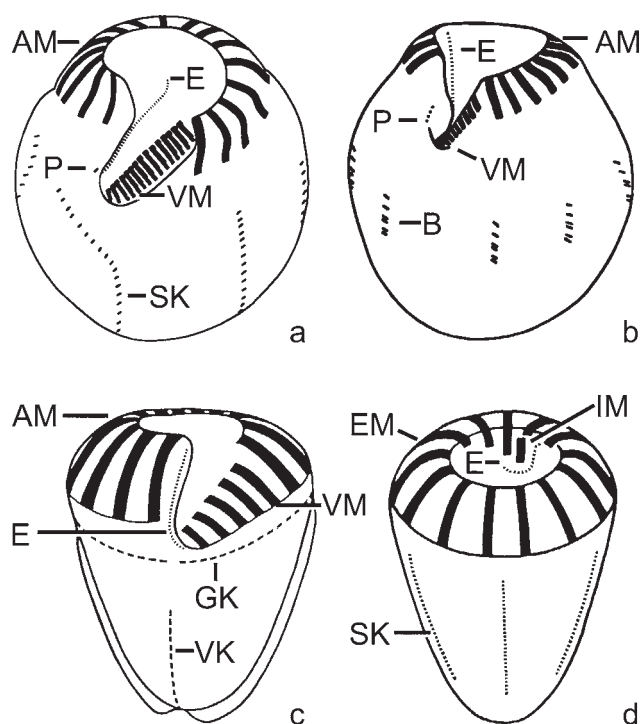
Figs 2a-d. Generalized ventral (a-c) and dorsal (d) views, illustrating some diagnostic features of the halteriid genera *Meseres* (a - modified from Petz and Foissner 1992) and *Halteria* (b - modified from Song 1993) as well as the oligotrichid genus *Strombidium* (c - from Agatha 2004) and the choreotrichid genus *Rimostrombidium* (d). The halteriids *Meseres* and *Halteria* have two undulating membranes, i.e., an outer paroral and an inner endoral, while the Oligotrichida and Choreotrichida possess only an endoral. AM - anterior polykinetids/membranelles, B - bristle complexes, E - endoral, EM - external polykinetids/membranelles, GK - girdle kinety, IM - internal polykinetids/membranelles; P - paroral, SK - somatic kineties, VK - ventral kinety, VM - ventral polykinetids/membranelles.

posed to have several longitudinal kineties, which were reduced to two ciliary rows in the Oligotrichida (Fig. 3b; Agatha 2004). The nature of the tail cilia in the tontoniids (Lynn and Gilron 1993, Suzuki and Song 2001) is uncertain; ontogenetic investigations are required.

Character 7: Dextral spiral of kineties. According to the proposed evolution of the ciliary patterns (Agatha 2004), the two remaining kineties (see Character 6) were located on the dorsal side and performed a dextral rotation parallel to the proximal portion of the adoral zone of membranelles (Fig. 3b). Further, the left kinety, i.e., the future ventral kinety, shortened anteriorly. Probably, this torsion of the oral apparatus is recapitulated during ontogenesis (see Character 20).

Character 8: Orientation of ventral kinety. Due to the dextral spiral of the posterior cell portion, both the ventral and girdle kinety were parallel to each other (Fig. 3b; Agatha 2004). Therefore, a longitudinal orientation of the ventral ciliary row is interpreted as an apomorphy (Fig. 3c).

Character 9: Girdle kinety patterns. Three patterns evolved from the dextrally spiralled course of the girdle kinety, as described by Agatha (2004) and briefly explained in the explanation of Fig. 3.
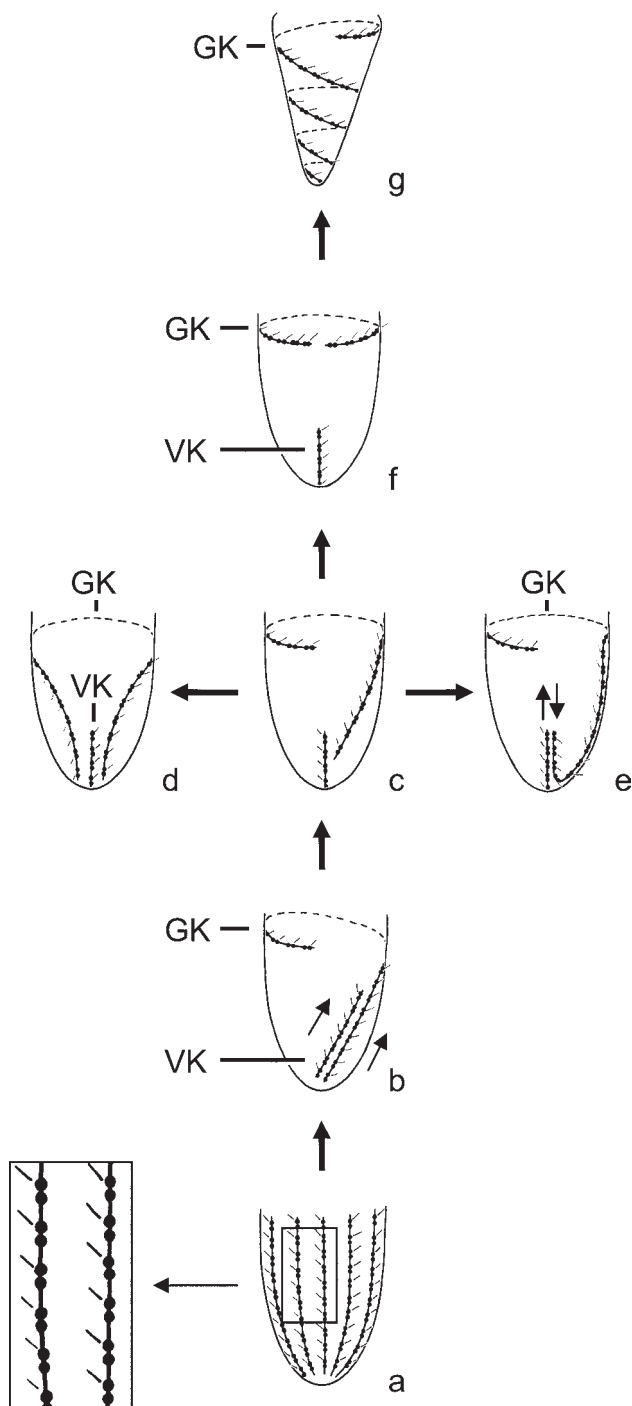
The lack of a ventral kinety in *Pelagostrombidium*, some *Strombidium* species, and probably also in *Laboea strobila* is difficult to interpret but is apparently only a species-specific feature and developed convergently several times.

Character 10: Shape of somatic cilia. Although detailed data are lacking for most Oligotrichia, the occurrence of clavate somatic cilia seems to be restricted to the freshwater genus *Limnostrombidium* (Kahl 1932; Krainer 1991, 1995; Foissner *et al.* 1999). Since cilia are usually rod-shaped or fusiform, clavate ones probably represent the derived state and developed convergently in the gymnostomatid ciliates.

Character 11: Lack of somatic cilia. Live observations, protargol impregnations, and ultrastructural studies show that the somatic kinetids are ciliated in the Hypotrichea, Halteriia, Choreotrichida, and Oligotrichida, except for those of *Pelagostrombidium* (Foissner *et al.* 1999). The latter state is therefore considered as an apomorphy.

Character 12: Bristle complexes. Separate cilia are the common state of the ciliature; accordingly, the bristles complexes of *Halteria* Dujardin, 1841 and *Pelagohalteria* Foissner, Skogstad and Pratt, 1988, that are composed of closely spaced dikinetids with one cilium each (Song and Wilbert 1989, Petz and Foissner 1992), likely represent the derived state.

Character 13: Fibrillar associates of somatic basal bodies. Hypotrichida have typical somatic dikinetids, i.e., with a kinetodesmal fibre, a transverse ribbon, and a postciliary ribbon, while the kinetodesmal fibres are resorbed during late ontogenetic stages in the Stichotrichida (Foissner 1996, Lynn and Small 2002). Data on the kinetid structure of Oligotrichia are only available for *Halteria* and three choreotrichids. While a kinetodesmal fibre is apparently lacking in *Halteria grandinella* (Grain 1972), *Strobilidium velox* (Grim 1987), and *Petalotricha ampulla* (Laval 1972), a short one occurs in *Cyttarocylis brandti* (Laval-Peuto 1994). In this cladistic approach, the lack of a kinetodesmal fibre is also assumed for morphostatic Oligotrichida and is regarded as the apomorphic state, that developed convergently in the Stichotrichida and Oligotrichea.

The distended cell surface in the posterior cell portion of the Oligotrichida is possibly correlated with the occurrence of the polysaccharidic cortical platelets.

Character 15: Perilemma. A perilemma, i.e., an additional layer probably covering the whole plasma membrane, was revealed by ultrastructural investigations of the Oligotrichida *Strombidium*, *Novistrombidium*, and *Tontonia* (Fauré-Fremiet and Ganier 1970, Laval-Peuto and Febvre 1986, Modeo *et al.* 2003), Tintinnina (Laval 1972, Laval-Peuto 1975, Hedin 1976), and several Stichotrichida (Bardele 1981, Wirnsberger-Aescht *et al.* 1989). A structure interpreted as perilemma was also recognized in TEM micrographs of *Laboea strobila* kindly provided by Per R. Jonsson (Tjärnö Marine Biological Laboratory, University of Göteborg, Sweden) and in the halteriid *Meseres corlissi* (Foissner; pers. commun.). Therefore, fixation problems might have caused the loss of the perilemma in *Halteria grandinella* (Grain 1972) and the choreotrichid ciliate *Strobilidium velox* where alveoli are also absent (Grim 1987). On the other hand, it is apparently lacking in the Hypotrichida (Bardele 1981). Bardele (1981) considered the perilemma as a temporary structure in stichotrichs, which is often renewed. Since the cyst wall is formed between the perilemma and the plasma membrane in stichotrichs, it might be a protection for the precursor of the cyst wall (Grimes 1973). Lynn and Corliss (1991) suggested that the perilemma might be a special preparation artifact of the glycocalyx. Nevertheless, its occurrence is apparently restricted to the Oligotrichea and Stichotrichida.

Character 16: Extrusomes. The trichites of strombidiids are extrusomes that differ distinctly in structure, size, and location from the extrusomes of hypotrichs,

Character 14: Cortical platelets. Alveolata are characterized by cortical alveoli, which occasionally contain platelets. Polysaccharidic cortical platelets are restricted to the Oligotrichida (Kahl 1932, Laval-Peuto and Febvre 1986), the heterotrich family Sicuophoridae (Tuffrau 1994), and the dinoflagellates (Taylor 1987); likely, they developed convergently.

Table 2. Distribution of none-hierarchical character states of the taxa cladistically analysed with the computer programs (Fig. 5). Note that multiple character states, such as the number of undulating membranes and the girdle kinety patterns (Table 1), have been separated and that (1) does not always mark an apomorphic state. In the PAUP program (Swofford 2002), the features 4, 6, 7, 20, and 28 have weight 2, features 5, 8, 17, 18, 22, 23, 24, 27, 30, 31 and 32 weight 3; the remaining features have weight 1. ? - character state unknown. A - Hypotrichida, B - Stichotrichida, C - *Meseres,* D - *Halteria/Pelagohalteria,* E - *Tontonia,* F - *Paratontonia,* G - *Pseudotontonia,* H - *Spirotontonia,* I - *Omegastrombidium,* J - *Parallelostrombidium,* K - *Novistrombidium,* L - *Spirostrombidium,* M - *Strombidium,* N - *Laboea,* O - *Limnostrombidium,* P - *Pelagostrombidium,* Q - *Cyrtostrombidium,* R - Choreotrichida.

		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
1	No or one undulating membrane	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2	No undulating membrane	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
3	Monostichomonad structure of undulating membranes	0	0	1	1	1	?	?	1	?	1	1	1	1	1	1	1	1	1
4	Adoral zone of membranelles apical	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
5	Adoral zone of membranelles closed	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
6	Ventral membranelles lacking	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
7	Cyrtos-like pharyngeal fibres	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
8	≤ 2 somatic kineties	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0
9	Girdle kinety's left end close to the cell's posterior	0	0	0	0	1	1	0	0	1	1	1	1	0	0	0	0	0	0
10	Ventral kinety dextrally spiralled	0	0	0	0	?	0	0	0	0	0	1	0	0	0	0	0	0	0
11	Girdle kinety ends near rear end	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
12	Posterior girdle kinety portion inversely orientated	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
13	Girdle kinety horizontal or sinistrally spiralled	0	0	0	0	0	0	1	1	0	0	0	0	1	1	1	1	1	0
14	Girdle kinety sinistrally spiralled	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0
15	Somatic cilia clavate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
16	Somatic kinetids bare	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
17	Bristle complexes	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
18	Cirri	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
19	Kinetodesmal fibres transient or lacking	0	1	?	1	?	?	?	?	?	?	?	?	?	?	?	?	?	1
20	Polysaccharidic cortical platelets	0	0	0	0	1	?	?	?	?	1	1	?	1	1	1	1	?	0
21	Perilemma	0	1	1	?	1	?	?	?	?	?	1	?	1	1	?	?	?	1
22	Oligotrichid extrusomes (trichites)	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0
23	Tail	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0
24	Enantiotropy	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
25	Epiapokinetal stomatogenesis	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
26	Hypoapokinetal stomatogenesis	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
27	Transient stomatogenic tube	1	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	1	0
28	Neoformation organelle	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0
29	Curvature of oral primordium's end	0	0	0	0	?	?	?	1	?	?	1	?	1	1	?	?	?	1
30	Undulating membranes originate *de novo*	0	0	1	1	?	?	?	1	?	?	1	?	1	1	?	?	?	1
31	Entire somatic ciliature originates *de novo*	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
32	Reorganization of somatic ciliature distinct	1	1	1	1	?	?	?	0	?	?	0	?	0	0	?	?	?	0

tintinnids, and strobilidiids (own observ.; Laval-Peuto and Barria de Cao 1987, Wirnsberger and Hausmann 1988, Modeo *et al.* 2001, Rosati and Modeo 2003, Agatha *et al.* 2004); thus, they are regarded as an autapomorphy.

Character 17: Tail. The contractile tail is an apomorphy of the tontoniids due to its complex and unique ultrastructure (Greuet *et al.* 1986, Agatha 2004).

Character 18: Division mode. The enantiotropic division mode is the most important autapomorphy of the Oligotrichea, although a modified (probably convergently developed) form is found in the prostomatid *Pseudobalanion* (Foissner *et al.* 1990, Petz and Foissner 1992, Foissner 1996). The Choreotrichida show a less

pronounced kind of enantiotropy compared to the Halteriia and Oligotrichida (Petz and Foissner 1992, 1993; Dale and Lynn 1998; Agatha 2003b). This difference is probably correlated with the formation of the oral primordium within a pouch and the circular arrangement of almost all membranelles on the oral rim, a structure restricted to the choreotrichids (Fig. 2d).

Character 19: Stomatogenic mode. When Petz and Foissner (1992) established their phylogenetic system, the general validity of the hypoapokinetal stomatogenic mode in the Oligotrichia was uncertain. However, recent studies on *Strombidium* (Petz 1994, Song and Wang 1996, Agatha 2003a), *Novistrombidium* (Agatha 2003a), *Laboea* (Agatha *et al.* 2004),

Table 3. Revised classification of the Oligotrichea (for further explanations, see "Classification of the Oligotrichea and diagnosis of some taxa").

Superclass Spirotricha Bütschli, 1889
  Class Hypotrichea Stein, 1859
     Order Hypotrichida Stein, 1859
     Order Stichotrichida Fauré-Fremiet, 1961
  Class Oligotrichea Bütschli, 1889
   Subclass Halteriia Petz and Foissner, 1992
    Order Halteriida Petz and Foissner, 1992
      Family Halteriidae Claparède and Lachmann, 1859
       Genus *Halteria* Dujardin, 1841
       Genus *Pelagohalteria* Foissner, Skogstad and Pratt, 1988
       Genus *Meseres* Schewiakoff, 1892
   Subclass Oligotrichia Bütschli, 1889
    Order Choreotrichida Small and Lynn, 1985
     Suborder Strobilidiina Jankowski, 1980[1]
      Family Strobilidiidae Kahl in Doflein and Reichenow, 1929
       Genus *Strobilidium* Schewiakoff, 1892
       Genus *Rimostrombidium* Jankowski, 1978
       Genus *Pelagostrobilidium* Petz, Song and Wilbert, 1995
      Family Strombidinopsidae Small and Lynn, 1985
       Genus *Strombidinopsis* Kent, 1881
      Family Leegaardiellidae Lynn and Montagnes, 1988
       Genus *Leegaardiella* Lynn and Montagnes, 1988
      Family Lohmanniellidae Montagnes and Lynn, 1991
       Genus *Lohmanniella* Leegaard, 1915
     Suborder Tintinnina Kofoid and Campbell, 1929
    Order Oligotrichida Bütschli, 1889
     "Family Strombidiidae Fauré-Fremiet, 1970"[2]
      Genus *Parallelostrombidium* Agatha, 2004
      Genus *Omegastrombidium* Agatha, 2004
      Genus *Spirostrombidium* Jankowski, 1978
      Genus *Novistrombidium* Song and Bradbury, 1998
      Genus *Laboea* Lohmann, 1908
      Genus *Strombidium* Claparède and Lachmann, 1859
     Family Tontoniidae Agatha, 2004
      Genus *Tontonia* Fauré-Fremiet, 1914
      Genus *Paratontonia* Jankowski, 1978
      Genus *Spirotontonia* Agatha, 2004
      Genus *Pseudotontonia* Agatha, 2004
     Family Cyrtostrombidiidae Agatha, 2004
      Genus *Cyrtostrombidium* Lynn and Gilron, 1993
     Family Pelagostrombidiidae Agatha, 2004
      Genus *Pelagostrombidium* Krainer, 1991
      Genus *Limnostrombidium* Krainer, 1995

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[1]Again established by Small and Lynn (1985). [2]The paraphyly is indicated by quotation marks and the genera are, as far as possible, arranged according to the sequencing convention (Ax 1984).

Strombidinopsis (Dale and Lynn 1998, Agatha 2003b), *Pelagostrobilidium* (own observ.), *Spirotontonia* (own observ.), and tintinnids from marine and freshwaters (own observ.; Petz and Foissner 1993) support their hypothesis. Thus, stomatogenesis takes place on the cell surface, except for the Oligotrichia (Anigstein 1913; Fauré-Fremiet 1912, 1953; Penard 1916, 1920, 1922; Buddenbrock 1922; Yagiu 1933; Kormos and Kormos 1958; Deroux 1974; Petz and Foissner 1992; Petz 1994; Song and Wang 1996; Agatha and Riedel-Lorjé 1997, 1998; Montagnes and Humphrey 1998; Suzuki and Song 2001), Hypotrichida (Ruffolo 1976, Song and Packroff 1993), and entodiniomorphids (Noirot-Timothée 1960); transitions to a subsurface development of the oral primordium are also found in some Stichotrichida (Foissner 1983). The hypoapokinetal stomatogenesis is therefore
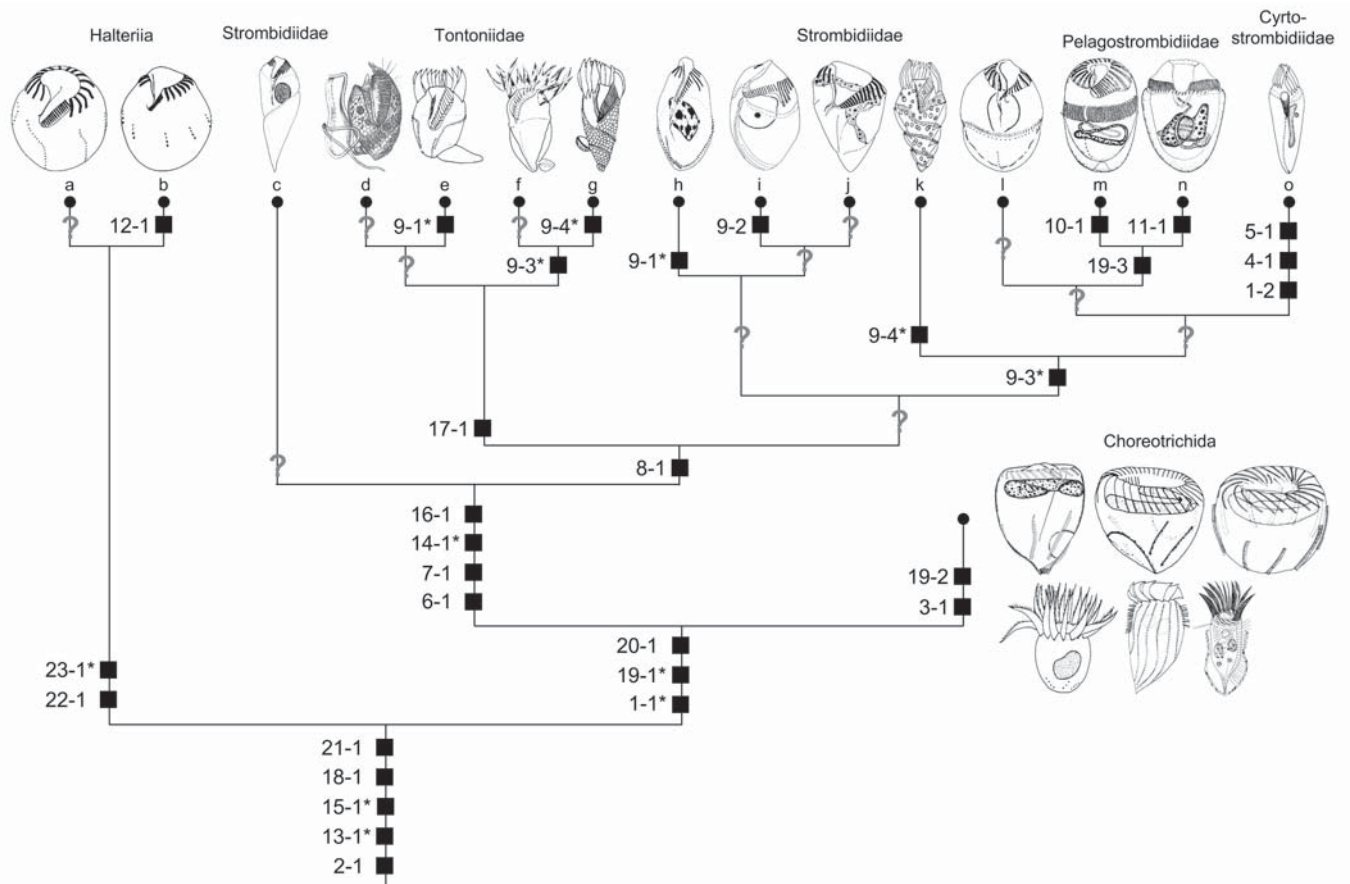
Fig. 4. Cladistic scheme generated by Hennig's argumentation method. For character coding, see Table 1 and section on character states. Apomorphies are marked by black squares, convergences are starred. **a** - *Meseres corlissi* Petz and Foissner, 1992 (modified from Petz and Foissner 1992); **b** - *Halteria grandinella* (Müller, 1773) Dujardin, 1841 (modified from Song 1993); **c** - *Parallelostrombidium rhyticollare* (Corliss and Snyder, 1986) Agatha, 2004 (modified from Petz *et al.* 1995); **d** - *Tontonia appendiculariformis* Fauré-Fremiet, 1914 (from Fauré-Fremiet 1924); **e** - *Paratontonia gracillima* (Fauré-Fremiet, 1924) Jankowski, 1978 (from Lynn *et al.* 1988); **f** - *Pseudotontonia cornuta* (Leegaard, 1915) Agatha, 2004 (modified from Suzuki and Song 2001); **g** - *Spirotontonia grandis* (Suzuki and Han, 2000) Agatha, 2004 (from Suzuki and Han 2000); **h** - *Omegastrombidium elegans* (Florentin, 1901) Agatha, 2004 (from Song *et al.* 2000); **i** - *Spirostrombidium urceolare* (Stein, 1867) Lei *et al.*, 1999 (from Lei *et al.* 1999); **j** - *Novistrombidium apsheronicum* (Alekperov and Asadullayeva, 1997) Agatha, 2003 (from Agatha 2003a); **k** - *Laboea strobila* Lohmann, 1908 (from Montagnes *et al.* 1988); **l** - *Strombidium sulcatum* Claparède and Lachmann, 1859 (from Song *et al.* 2000); **m** - *Limnostrombidium pelagicum* (Kahl, 1932) Krainer, 1995 (from Krainer 1991); **n** - *Pelagostrombidium mirabile* (Penard, 1916) Krainer, 1991 (from Krainer 1991); **o** - *Cyrtostrombidium longisomum* Lynn and Gilron, 1993 (from Lynn and Gilron 1993).

regarded as derived state and developed probably convergently in the taxa mentioned above, as other argumentations are less parsimonious (Petz and Foissner 1992). The assumption by Kahl (1932), that the subsurface development of the new oral apparatus became necessary when the membranelles undertook the cell's movement, cannot be supported; some data even indicate that this is not so: (i) in the related planktonic Halteriia, the new oral apparatus originates on the cell surface and (ii) a subsurface development of the new oral apparatus occurs in the benthic Hypotrichida and the endocommensalic Entodiniomorphida. The rigid cortex (polysaccharidic or proteinous cortical platelets in the Hypotrichida and Oligotrichida and skeletal plates in the

Entodiniomorphida) possibly causes the special mode of stomatogenesis in these taxa.

The shape of the subsurface organelle, in which the oral primordium originates, probably depends on the shape of the adoral zone of membranelles, i.e., a C-shaped zone necessitates a tube, while a closed zone requires a pouch. Accordingly, it is reasonable to assume a parallel development of the closed zone and the subsurface pouch (cp. Character 3). In contrast to the suggestion by Petz and Foissner (1992), the pouch, not the tube, thus represents the derived state.

Since a temporary structure in which stomatogenesis occurs, as in the Hypotrichida and the Oligotrichia, is considered as plesiomorphic, a permanent one
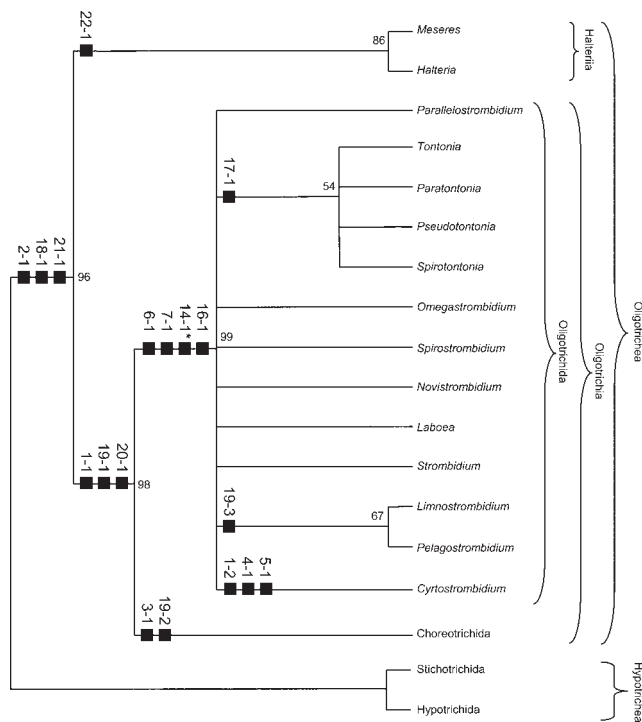
Fig. 5. 50% majority-rule consensus tree (tree length = 74, retention index = 0.85, rescale consistency index = 0.68) computed with the maximum parsimony analysis of PAUP* version 4.0b10 (Swofford 2002), using the Hypotrichea, i.e., the hypotrichs and stichotrichs, as out-group (for feature matrix, see Table 2). The numbers represent the bootstrap values out of 100 re-samplings of the data set. The main apomorphies are marked by black squares; convergences are starred.

(neoformation organelle) is a strong synapomorphy of the genera *Limnostrombidium* Krainer, 1995 and *Pelagostrombidium* Krainer, 1991.

Character 20: Rotation of oral primordium. Although stomatogenesis of the Halteriia and Oligotrichia is similar at first glance, there is a difference, supporting a closer affiliation of the former with the Hypotrichea, viz., a pronounced anticlockwise rotation of the anterior end of the oral primordium (Fauré-Fremiet 1953, Ruffolo 1976, Petz and Foissner 1992, Song 1993, Berger 1999, Agatha 2004). This rotation is apparently lacking in the Oligotrichia or is, at least, less pronounced (Fauré-Fremiet 1953; Deroux 1974; Petz and Foissner 1992; Petz 1994; Song and Wang 1996; Dale and Lynn 1998; Agatha 2003a, b; Agatha *et al.* 2004). On the other hand, the posterior end of the oral primordium performs a distinct clockwise torsion, which is absent or less conspicuous in the Halteriia and the outgroup Hypotrichea. Accordingly, the distinct clockwise torsion is assumed to be apomorphic.

Character 21: Origin of undulating membranes. The undulating membranes of the outgroup Hypotrichea are generated by the oral primordium or cirral anlagen (Song and Packroff 1993, Berger 1999, Foissner *et al.* 2002, Song 2003), while they originate *de novo* in the Oligotrichea (Petz and Foissner 1992, 1993; Petz 1994; Song and Wang 1996; Dale and Lynn 1998; Agatha 2003a, b; Agatha *et al.* 2004). Since the oral anlage usually derives from the parental somatic or oral ciliature (Foissner 1996), the *de novo*-origin is regarded as apomorphy.

Character 22: Origin of somatic ciliature. The entire somatic ciliature of the Oligotrichia as well as the marginal and dorsal rows of the Hypotrichea are usually generated by intrakinetal proliferation of kinetids (Petz and Foissner 1992, 1993; Petz 1994; Song and Wang 1996; Dale and Lynn 1998; Berger 1999; Agatha 2003a, b; Agatha *et al.* 2004); only very rarely, *de novo*-formation occurs, e.g., in *Engelmanniella* (Wirnsberger-Aescht *et al.* 1989). Thus, the development of the girdle kinety within the neoformation organelle, as mentioned for *Pelagostrombidium fallax* (Petz and Foissner 1992), is considered to be a misobservation. In contrast to the intrakinetal proliferation, the *de novo*-generation of the entire somatic ciliature is regarded as the autapomorphy of the Halteriia.

Character 23: Reorganization of somatic ciliature. The somatic ciliature is usually not distinctly reconstructed during ontogenesis (Foissner 1996); thus, the extensive reorganization in the Hypotrichea is regarded as apomorphy, and the reorganization of the entire somatic ciliature in the Halteriia as a convergence (Petz and Foissner 1992, Song 1993). This explanation is more parsimonious than to assume a common ancestor of the Hypotrichea and Halteriia, which would require the assumption of several convergences in the Halteriia and the Oligotrichia (the enantiotropy, the *de novo*-origin of the undulating membranes, and the apical membranellar zone).

Characters not considered

Although occasionally mentioned in discussions, the following features were not included in this approach as they are plesiomorphies, convergences or require further investigations: structure of the membranellar zone, chromosomal fragmentation, arrangement of the extrusomes and their fibrillar associates, shape of the neoformation organelle, ontogenetic behaviour of the macronuclei, number of anlagen per somatic kinety, reorganization of the parental oral ciliature, arrangement of the cortical

platelets, resting cysts, and fate of the somatic ciliature in encysted cells.

Comparison of morphological cladograms

There are few morphologic phylogenetic systems available for the oligotrichs, and all are confined to higher taxonomic levels (Puytorac *et al.* 1984, 1994; Petz and Foissner 1992). Although several new features (Characters 1, 2, 4-17, 20, 21, 23; Table 1) are included, the Hennigian tree matches that of Petz and Foissner (1992) very well (cp. Fig. 1a and Figs 4, 5). The monophyly of the Hypotrichea (hypotrichs and stichotrichs) and Oligotrichea bases on the macronuclear replication band; the apokinetal stomatogenesis is a newly introduced strong synapomorphy. Since the perilemma is apparently absent in the Hypotrichida, it is not a synapomorphy of the Hypotrichea and Oligotrichea, as suggested by Petz and Foissner (1992), but possibly developed convergently in the Stichotrichida and Oligotrichea. Otherwise, it is a synapomorphy of the Oligotrichea and Stichotrichida, and the cirri are either a convergence in the Hypotrichida and Stichotrichida or a symplesiomorphy which was lost in the Oligotrichea. However, there are no morphologic or ontogenetic data that support these two latter explanations. The Oligotrichea are mostly characterized by the enantiotropic division mode and the *de novo*-formation of the undulating membranes (a newly included character). With respect to the position of *Halteria*, the tree is supported by the parsimony analyses chiefly of ultrastructural data (Puytorac *et al.* 1984, 1994), in that the cluster of *Halteria* and the tintinnid *Petalotricha ampulla* forms a sister group with the monophyletic Hypotrichea. The unique feature of the Halteriia is the *de novo*-origin of the entire somatic ciliature, whereas the Oligotrichia are characterized by convergences (the hypoapokinetal stomatogenesis and the absence of a paroral), except for the rotation of the oral primordium, which is a potentially useful feature; more data are, however, required to support its significance. Since a concomitant development of the closed adoral zone of membranelles and the subsurface pouch is assumed (see Characters 2 and 19), two apomorphies characterize the Choreotrichida, instead of only one, as in the scheme of Petz and Foissner (1992).

More detailed tree comparisons are impossible because the present cladistic approach is the first that investigated the genealogy of the families and genera of the Oligotrichida. Although the tail cilia in tontoniids might represent a third kinety besides the ventral and girdle kinety, the reduction of the somatic ciliature is distinct and represents together with the extrusomes (trichites) and the proposed dextral spiral of the kineties the main autapomorphy of the Oligotrichida. Like the neoformation organelle in the Pelagostrombidiidae and the oral structures of the Cyrtostrombidiidae (cyrtos-like pharyngeal fibres, no undulating membrane and ventral membranelles), the tontoniid tail is a good apomorphy due to its unique ultrastructure. The remaining apomorphies within the Oligotrichida, such as the ciliary patterns, are interpreted as convergences, or further ultrastructural data are needed to evaluate their importance and distribution. The cladistic relationships of the Oligotrichida on generic and familial level are mainly based on the evolution of the ciliary patterns proposed by Agatha (2004).

The attempt to reconstruct a phylogenetic tree for the Oligotrichida, using the Hennigian method, revealed that the family Strombidiidae is paraphyletic, which might be due to the scarce knowledge of the group (Fig. 4). The Hennigian scheme postulates that every split in the cladogram produces two new clades; diverging from each other and the parental phenotype multiple speciation and budding processes are excluded. Thus, the many unknown apomorphies in the cladistic scheme of the Oligotrichida are not only caused by the lack of data but also by the applied method. There are many good examples for the separation of new lineages, while the parental persists virtually/essentially unchanged (Mayr and Bock 2002). Furthermore, the Hennigian method uses the principle of parsimony as a methodological instrument, while the assumption of a parsimonious evolution is unfounded and many more than only the nine convergences in the evolution of the Oligotrichida might exist (Ax 1984, Moore and Willmer 1997). Therefore, phylogenetic trees do not represent reality, but can merely be a theorem of probability (Bachmann 1995, Haszprunar 1998).

All computer-generated cladograms support the monophyly of the Hypotrichea, Oligotrichea, Halteriia, Oligotrichia, Oligotrichida, and Choreotrichida found in the Hennigian scheme (Figs 4, 5; trees from HENNIG86 and FreeTree not shown). Likewise, they show a sister group relationship of the Halteriia and Oligotrichia. The classical and PAUP tree reveal a monophyly of the Tontoniidae and Pelagostrombidiidae due to the tail and the neoformation organelle, respectively. The cladograms generated with the unweighted data and the programs FreeTree and HENNIG86, however, place the genera

mainly according to girdle kinety patterns, which have been developed convergently, according to the Hennigian scheme.

Comparison with cladograms inferred from gene sequence data

The Oligotrichia comprise at least 19 sufficiently known genera with about 180 species (Table 3; Fig. 4), while gene sequences are available from about twenty Choreotrichida and ten Oligotrichida species from the genera *Strombidium*, *Laboea*, *Novistrombidium*, and *Spirostrombidium*. Due to undersampling and unequal sampling, the gene trees are not comparable with the morphology based cladistic approach at familial and generic level (Agatha *et al.* 2004).

All molecular trees differ from the morphological cladograms in the position of the halteriids. They consistently reveal *Halteria grandinella* not as an early branch of the stichotrichs, but within this taxon as sister group to *Oxytricha granulifera* (Baroin-Tourancheau *et al.* 1992, Hoffman and Prescott 1997, Shin *et al.* 2000, Bernhard *et al.* 2001, Snoeyenbos-West *et al.* 2002, Croft *et al.* 2003, Hewitt *et al.* 2003, Modeo *et al.* 2003, Strüder-Kypke and Lynn 2003, Agatha *et al.* 2004). A placement of the Halteriia within the Stichotrichida is less parsimonious than the assumption presented above, as it requires several other convergences: (i) the diplo-/polystichomonad undulating membrane structure and the cirri in the Stichotrichida and Hypotrichida; and (ii) the enantiotropy, the apical adoral zone of membranelles, and the *de novo*-origin of the undulating membranes in the Halteriia and Oligotrichia. Shin *et al.* (2000) as well as Strüder-Kypke and Lynn (2003) argued that the enantiotropic division mode may be an adaptation to the planktonic lifestyle. Indeed, this might be true, although there are no evidences for this assumption (Foissner *et al.* 2004). Additionally, this argument does not favour an arrangement of the Halteriids within the Stichotrichida but also supports my cladistic scheme because it is more parsimonious to assume that the ancestor of the Oligotrichea was possibly a planktonic ciliate that developed enantiotropic division as an adaptation to this habitat.

Besides the sequence of the small subunit rRNA gene, there are morphological features suggesting a close relationship between the halteriids and stichotrichs: (i) the stomatogenesis on cell surface (plesiomorphic as it is present in most other ciliates); (ii) the four-rowed ventral membranelles (probably a plesiomorphy); (iii) the two undulating membranes (probably a plesiomorphy);

(iv) the bristle complexes, which are absent in the halteriid *Meseres* (Fig. 2a; Petz and Foissner 1992) and whose homology to cirri has yet to be tested; and (v) the two distinct anlagen per somatic kinety in dividers, which also occur in some Hypotrichida, e.g., *Diophrys* (Song and Packroff 1993), and possibly the Oligotrichida. Finally, there are no derived morphologic, ontogenetic, or ultrastructural characters left, that support the position of *Halteria* within the stichotrichs. In agreement with Petz and Foissner (1992) and my results, the halteriids are, however, still the closest oligotrich relatives to the hypotrichs and stichotrichs. As discussed by Foissner *et al.* (2004), even the assumption that halteriids have developed from stichotrichs by an involution of the ventral and an extension of the dorsal side connected with a reduction of all cirri does not explain the enantiotropic division mode and the *de novo*-origin of the undulating membranes. Thus, the topologies of the gene and traditional trees concerning the position of the halteriids cannot be reconciled, especially, as nothing is known about the possible correlation between the evolution of the rRNA molecules and the selection of the phenotypes in ciliates (Puytorac *et al.* 1994). Accordingly, an exclusion of the Halteriia from the Oligotrichea, as suggested by Modeo *et al.* (2003) and Strüder-Kypke and Lynn (2003) seems to be unfounded, as genealogical analyses of the α-tubulin nucleotide sequences corroborate the cladistic scheme presented here by showing a closer relationship of *H. grandinella* to the Oligotrichia than to the stichotrichs (Snoeyenbos-West *et al.* 2002). Furthermore, molecular homologies are not always more accurate than morphological ones (Puytorac *et al.* 1994, Moore and Willmer 1997), and morphological characters, the product of a large number of genes, are usually quite reliable in phylogenetic analyses (Mayr and Bock 2002). However, the gene trees match the cladistic approach in other cases very well, e.g., in the close relationship between *Meseres* and *Halteria* (Katz and Foissner, pers. commun.) and between *Novistrombidium* and *Spirostrombidium* (both with a dextrally spiralled girdle kinety); the latter form a cluster separate from the Strombidiidae with a horizontal or sinistrally spiralled girdle kinety (Strüder-Kypke, pers. commun.). In summary, the SSrRNA trees alone do not solve all evolutionary problems, but together with other characters, such as morphologic, ontogenetic, and ultrastructural ones as well as other gene loci, they contribute to a better understanding of the phylogenetic relationships in ciliates (Moore and Willmer 1997, Hewitt *et al.* 2003).

Classification of the Oligotrichea and diagnosis of some taxa

The results obtained by the present cladistic approach match the findings of Petz and Foissner (1992). Accordingly, I follow mainly their classification and add all sufficiently known families and genera (Table 3). The permissibility of paraphyletic taxa in a classification is controversially discussed: it is supported by the evolutionary systematics, but rejected by the cladistic systematics (Sudhaus and Rehfeld 1992). In favour of simplicity and to provide a "user-friendly" classification, I follow the evolutionary systematics, i.e., the paraphyletic family Strombidiidae is not eliminated, however, it is marked as such. The members of the Strombidiidae are easily recognized, although the family is characterized only by plesiomorphies, as revealed by the cladistic analyses. The included genera are mostly arranged, following the sequencing convention (the first taxon represents the sister group to the remaining taxa and so on; Ax 1984). Additionally, no taxa have been established for the newly recognized sister groups, viz., for *Tontonia* and *Paratontonia* or *Spirotontonia* and *Pseudotontonia*.

The fact, that the ICZN (1999) does not govern the nomenclature above the familial level, causes some confusion within the Class Oligotrichea. Small and Lynn (1985) introduced the order Choreotrichida for taxa with a closed adoral zone of membranelles (strobilidiids and tintinnids) and assumed that the Halteriidae and Strombidiidae are adelphotaxa. The phylogenetic results of Petz and Foissner (1992), however, suggest a closer relationship of the strombidiids to the tintinnids and strobilidiids than to the halteriids; thus, the authors excluded the halteriids from the subclass Oligotrichia and established the subclass Halteriia (Fig. 1a). They summarized the Oligotrichia with a C-shaped membranellar zone in the order Strombidiida Jankowski, 1980. Petz and Foissner (1992) argued that the order Choreotrichida is superfluous and summarized the Oligotrichia with a closed membranellar zone in the order Oligotrichida. However, the terms "oligotrichs" for ciliates with a C-shape zone and "choreotrichs" for those with a closed zone have widely been accepted, not only in ecological papers but also in taxonomic publications. To avoid further confusion, and to be in accordance with the principle of an ascending and descending nomenclature used on subordinal levels (ICZN 1999), I reject the order Strombidiida Jankowski, 1980 and use the order

Oligotrichida Bütschli, 1889. This seems especially justified as Jankowski (1980) only established a suborder Strombidiina, which is a junior synonymy to the suborder Oligotrichina Bütschli, 1889 used by Corliss (1979).

Based on the new characters, the diagnosis of some taxa are improved. Moreover, the lack of a type species in the genus *Meseres*, as recognized by Aescht (2001), is remedied by designating a type.

Class Oligotrichea Bütschli, 1889

Improved diagnosis: Cell usually globular to obconical. Macronucleus with replication band. Adoral zone of membranelles conspicuous, occupies apical cell end. Kinetodesmal fibres of somatic kinetids absent in at least morphostatic specimens. Undulating membranes monostichomonad, originate *de novo*. Stomatogenesis apokinetal, division enantiotropic. Mainly planktonic species.

Comparison with related taxa: The members of the class Hypotrichea, i.e., the hypotrichs and stichotrichs, are mainly dorsoventrally flattened benthic organisms, which can be distinguished from the Oligotrichea by the cirri, the division mode (homothetogenic *vs.* enantiotropic), the arrangement of the membranellar zone (mainly ventral *vs.* apical) as well as by the origin and structure of the undulating membranes (diplo-/polystichomonad structure and originating from the oral primordium or cirral anlagen *vs.* monostichomonad structure and originating *de novo*).

According to Lynn and Small (2002), the spirotrich ciliates comprise five subclasses (Fig. 1b): the Hypotrichia, Stichotrichia, Oligotrichia, Choreotrichia, and the Protocruziidia. The affiliation of the last of these with the spirotrichs is based only on gene sequence analyses as the morphologic and ontogenetic features (unusual nuclear complex, stichodyad undulating membrane, kinetid ultrastructure; Ammermann 1968, Ruthmann and Hauser 1974, Grolière *et al.* 1980, Song and Wilbert 1997) indicate rather a relationship to the heterotrichs; thus, a morphologic comparison of the Protocruziidia and the Oligotrichea is not necessary.

Subclass Halteriia Petz and Foissner, 1992

Improved diagnosis: Oligotrichea with endoral and minute paroral. Somatic ciliature comprises more than three kineties or bristle complexes, develops *de novo*,

and reorganizes completely during ontogenesis. Oral primordium originates epiapokinetally and its anterior end rotates rightwards.

Comparison with related taxa: The Halteriia differ from the Hypotrichea and Oligotrichia in the origin of the somatic ciliature (entirely *de novo vs*. partially or completely by intrakinetal proliferation). The Oligotrichia are also distinguished by the stomatogenic mode (hypo- *vs*. epiapokinetal), the number of undulating membranes (one *vs*. two), the reorganization of the somatic ciliature (entire *vs*. none or indistinct), and the shaping of the new membranellar zone (rightwards rotation of proximal *vs*. distal end).

Order Halteriida Petz and Foissner, 1992

Improved diagnosis: With character of the subclass.

Type family: Halteriidae Claparède and Lachmann, 1859.

Family Halteriidae Claparède and Lachmann, 1859

Improved diagnosis: With characters of the order.
Type genus: *Halteria* Dujardin, 1841.

Genus *Meseres* Schewiakoff, 1892

Diagnosis: Halteriids with somatic kineties composed of dikinetids each with a cilium only at the anterior basal body. With perilemma.

Type species: *Meseres cordiformis* Schewiakoff, 1892.

Remarks: In 1892, Schewiakoff established the genus with *M. cordiformis* and *M. stentor* but did not fix any as type. Nevertheless, the genus name is available as it is accompanied by an indication, i.e., satisfies article 12.2.5. of the ICZN (1999). Following the recommendations of the Code, concerning the eligibility of species for type fixation (article 69A.10.), *Meseres cordiformis* is selected because it is the first species cited not only in Schewiakoff (1892) but also in Schewiakoff (1893), which includes drawings of the species.

Comparison with related genera: *Meseres* differs from *Halteria* Dujardin, 1841 and *Pelagohalteria* Foissner, Skogstad and Pratt, 1988 in the arrangement of the somatic ciliature (in long kineties *vs*. bristle complexes).

Subclass Oligotrichia Bütschli, 1889

Improved diagnosis: Endoral on inner wall of buccal lip in Oligotrichida or extending across peristomial field into oral funnel in Choreotrichida. With perilemma. Somatic ciliature entirely generated by intrakinetal proliferation, parental one not reorganized or without special anlagen. Oral primordium originates hypoapokinetally and its posterior end rotates rightwards.

Comparison with related subclass Halteriia: See discussion of Halteriia.

Order Oligotrichida Bütschli, 1889

Improved diagnosis: Adoral zone of membranelles C- shaped with ventral gap. Endoral on inner wall of buccal lip. Somatic ciliature reduced to usually a girdle kinety and a ventral kinety. Kineties composed of dikinetids each with a cilium only at the left, respectively, anterior basal body. Stomatogenesis in a subsurface tube. Polysaccharidic cortical platelets.

Comparison with order Choreotrichida: In contrast to the Oligotrichida, the Choreotrichida have a circular adoral zone of membranelles which originates in a subsurface pouch. Their endoral extends across the peristomial field into the oral funnel and their somatic ciliature usually comprises more than three kineties.

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ACTA
PROTOZOOLOGICA

Rapid Identification of Rumen Protozoa by Restriction Analysis of Amplified 18S rRNA Gene

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Summary. A rapid method has been developed for molecular identification of rumen ciliates without the need for cultivation. Total DNA was isolated from single protozoal cells by the Chelex method and nearly complete protozoal 18S rRNA genes were amplified and subjected to restriction fragment length polymorphism analysis. On the basis of restriction patterns generated a molecular key was elaborated allowing identification of protozoa solely by a molecular technique without prior knowledge of morphology. No differences were observed between identical species originating from different animals or geographic locations, or between morphological variants of the same species. The ARDREA analysis described here provides a rapid and convenient way for identification and diversity studies of rumen protozoa.

Key words: 18S rRNA, ARDREA, ciliate, identification, protozoa, rumen.

Abbreviations: ARDREA - Amplified Ribosomal DNA Restriction Analysis, PCR - Polymerase Chain Reaction.

INTRODUCTION

Rumen protozoa represent a substantial part of the rumen microbial population contributing up to 50 % of the total microbial biomass (Williams and Coleman 1992). However, due to highly specific growth requirement and their complex morphology our understanding

of the role of protozoa in rumen fermentation is still limited. Ciliate protozoa in the rumen are classified on the basis of the micro- and macronucleus and the presence and morphology of exterior spines and lobes or internal skeletal plates as well as the shape and size of cells (Dogiel 1927, Ogimoto and Imai 1981, Williams and Coleman 1992). Based on such morphological characteristics a large number of genera and species have been described, but it is not clear to what extent these represent true species. Furthermore, morphological classification and identification of protozoa is made more complex as many of the morphological traits rapidly

change or completely disappear under *in vitro* conditions (Williams and Coleman 1992). Molecular methods based on DNA analysis and fingerprinting provide a rational alternative to the classic morphology. In recent years the PCR amplification and sequencing of small subunit (SSU) rRNA genes from rumen bacteria and fungi have revolutionised studies on rumen microbial ecology. However, relatively few studies have applied this methodology to study the rumen protozoa (Hori and Osawa 1987, Lee and Kugrens 1992, Wright *et al.* 1997, Hirt *et al.* 1998), primarily due to the demanding growth requirement of rumen protozoa. The aim of this study was to develop a PCR fingerprinting method, which is independent of cultivation, for rapid identification of predominant rumen protozoa.

MATERIALS AND METHODS

Collection of protozoa. Twenty protozoal species were included in the study. The cultures came from monofaunated sheep obtained during the ERCULE project and species included (country of original place of isolation is shown in parentheses) *Dasytricha ruminantium* (France), *Diplodinium dentatum* (Poland), *Diploplastron affine* (Poland), *Enoploplastron triloricatum* (Poland), *Entodinium bursa* (Slovakia), *Ent. caudatum* (Scotland and Slovakia), *Ent. furca monolobum* (Slovakia), *Ent. nanellum* (Slovakia), *Ent. simplex* (Poland), *Epidinium ecaudatum* (Poland), *Eudiplodinium maggii* (France and Poland), *Isotricha intestinalis* (Poland), *I. prostoma* (France and Slovakia), *Metadinium medium* (Poland), *Ophryoscolex caudatus* (Poland), *Ophr. purkynjei* (Poland), *Ostracodinium gracile* (Poland), *Ostr. dentatum* (Poland), *Polyplastron multivesiculatum* (France and Poland). For study of protozoal variability *Ophr. caudatus* (forma *tricoronatus*) cells were directly picked out from fresh rumen fluid of goat and sheep from Slovakia. The morphological variants of *Dipl. dentatum* were picked from an *in vitro* culture kept in Kielanowski Institute of Animal Physiology and Nutrition of Jablonna. *Entodinium caudatum* type and forma *dubardii* cells were from an *in vitro* culture kept in Institute of Animal Physiology, Kosice, Slovakia.

DNA isolation and analysis. A single protozoal cell was picked either from rumen fluid or from *in vitro* culture under the microscope, washed twice in drop of sterile water and put into 50 μl of 5% Chelex-100 (BioRad, California, USA) in water. Pre-incubated proteinase K (Merck, Germany) was then added to the reaction mixture to a final concentration of 20 μg/ml. After proteinase treatment (55°C for 30 min) DNA was released from cell by heating the sample at 98°C for 5 min. After rapid cooling to 0°C and centrifugation (3000g for 5 min) DNA containing supernatant was directly used for PCR amplification. All isolation and manipulation steps were done under aerobic conditions.

Polymerase chain reaction. Five μl of isolated DNA was amplified using a Techne Thermal Cycler Progene in a 50 μl reaction mix containing 0.04 mM of each deoxynucleosidetriphosphate, 20 pmol of each primer, PCR reaction buffer (Perkin Elmer), and 0.5 U of

AmpliTaq DNA polymerase (Perkin Elmer). An initial denaturation step at 95°C for 5 min was followed by 35 cycles of (94°C for 1 min, 52°C for 1 min and 72°C for 1 min), and a final incubation at 72°C for 10 min. Primers based on conserved regions in eukaryotic 18S rDNA genes were used in the PCR amplification: EukFor (5'-AATATGGTTGATCCTGCCAGT-3' and EukRev (5'- TGATCCTTCTGCAGGTTCACCTAC -3'. Quality and quantity of amplified DNA was determined by electrophoresis in 1 % agarose gel (Maniatis *et al.* 1982).

Amplified DNA (about 0.5 μg per reaction) was digested by 18 restriction endonucleases - *Acc*I, *Ase*I, *Ava*II, *Ban*I, *Bst*UI, *Cfo*I, *Eco*RV, *Hae*III, *Hin*dIII, *Mae*II, *Mlu*I, *Msp*I, *Nru*I, *Sac*II, *Spe*I, *Stu*I, *Sty*I and *Xma*I (New England Biolabs and Gibco BRL), according to manufacturer's instructions for 1 h. Digestion products were separated by horizontal 2% agarose gel electrophoresis in Tris-acetate buffer (Maniatis *et al.* 1982).

RESULTS

A rapid method has been developed for molecular identification of rumen protozoa without the need for cultivation. Total DNA was isolated from single protozoal cells by the Chelex method and used as a target for PCR amplification using primers directed to the 18S rDNA gene. The nearly complete SSU rDNA gene was obtained by PCR amplification from all tested samples. Amplified DNA was then subjected to cleavage by several (18) restriction endonucleases recognizing tetra- or hexa-nucleotide sequences and fragments generated were resolved by agarose gel electrophoresis. Specific DNA fingerprints were obtained after agarose gel electrophoresis (Fig. 1). While for example all tested species produced identical banding patterns after cleavage by *Ban*I and *Cfo*I restriction endonucleases, substantial variability was observed after the cleavage by *Acc*I (four different profiles) or *Ase*I endonuclease (five different profiles). The highest discriminatory power was observed for *Msp*I and *Bst*UI endonuclease (six profiles). The restriction fragment length polymorphism (RFLP) analysis of amplified 18S rDNA genes was found to clearly discriminate between all species studied, with DNA from each species giving unique sets of patterns. The fingerprints were recorded and species were grouped into similarity groups. The data obtained from DNA cleavage were then correlated with data obtained by computer-aided analysis of available 18S rDNA sequences from GenBank and a molecular key was designed. Identification starts by *Ava*II cleavage and by using six selected endonucleases any from 20 protozoal species be can unambiguously identified (Table 1).
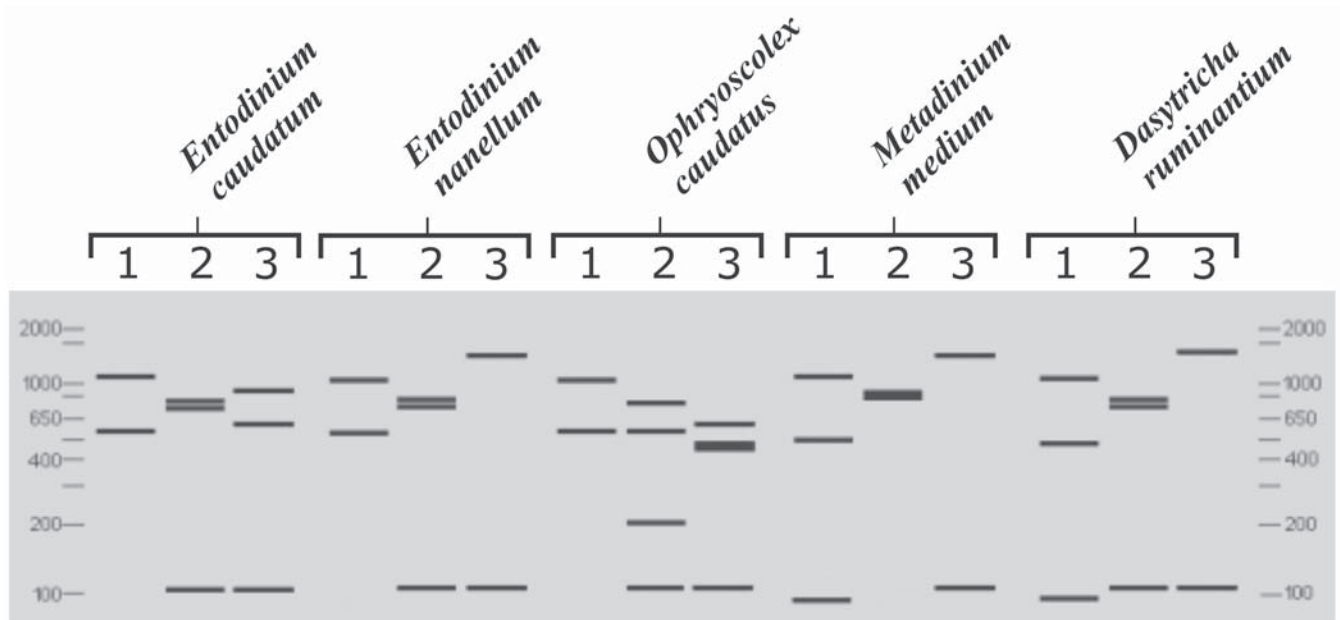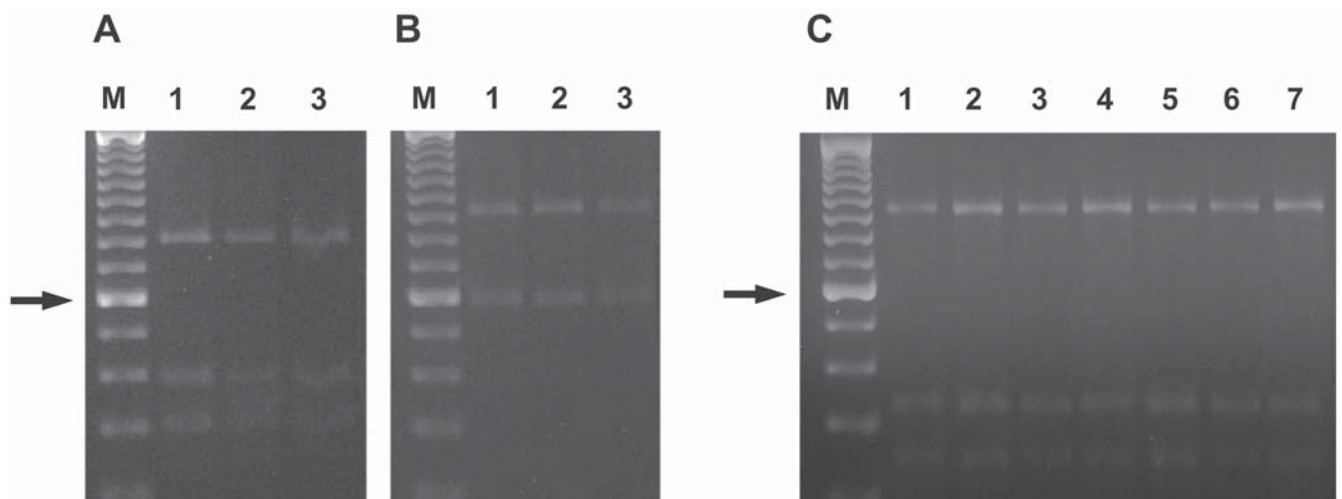
Fig. 1. Schematic representation of ARDREA profiles of selected protozoal species obtained by *Ava*II (lane 1), *Ase*I (lane 2) and *Msp*I (lane 3) restriction endonucleases.

Figs 2A-C. Geographic and morphological invariability of ARDREA profiles of *Ophryoscolex caudatus* f. *tricoronatus* strains isolated from Slovak sheep - lane 1, Slovak goat - lane 2 and Polish sheep - lane 3 obtained by *Bst*UI (part **A**) and *Msp*I (part **B**) restriction endonuclease. Part **C** - ARDREA profiles of *Entodinium caudatum* type strains (lanes 1-3) were compared to those of f. *dubardii* (lanes 4-7) obtained by *Bst*UI restriction endonuclease. Lane M - 100 bp DNA ladder (Gibco BRL, California USA), standard of molecular weight. The arrow indicates marker band of 600 bp.

No differences were observed between identical species originating from different animals or geographic locations, or between morphological variants of the same species. Analysis of animal-to-animal variability did not reveal any variability in *Ophr. caudatus* f. *tricoronatus* (Figs 2A, B). No differences were found in morphologically different *Dipl. dentatum* strains (data not shown) and similarly no differences were found in several

Table 1. Key for RFLP analysis of rumen protozoa.

1. Digestion with *Ava*II:		
a) (fragments 1080, 550)		2
b) (fragments 1080, 460, 90)		10
2. Digestion with *Nru*I		
a) (no cleavage, fragment 1650)	*Entodinium* sp.	3
b) (fragments 1100, 450)		7
3. Digestion with *Msp*I		
a) (fragments 1530, 100)		4
b) (fragments 920, 610, 100)		5
4. Digestion with *Acc*I		
a) (fragments 910, 620)	*Ent. nannellum*	
b) (no cleavage, fragment 1650)	*Ent. bursa*	
5. Digestion with *Eco*RV		
a) (fragments 910, 620)	*Ent. caudatum*	
b) (no cleavage, fragment 1650)		6
6. Digestion with *Acc*I		
a) (fragments 910, 620)	*Ent. simplex*	
b) (no cleavage, fragment 1650)	*Ent. furca monolobum*	
7. Digestion with *Acc*I		
a) (fragments 750, 600, 300)	*Ophryoscolex* sp.	8
b) (no cleavage, fragment 1650)	*Epidinium* sp.	9
8. Digestion with *Msp*I		
a) (fragments 920, 610, 100)	*Ophr. purkynjei*	
b) (fragments 610, 480, 400, 100)	*Ophr. caudatum*	
9. Digestion with *Msp*I		
a) (fragments 1330, 200, 100)	*Epid. caudatum*	
b) (fragments 920, 610, 100)	*Epid. ecaudatum*	
10. Digestion with *Sty*I		
a) (fragments 800, 530, 300)		11
b) (fragments 800, 530, 150)		12
11. Digestion with *Acc*I		
a) (fragments 750, 600, 300)	*Enoploplastron triloricatum*	
b) (no cleavage, fragment 1650)	*Isotricha intestinalis*	
12. Digestion with *Msp*I		
a) (fragments 1530, 100)		13
b) (more fragments)		16
13. Digestion with *Nru*I		
a) (fragments 1100, 450)		14
b) (no cleavage, fragment 1650)		15
14. Digestion with *Acc*I		
a) (fragments 1000, 650)	*Metadinium medium*	
b) (fragments 1350, 300)	*P. multivesiculatum*	
c) (no cleavage, fragment 1650)	*Diploplastron affine*	
15. Digestion with *Acc*I		
a) (fragments 1350, 300)	*Dasytricha ruminantium*	
b) (no cleavage, fragment 1650)	*Isotricha prostoma*	
16. Digestion with *Nru*I		
a) (fragments 1100, 450)		17
b) (no cleavage, fragment 1650)	*Ostracodinium dentatum*	
17. Digestion with *Ase*I		
a) (fragments 840, 800)	*Eudiplodinium maggii*	
b) (no cleavage, fragment 1650)		18
18. Digestion with *Acc*I		
a) (fragments 1350, 300)	*Ostracodinium gracile*	
b) (no cleavage, fragment 1650)	*Diplodinium dentatum*	

morphotypes of *Ent. caudatum* (Fig. 2C), indicating that morphological variability observed is not due to genetic heterogeneity.

DISCUSSION

The rumen ciliates are the most abundant protozoa in the rumen and are involved in host metabolism and digestion of plant material. By classical morphological criteria more than 250 species of ciliates have been described which live in the rumen of various feral and domesticated ruminants (Williams and Coleman 1992). Identification of protozoa by these criteria is extremely tedious and requires extensive special knowledge and skills. Moreover, the validity of classical identification is questioned, since many of the "species" described exhibit a substantial morphological plasticity (Dehority 1994). Introduction of modern molecular methods based on DNA analysis and fingerprints, especially the methods targeted at ribosomal RNA operon provide exact insight into similarity studies of micro-organisms. Recent research using molecular characterisation has suggested that the protozoal diversity within the rumen is even greater than that first anticipated, but despite recent progress with molecular ecological studies (Karnati *et al.* 2003), the level of diversity present between individuals remains unclear.

While there are numerous examples of application of molecular methods for identification of bacteria (e.g. Blanc *et al.* 1997), only a few papers deal with identification of protozoa (Yang *et al.* 2002). The primary objective of the present work was to develop a rapid method for identification of predominant rumen protozoa. Restriction analysis of amplified ribosomal DNA method described here was found to be able to clearly discriminate between all strains studied. Use of the Chelex method enabled isolation of DNA from single protozoal cell thus reducing possible contamination by foreign DNA from feeds or fungi. On the basis of DNA fingerprints obtained a molecular key was designed. While all 18 restriction endonucleases provided some discrimination between strains, the key was optimized in order to minimize the number of cleavage steps and endonucleases used. While small *Entodinia* could be identified in four steps, up to six restriction endonucleases cleavage steps had to be used to unequivocally identify *Ostr. dentatum* and *Diplod. dentatum* species. RFLP patterns were found to be stable and reproducible.

In addition, to providing a simple method for discrimination of morphologically similar species, the RFLP technique also demonstrated that new morphologically different variants (e.g. Dehority 1994) are not necessarily new species. No differences were observed between identical protozoal species originating from different animals or geographic locations, or between morphological variants of the same species, indicating limited intraspecies variability of studied protozoa. These data are in correlation with a previous report on very limited intraspecies sequence variation among eight isolates of the rumen ciliate *I. prostoma* (Wright 1999).

In conclusion, the methodology and molecular key described here provides a rapid and convenient way for identification of rumen protozoa. Furthermore, it enables the examination of the diversity of rumen protozoa without requiring specialist knowledge regarding the morphological characteristics of the ciliates being studied. The technique may be performed in even a basic molecular laboratory and by a researcher unskilled in the identification of rumen protozoa by traditional means.

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ACTA
PROTOZOOLOGICA

Phylogenetic Relationship of *Trypanosoma corvi* with Other Avian Trypanosomes

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Summary. The phylogenetic relationships of avian trypanosomes, common parasites of birds, remain ambiguous and validity of many species is questionable. Analyses based on 18S rRNA sequences, dimensions of the kinetoplast disc and the size of kinetoplast (k) DNA minicircles were used to differentiate among large trypanosomes parasitizing birds of the Old World. These trypanosomes with typical striated appearance formed two well-supported groups - the "*Trypanosoma avium*" clade and the "*Trypanosoma corvi*" clade. Interestingly, the isolate derived from the Central European hipboscid fly (*Ornithomyia avicularia*) is closely related to *T. corvi* from a raven captured in the U.K., whereas a trypanosome obtained from the blood of a raven of the Central Europe origin (Czech Republic) is a typical member of the "*T. avium*" clade.

Key words: 18S rRNA, hippoboscid fly, insect vectors, kinetoplast DNA, phylogeny, *Trypanosoma avium*, *T. corvi*.

INTRODUCTION

Avian trypanosomes, transmitted by various blood-sucking invertebrates, are widespread parasites of birds (Apanius 1991). About one hundred species of bird trypanosomes have been described worldwide, mostly on the basis of "one host - one species" paradigm, according to which a new species was assigned for every "new" bird host (Bishop and Bennett 1992). However, clear-cut evidence for strict host specificity of bird trypanosomes is lacking, while several experimental transmissions of trypanosomes from one host

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species to another (sometimes across a family or an order) have been described (Baker 1956a, c; Bennett 1970; Woo and Bartlett 1982; Chatterjee 1983). Since the validity of most species has been questioned, many taxa are considered *nomina dubia* and the taxonomic status of avian trypanosomes remains generally controversial.

In 1885 Danilewsky described *T. avium* from birds of the families Corvidae, Accipitridae and Laniidae, and in 1889 included *Asio otus* (Strigiformes) and *Coracias garrulus* (Coraciiformes) as additional hosts. Later, *Strix aluco* obtained from a street market in Paris enlarged the list of *T. avium* hosts, while Laveran proposed that trypanosomes from *C. garrulus* belong to a different species (Laveran 1903). Since these early works, avian trypanosomes from many bird species

captured in Europe and Asia have been labelled as *T. avium*, but the descriptions and illustrations are (mostly) inadequate. According to Novy and McNeal (1905), *T. avium* also parasitizes a wide range of hosts in North America (*Agelaius phoeniceus*, *Colaptes auratus*, *Cyanocitta cristata*, *Icterus galbula*, *Lophotrix* sp., *Melopiza melodia*, *Passer domesticus*, *Sialia sialis*, *Turdus migratorius*, *Zenaidura macroura*). A more recent example of the concept that *T. avium* is a widespread parasite of birds is exemplified by its description from a fish crow (*Corons ossifragus*) in Florida (Dusek and Forrester 2002). Quite early, Lühe (1906) used the name *Trypanosoma confusum* for what he considered a "species mix". Many authors accepted this taxonomy of the New Word avian trypanosomes, while according to others *T. confusum* is regarded as a junior synonym of *T. avium* (Baker 1976).

Based on several studies (Bennett 1961, Baker 1976, Bishop and Bennett 1992), large trypanosomes with typical striated appearance (so-called myonemes) have been grouped together as species of the *T. avium* complex or the "*avium*" group (Baker 1976, Woo and Bartlett 1982, Apanius 1991, Bennett *et al.* 1994, Sehgal *et al.* 2001). The flag species of this group are *T. avium* and *T. corvi* from the Old World and *T. confusum* from the New World avian hosts. Baker (1976) proposed a concept according to which there are no more than 12 valid species of trypanosomes in birds world-wide, with *T. avium* restricted to trypanosomes of the Old World owls (Strigiformes), and morphologically similar protozoans parasitizing birds of the New World belonging to *T. confusum*.

Trypanosoma corvi Stephens et Christophers, 1908, originally described from a house crow (*Corvus splendens*) in India, was re-described from the material obtained from a raven (*Corvus frugilegus*) captured in the U.K., and emended for the large striated trypanosomes of corvids and blackbirds (*Turdus merula*) (Baker 1956a, c, 1976). Finally, flagellates obtained from the blood of a tree pic (*Dendrocitta vagabunda*) captured in India served as material for another morphology-based re-description of *T. corvi* (Nandi and Bennett 1994). At present, *C. splendens* is the type host of *T. corvi*, with *C. frugilegus, C. monedula spermologus, D. vagabunda*, and *Turdus* spp. being additional hosts. Generally, all large trypanosomes found in Corvidae and other families (e.g. Turdidae) of the Old World birds are considered to be conspecific into this species (Baker 1976).

The classification summarised above proved helpful for distinguishing trypanosomes from different bird hosts and continents; however, several taxonomic problems with the avian trypanosomes remain unsettled. The lack of morphological features among different isolates is out weighted by biochemical and molecular data. Biochemical characterization of trypanosome strains obtained from the American raptors (*Buteo jamaicensis*, *Accipiter striatus*) and owls (*Aegolius acadicus*) led Kirkpatrick and Terway-Thompson (1986) to conclude that they are closely related. The electrophoretograms of *T. corvi* from ravens were similar to those of trypanosomes isolated in the U.K. from sparrowhawks (*Accipiter nisus*) and black flies (*Eusimulium latipes*) (Dirie *et al.* 1990) indicating that this blood-sucking insect is the vector of *T. corvi*. However, according to Baker (1956b) and Mungomba *et al.* (1989), *T. corvi* is transmitted by the hippoboscid fly (*Ornithomyia avicularia*).

The 18S rRNA gene sequences are available for dozens of trypanosome species and are widely used to infer their relationship. Our previous study based on the sequences of the 18S rRNA genes showed that isolates from four raptor species (*Buteo buteo*, *Accipiter nisus*, *Falco tinnunculus*, *Aquila pomarina*), a chaffinch (*Fringilla coelebs*), a raven (*Corvus frugilegus*), and a black fly (*Eusimulium securiforme*), all originating from Central Europe, belong to a single robustly monophyletic "*T. avium*" clade (Votýpka *et al.* 2002). Furthermore, we have shown that the different bird trypanosomes are diverse in the size of kinetoplast (k) DNA minicircles and that this feature is reflected in a unique and characteristic shape of their kinetoplast (Lukeš and Votýpka 2000, Votýpka *et al*. 2002). In this study we have used molecular techniques (sequencing of the 18S rRNA gene and determination of minicircle size) and electron microscopy (measurement of kinetoplast thickness) to compare trypanosomes originating from a raven captured in the U.K. and described as *T. corvi* with the other Old World avian trypanosomes including isolates from a raven (*C. frugilegus*) captured in the Czech Republic and a hippoboscid fly (*Ornithomyia avicularia*).

MATERIALS AND METHODS

Strain origin and cultivation of trypanosomes. The *Trypanosoma corvi* strain "LUM-LSHTM" (ITMAP 180795) was isolated by J. R. Baker from the blood of an adult raven (*C. frugilegus*)

captured in Fordingbridge, Hampshire, U.K. in June 1970. Cultivation was performed on blood agar with RPMI 1640 medium (Sigma) and supplemented with 10 % (v/v) foetal calf serum (Sigma) and gentamicin (80 µg/ml) at 23°C.

DNA processing. Isolation of total cellular DNA, PCR amplification of the 18S rRNA gene using primers S762 and S763, cloning, and sequencing with a set of conserved primers were performed as described previously (Maslov *et al.* 1996). Isolation of the kDNA network and enzyme digestion of minicircles was done according to Votýpka *et al.* (2002).

Electron microscopy. For transmission electron microscopy, cells collected from culture in the exponential phase (6-day-old culture) were washed in 0.1 M phosphate-buffered saline solution and fixed in 2.5 % glutaraldehyde in the same buffer at 4°C. Samples were further processed and the kDNA structure was analyzed as described elsewhere (Lukeš and Votýpka 2000).

Sequence availability. 18S rRNA sequences of *T. corvi* and *T. "avium"* FT2 were deposited in GenBank under the accession numbers AY461665 and AY099319, respectively. Nucleotide sequences used in phylogenetic analysis are available from the GenBank/EMBL databases under the following accession numbers: *T. cruzi* PERU (X53917), *T. cruzi marinkellei* (AJ009150), *T. dionisii* PJ (AJ009152), *T. dionisii* P3 (AJ009151), *T. rangeli* (AJ012416), *T. vespertilionis* (AJ009166), *Trypanosoma* sp. "kangaroo" (AJ009168), *T. lewisi* (AJ223566), *T. microti* (AJ009158), *Trypanosoma* sp. OA6 (AF416562), *Trypanosoma* sp. CUL1 (AF416561), *T. bennetti* (American kestrel), *T. grayi* (AJ223565), *T. "avium"* A1412 (U39578), *T. "avium"* SIM3 (AF416563), *T. "avium"* APO1 (AF416559), *T. "avium"* LSHTM144B (AJ009140), *T.* cf. *paddae* N335 (AJ223570), *T. varani* (AJ223572), *T. scelopori* (U67182), *Trypanosoma* sp. "wombat" (AJ009169), *T. pestanai* (AJ009159), *T. theileri* (AJ009164), *Trypanosoma* sp. D30 (AJ009165), *T. theileri* (AJ009163), *T. cobitis* (AJ009143), *Trypanosoma* sp. "fish" (L14841), *Trypanosoma* sp. "leech" (AJ009167), *T. boissoni* (U39580), *T. triglae* (U39584), *T. rotatorium* B2-II (AJ009161), *T. rotatorium* B21(U39583), and *T. mega* (AJ009157). Information on avian trypanosomes (for which molecular data are available) has been summarized in Table 1.

Phylogenetic analyses. The entire 18S rRNA gene sequences from 35 trypanosomes were aligned using the ClustalX program (Thompson *et al.* 1997), alignment was manually corrected, and gaps and ambiguously aligned regions were excluded from phylogenetic analyses. Maximum parsimony (MP) and maximum likelihood (ML) trees, as well as trees based on LogDet-distances (D) were constructed. MP trees were constructed using PAUP* program (Swofford 2000) with TBR as a branch swapping method and random addition of sequences with 10 replicates. MP bootstrap support was computed out of 1000 replicates with random addition of sequences with 10 replicates. ML tree was computed using the DNAml program in Phylip package (Felsenstein 2001) version 3.6a3, using HKY85 model with discrete gamma distribution in 8+1 categories. Gamma distribution parameter α and portion of invariable sites were estimated from dataset using Tree-Puzzle program (Strimmer and von Haeseler 1996), version 5.0. Maximum-likelihood puzzle support was computed in 10,000 puzzling steps using Tree-Puzzle 5.0 with discrete gamma distribution in 8+1 categories. Distance tree was constructed using LogDet/paralinear distances as implemented in PAUP* version 4b10, with constant positions excluded from the analysis. Consistent with previously published phylogenies of trypanosomes (Stevens and Gibson 1999, Votýpka *et al.* 2002), all constructed trees were rooted using *T. mega* as an outgroup.

RESULTS AND DISCUSSION

Alignment used for phylogenetic analysis contained 35 taxa and 2031 characters and is available from the authors upon request. In this alignment, 441 characters are variable, with 281 characters parsimony informative. Maximum parsimony analysis resulted in construction of 22 equally parsimonious trees with TL (tree length) = 1039 steps; CI (consistency index) = 0.5987; RI (retention index) = 0.6936; RC (rescaled consistency index) = 0.4152. Although the general topology of the MP tree shown in Fig. 1 is unstable, the cluster composed of *T. varani*, *T. scelopori, T. pestanai*, and *Trypanosoma* sp. "wombat" branches together with the "*T. avium*" clade in 20 out of 22 equally parsimonious trees constructed. These 20 trees differ only in the internal

Table 1. Summary details of avian trypanosomes analysed.

| Species	Acc. No.	Strain	Host species		Origin
T. "avium"	AF416559	AAQU/SK/97/APO1	lesser-spotted eagle	*Aquila pomarina*	Slovakia
T. "avium"	AF416563	IEUS/CZ/99/SIM3	black fly	*Eusimulium securiforme*	Czech Rep.
T. "avium"	AY099319	AFAL/CZ/99/FT2	European kestrel	*Falco tinnunculus*	Czech Rep.
T. "avium"	AJ009140	LSHTM144B	chaffinch	*Fringilla coelebs*	Czech Rep.
T. "avium"	U39578	A1412	raven	*Corvus frugilegus*	Czech Rep.
T. cf. *paddae*	AJ223570	N335	Java sparrow	*Padda oryzivora*	Asia (?)
T. corvi	AY461665	ITMAP 180795	raven	*Corvus frugilegus*	England (U.K.)
Trypanosoma sp.	AF416562	IORN/CZ/99/OA6	hippoboscid fly	*Ornithomyia avicularia*	Czech Rep.
Trypanosoma sp.	AF416561	ICUL/CZ/98/CUL1	mosquito	*Culex pipiens*	Czech Rep.
T. bennetti	AJ223562	KT-2 (ATCC 50102)	American kestrel	*Falco sparverius*	USA, New Jersey
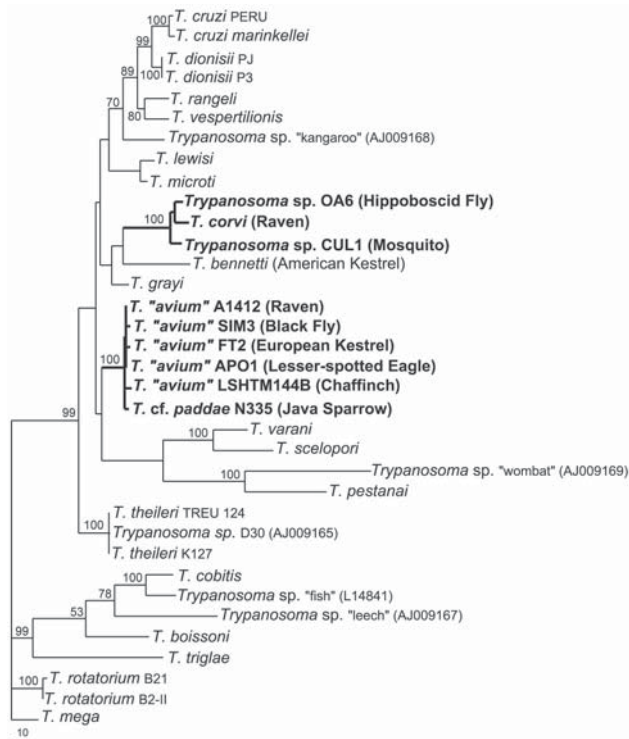
Fig. 1. Maximum parsimony (MP) phylogenetic tree (one of 22 equally parsimonious trees) as inferred from 18S rRNA gene sequences. Tree was computed out of 2031 characters, from which 281 were parsimony informative, using heuristic options in the PAUP* program (Swofford 2000) version 4b10, with random addition of sequences with 10 replicates. Tree is 1039 steps long, CI = 0.5987; RI = 0.6936; RC = 0.4152. Numbers above branches indicate MP bootstrap support (higher than 50%) in 1000 replicates with random addition of sequences in 10 replicates. Tree was rooted using *T. mega* as an outgroup.
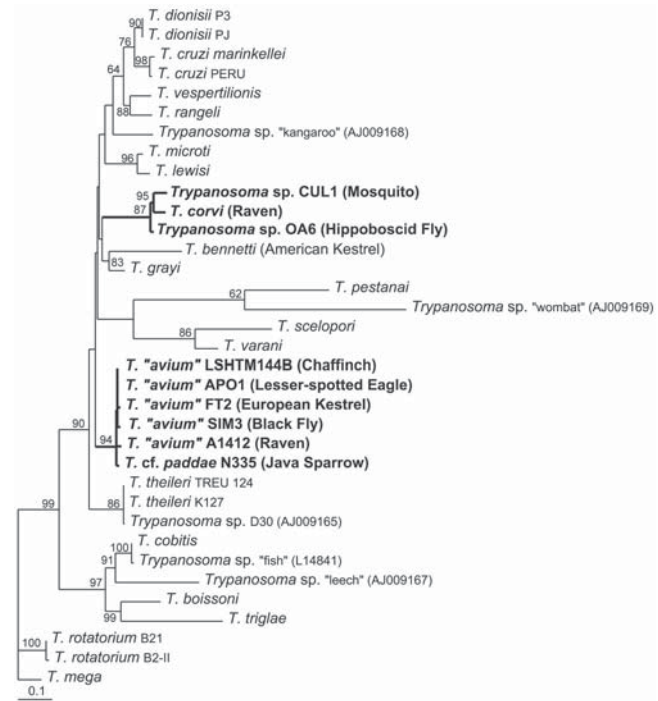
Fig. 2. Maximum likelihood (ML) phylogenetic tree as inferred from 18S rRNA gene sequences. Tree was constructed using DNAml from the Phylip package version 3.6a3 (Felsenstein 2001) with HKY85 model with discrete gamma distribution in 8+1 categories. Gamma distribution parameter α, as well as portion of invariant sites, were estimated from the dataset using the Tree-Puzzle program version 5.0 (Strimmer and von Haeseler 1996). Numbers above branches indicate ML puzzle support (higher than 50%) as determined by Tree-Puzzle (discrete gamma distribution in 8+1 categories). Tree was rooted using *T. mega* as an outgroup.

topologies of particular clusters. In the remaining two trees, this cluster does not form a sister group to the "*T. avium*" clade, which is more closely related to *T. theileri*. Moreover, in these two trees, *T. grayi* does not appear on the root of the "*T. corvi*" cluster, as it does in most MP trees, but becomes affiliated to *T. theileri*. However, bootstrap support for these topological variations is low (data not shown). In ML tree (Fig. 2), the *T. varani, T. scelopori*, *T. pestanai* and *Trypanosoma* sp. "wombat" cluster appears between the "*T. corvi*" and "*T. avium*" clades, but similarly to the MP bootstrap analysis, this position is not supported by the ML-puzzle tree. Finally, in phylogenetic trees based on the LogDet/ paralinear distances, position of the above-mentioned cluster differs from that found in the ML and MP trees, since it is related to *Trypanosoma* sp. "kangaroo" (Fig. 3), while in the same tree, *T. theileri* appears on the

root of the clade composed of *T. cruzi*, *T. dionisii*, *T. rangeli*, and *T. vespertilionis*. The four equal LogDet trees constructed differed only in the internal topology of the *T. avium* cluster. Importantly, in all constructed trees, *T. corvi* and *T. avium* formed highly supported separate clusters (see Figs 1-3), however, their mutual relation has not been resolved. In all trees, except the two MP trees, *T. grayi* and *T. bennetti* appear on the root of the *T. avium* cluster, although this relationship is not supported by bootstrap or puzzle analyses.

Several strains of *T. corvi* isolated from ravens in the U.K. have been studied so far: (i) unnamed strains isolated by J. R. Baker from ravens in Hertfordshire (England) between the years 1953 and 1955 were used to reveal the incidence of parasites in birds and transmission experiments using hippoboscid flies (Baker 1956a, b, c) and, (ii) together with our model strain ITMAP
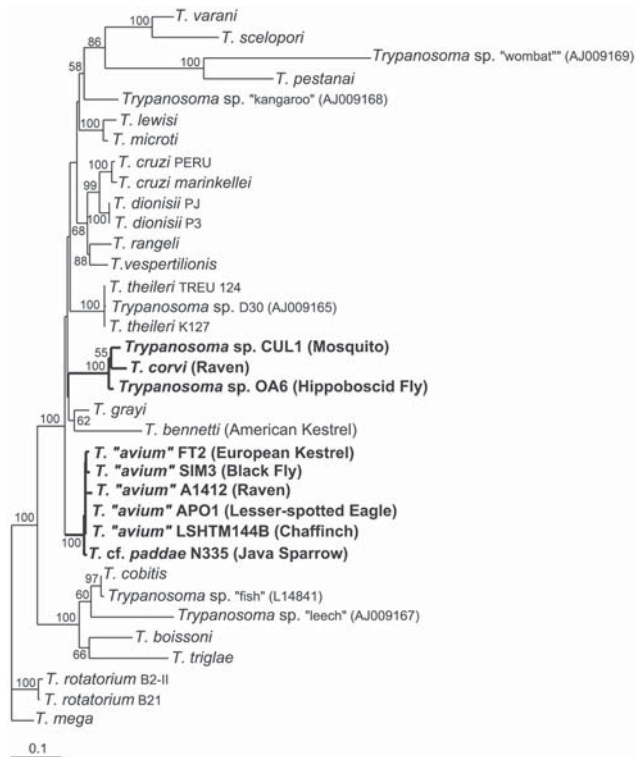
Fig. 3. Phylogenetic tree (one of four trees) based on LogDet distances, as inferred from 18S rRNA gene sequences. Tree was constructed using PAUP* program (Swofford 2000) and alignment containing 441 variable positions (constant positions excluded). Minimum evolution score was 2.57308. Numbers above branches indicate bootstrap support (higher than 50%) computed with 1000 replicates. Tree was rooted using *T. mega* as an outgroup.

180795, have been used for species re-description of *T. corvi* (Baker 1976); (iii) strain no. 85 from a bird captured in 1973 in the same locality (Hertfordshire) as the above-mentioned strains was used for immunity studies (Molyneux and Gordon 1975); and (iv) strain from Cheshire (England) was subjected to electron microscope observation of development in the gut of its insect vector (Mungomba *et al.* 1989). We assume that all these strains belong to the same species, of which the one available to us (ITMAP 180795) is considered to be most representative.

Unexpectedly, based on the 18S rRNA sequence, the trypanosome strain obtained from the blood of a raven captured in the Czech Republic (strain A1412) branched within the well-supported "*T. avium*" clade, in the company of strains parasitizing a chaffinch, lesser-spotted eagle, java sparrow and black fly (Votýpka *et al.* 2002). However, when features like minicircle size (10 kb) and

kinetoplast thickness (1.2 μm) are considered, the strain A1412 is clearly different from other avian trypanosomes (Lukeš and Votýpka 2000, Votýpka *et al.* 2002). These results imply that the studied European populations of ravens are infected by at least two different trypanosome species, which may possibly be transmitted by different vectors.

Previous studies identified the hippoboscid fly *Ornithomyia avicularia* as the vector of *T. corvi* (Baker 1956b, Mungomba *et al.* 1989). When we included the newly obtained 18S rRNA gene sequence of *T. corvi* ITMAP 180795 into the available 18S rRNA dataset, it clustered together with the trypanosomes isolated from a hippoboscid fly (strain OA6) and a mosquito (CUL1) collected on raptor nestlings in the Czech Republic, forming a highly supported clade (Figs 1-3). Analyses of the kinetoplast structure and the kDNA minicircle size further supported this relationship. In fact, the low-pitched and elongated kinetoplast observed by electron microscopy in the *T. corvi* cells (thickness 0.39 ± 0.35 μm; n = 38) was almost indistinguishable from the kinetoplast disc of the strain OA6 obtained from a hippoboscid fly (Fig. 4; see also Votýpka *et al.* 2002). This is in contrast to the cylindrical-shaped kinetoplasts characteristic of trypanosomes from Central European raptors, raven, black bird, and black fly (Lukeš and Votýpka 2000, Votýpka *et al.* 2002).

In our recent work (Votýpka and Svobodová 2004) we show that kinetoplast thickness is a character stable for a particular trypanosome species in its host, vector and derived culture stages. Moreover, the thickness of the kinetoplast disc correlates with the size of minicircles that constitute the kDNA network (Lukeš and Votýpka 2000). The thin disc of *T. corvi* is composed of 3.0 kb long minicircles (data not shown) similar in size to minicircles of the strain OA6 from the hippoboscid fly (Votýpka *et al.* 2002). In addition, the hindgut and rectum of infected hippoboscid flies harbour a heavy infection of slender epimastigotes (data not shown), the localization and morphology of which is reminiscent of flagellates described from vectors by Baker (1956b) and Mungomba *et al.* (1989). Molecular and morphological data thus provide evidence that *T. corvi* from a raven captured in the U.K. is either identical with, or very closely related to, the trypanosome isolated from a hippoboscid fly in central Europe three decades later.

Close relatedness of *T. corvi* isolated from a raven with trypanosomes originating from a hippoboscid fly (strain OA6) and a mosquito (strain CUL1), based on the 18S rRNA sequences as well as morphological
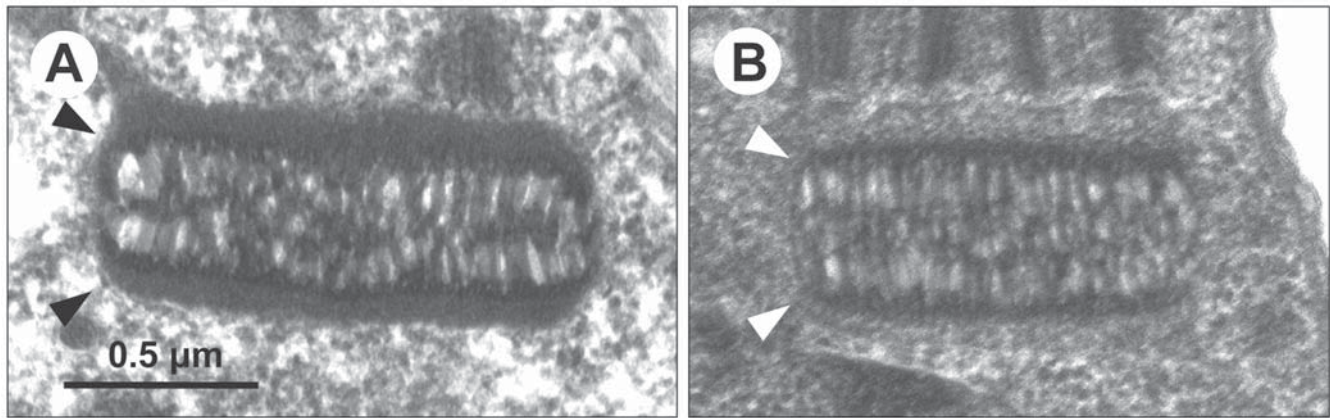
Fig. 4. Transmission electron microscopy of low-pitched and elongated kinetoplasts from *Trypanosoma corvi* (strain ITMAP 180795) (**A**) and trypanosome isolated from the hippoboscid fly (*Ornithomyia avicularia*) (strain OA6) (**B**). Arrowheads indicate thickness of the kinetoplasts. Both micrographs are to the same scale.

features, prompted us to speculate that *T. corvi* is transmitted *via* different vectors among its avian hosts. However, important differences exist between the infection in the hipposcids, for which a contaminative transmission has been proposed (Mungomba *et al*. 1989), and that in the mosquitoes. In the latter insects, a massive plug of parasites attached to the stomodeal valve in the foregut region was observed, as well as accompanying degenerative changes of the valve, reminiscent of the sand fly infections by *Leishmania* spp. (Hajmová, Votýpka and Volf, unpubl. results). Therefore, we conclude that cycling of trypanosomes between mosquitoes and birds is carried out *via* inoculation or regurgitation rather than by the contaminative way described in the hipposcids. Such a dissimilar type of transmission is an important feature of the biology of kinetoplastids, and should be considered incompatible with their belonging to one species. On the basis of this difference, we propose that avian trypanosomes isolated from hipposcids fly and mosquito belong to two different but closely related species.

Questions concerning the host specificity of *T. corvi* still remain open. Baker (1956a) found trypanosomes in ravens (*Corvus frugilegus frugilegus*), jackdaws (*C. monedula spermologus*), and blackbirds (*Turdus merula merula*) and suggested that flagellates from these birds belong to the same species. Furthermore, he transmitted them experimentally to canaries (*Serinus canaries*). After the emendment of *T. corvi* by Stephens and Christophers (1908), Baker restricted the use of the name *T. corvi* to large trypanosomes from non-American corvids and also from other bird families (e.g. Turdidae) (Baker 1976). While our trypanosome strain isolated from a blackbird (strain A493) appears to be a typical member of the '*avian*' group (Lukeš and Votýpka 2000; this work), we could not exclude the possibility that *T. corvi* is able to infect other bird species than corvids.

With the exclusion of *T. bennetti*, all other trypanosomes isolated from birds, for which the 18S rRNA sequence is available (Table 1), constitute a homogeneous group, the monophyly of which is supported by 100% bootstraps. Moreover, this compact group contains one trypanosome isolated from a black fly (strain SIM3). It is hard to find support in our dataset for the existence of more than one species - *T. avium*.

Trypanosoma avium is apparently able to invade many species of birds belonging to distant orders (Bennett 1970, Chatterjee and Ray 1971). While for *T. paddae* (*T.* cf. *paddae*; strain N335) a feature that distinguishes this species from other avian trypanosomes has been described (kinetoplast situated closer to the nucleus) and since it constitutes the earliest branch within the "*avian*" clade, its separate status should be retained until more sequence data is available. *T. paddae* was originally described from the Asian Java sparrow (*Padda oryzivora*) and Baker (1976) speculated that this name is valid for most of the trypanosomes of Fringillidae recorded from Europe and Asia. Our results do not support this view, since trypanosome strain LSHTM144B from chaffinch (*F. coelebs*) has the 18S rRNA sequence more related to *T. avium* from birds of prey and the black fly (Figs 1-3). The inclusion of strains isolated

from small passerine birds into the phylogenetic analysis is necessary to settle the taxonomic status of *T. brimonti*, *T. bakeri* (Baker 1976, Chatterjee 1983) and other avian trypanosomes.

Based on a combined analysis of molecular data and morphologic features, we provide the evidence that avian trypanosomes form two well-supported monophyletic clades: the "*T. avium*" clade and the "*T. corvi*" clade. While no significant differences have been found in their 18S rRNA sequences, avian trypanosomes of the "*T. corvi*" clade isolated from a mosquito and a hippoboscid fly undergo such a different development in the vector that their provisional placement into two different species is unavoidable. The data available for flagellates belonging to the "*T. avium*" clade is consistent with the notion that trypanosomes parasitizing unrelated bird hosts may indeed belong to one species with a low host specificity that is transmitted by black flies.

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ACTA
PROTOZOOLOGICA

Relationship between Atmospheric Pollution Characterized by NO$_2$ Concentrations and Testate Amoebae Density and Diversity

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Summary. To assess the potential use of testate amoebae as biomonitors of atmospheric pollution we studied the relationship between atmospheric nitrogen dioxide (NO$_2$) pollution and testate amoebae density, diversity, and community structure (Protista: Rhizopoda) in (zone 1) and around (zone 2) the city of Besançon, France. NO$_2$ concentrations were on average significantly lower in the city zone (mean: 34.8 ± 9.5 µg/m^3) than in the periphery zone (mean: 14.6 ± 4.7 µg/m^3). The density of living amoebae was correlated with that of empty tests ($0.001 < p < 0.043$ depending on the species), therefore we used the total of dead and living amoebae in all analyses. Testate amoebae species richness was significantly lower in the city (4.7 species) than in the less polluted surrounding areas (6.0 species) but the total density did not vary between the two zones. Of the nine recorded taxa, the density of only one, *Paraquadrula irregularis* differed significantly between the two zones (p = 0.017), being present in all periphery samples and absent from all city samples. These results are interesting because the pollution level recorded was very low. Although further work is needed before testate amoebae can be used as a monitoring tool for atmospheric pollution, these results suggest they may have a potential for such a use. Further work should focus on potential effects of other pollutants and studies under controlled conditions.

Key words: air pollution, bioindication, bryophyte, nitrogen dioxide, protist, testate amoebae.

INTRODUCTION

The degradation of air quality in urban areas is a major problem and many countries have developed programs to monitor and control its intensity. Atmospheric pollution is a complex notion that is mainly defined by its negative consequences. The perception of this threat changes as progresses are made in atmospheric physics and chemistry, toxicology (Sandström 1995, Sega 1995) and epidemiology (Last *et al*. 1994, Nakai *et al*. 1995, Société Française de Santé Publique 1996, Bernard *et al*. 1998). In addition, atmospheric pollution varies temporally in relation to climate, especially wind and temperature. For this reason, measurement must be done almost on a continuous basis. A complementary option is to use the sensitivity of organisms to pollution level. This biomonitoring approach

integrates the pollution level over a long period of time and therefore provides data on an average pollution level for a given place. It may also allow the detection of extreme or catastrophic events than may not be recorded by non-continuous pollution monitoring. The sensitivity of an organism to environmental stress depends on its ability to recover from stress, its mobility and its live cycle pattern.

Several groups or organisms have been used as bioindicators of atmospheric pollution. The most commonly used are mosses (Palmieri *et al*. 1997, Pearson *et al*. 2000), and lichens (Hamada and Miyawaki 1998, Conti and Cechetti 2001). Farmer *et al*. (1992) performed fumigation experiments with bryophytes and lichens and criticized the utilization of unrealistically high concentration of NO_2 used in many experiments. The effects of a realistic NO_2 concentration (122.2 µg/m^3 = 64 ppb - using for NO_2 1 ppb = 1.91 µg/m^3 at 21.1°C) on the endohydric moss *Polytrichum formosum* were varied: the growth of existing shoots was a first stimulated but later these shoots suffered a 46% reduction in number, and there was a 36% reduction of new shoot production (Bell *et al*. 1992). In a fumigation experiment, Morgan *et al*. (1992) showed that, at about half that concentration (35 ppb = 66.9 µg/m^3), NO_2 stimulated nitrate reductase activity in four mosses. In another laboratory experiment nitrite was showed to affect the shoot respiration of two terricolous (i.e. growing on the soil) mosses *Pleurozium schreberi* and *Rhytidiadelphus triquetrus* (Bharali 1999).

By comparison to bryophytes and lichen, only few studies have focused on microorganisms. The rare exceptions concern fungi and algae. For example, Garcia *et al*. (1998) showed that the lead concentration of *Coprinus comatus* (Fungi) was correlated to pollution levels. Green algae have also been shown to be good indicators of nitrogen, sulphur and metal pollution (Poikolainen *et al*. 1998, Shubert *et al*. 2001). To our knowledge, although testate amoebae were shown to be valuable indicators for soil conditions (Foissner 1987, 1999; Tolonen *et al*. 1992, 1994; Gilbert *et al*. 1998; Muqi and Wood 1999), only two studies have focused on using these protists as bioindicators of atmospheric pollution (Lüftenegger and Foissner 1989, Balik 1991).

In a study on the effect of road pollution on testate amoebae in Warsaw (Poland), Balik reported a decrease in abundance, species richness, species diversity (Shannon Weaver index), and the index of equitability (Balik 1991). Furthermore a shift in community composition was also observed. Of a total of 42 species and subspe-

cies recorded, 16 were found only in the less polluted sites, while three were found only in the more polluted sites, but two of these each in a single sample and in relatively low abundance. Unfortunately, no data on the atmospheric or soil pollution were given in this study and therefore the information is qualitative.

Lüftenegger and Foissner (1989) also studied the effect of road traffic pollution on soil testate amoebae by analysing two 100 m transects on both sides of a high-traffic road. In this study several pollutants were measured: lead, cadmium, chloride, and polycyclic aromatic hydrocarbons. The lowest density, biomass and species richness of testate amoebae occurred near the road, but were not correlated with the highest concentrations of the measured pollutants, most of which peaked at 50 m from the road. Testate amoebae were thus correlated with higher lead, total organic carbon, and polycyclic aromatic hydrocarbon concentrations but the reason for their lower abundance near the road could not be established.

Testate amoeba may be interesting candidates for the monitoring of air pollution for several reasons: (1) they can live in a sub-aerial environment where they are directly exposed to atmospheric pollutants, (2) they are very abundant, diverse (about 100 potential species in mosses alone although the number in any given samples is much lower), and most species are cosmopolitan (although exceptions exist) (Bonnet 1973), (3) their identification is relatively easy based on the morphology of their test (shell) that remains even after the death of the organism and (4) they are good integrators of perturbations because of their trophic position at the end of the microbial food webs (Gilbert *et al*. 1998, 2000). Mosses growing on vertical surfaces, such as trees and walls, where water drains fast, represent an extreme environment for aquatic microorganisms including amoebae. Water availability appears to be the main limiting factor for the amoebae, and accordingly, the species found in these mosses often have adaptations such as small size or a flattened test (shell) with a ventral aperture (Bonnet 1973).

The aim of this study was to evaluate the relationship between anthropogenic atmospheric NO_2 pollution and the density, diversity and community structure of testate amoebae living in *Tortula ruralis* (Hedw), a common moss species in urban and suburban areas. Testate amoebae are likely to be indirectly affected by NO_2 pollution through the bacteria and other microorganisms on which they feed if these were themselves feeding on contaminated remains of mosses. In addition, gases may

directly affect amoebae as they diffuse in the water film and change the water chemistry. Our working hypotheses were: (1) NO_2 pollution levels would be higher in the city than in the surrounding areas. (2) The density and diversity of testate amoebae and the structure of communities would differ between the city and the surrounding area, and along the NO_2 pollution gradient.

METHODS

Study sites. This study took place between April 20th and May 3rd 2001 in 15 sites located in the town of Besançon (Franche-Comté, France) and surrounding villages (Fig. 1). The sites were selected along an East-West axis in two zones: (1) city: seven sites located within the limits of Besançon and (2) periphery: eight sites located in the surrounding outskirts and rural area. The periphery zone included the city outskirts where shopping areas are located but with no industrial source of pollution, and rural areas. As the dominant winds blow from the Southwest, the peripheral sites were presumably not contaminated by the "plume" of higher pollution levels produced in the city, and therefore the pollution gradient was maximized.

Meteorological data. Data from MeteoFrance stations located in Besançon (temperature, rain, humidity and wind speed) and in Marnay (rain) were used. In Besançon, the minimal temperature varied from -0.9 to 11.4°C and the maximal from 7.2 to 20.4°C. The sum of rain during this period was 38.4 and 36.1 mm of water respectively in Besançon and Marnay. The humidity varied from 67.4 to 90.5% (mean = 78.7 ± 7.3%). The wind speed was very low for the whole period (mean = 2.4 ± 0.9 m/s; data from Meteo France). These data were similar to those usually observed at the beginning of spring in Besançon.

Sampling for testate amoebae. For each site, two individual moss cushions were collected on hard substrate and in non-trampled places (walls, large rocks, roofs). The same bryophyte species, *Tortula ruralis*, a cosmopolitan and common bryophyte growing on rocks, walls, and calcareous-rich substrates (Jahns 1996), was sampled in all sites. The top part of the mosses (living, green) was separated from the lower part (brown, dead) in the laboratory. Only the top part was used for testate amoebae analyses. For each sample we measured 20 shoots to determine the average thickness of the green part. For each sample, approximately 0.3 g (fresh weight) of the living part was inserted in a glass vial with 7 ml of a 4% formaldehyde solution.

Testate amoebae extraction and analyses. To extract testate amoebae, the moss samples were shaken with a vortex mixer, filtered through a 40 µm mesh, and washed with deionised water. The fraction remaining on the filter contained no testate amoebae. Testate amoebae larger than 40 µm were recovered in the filtrate. This suggests that the tests of species such as *Arcella catinus*-type were flexible enough to be forced through the filter by the water pressure, and/or that the filter itself was flexible. The fraction remaining on the filter was dried at 80°C during 48h and weighed. The filtrate containing the testate amoebae was placed in a plankton-settling chamber and left to sediment for 24h. The slides were then analysed at a magnification of 200× and 400× with an inverted microscope following Uthermöhl's method (Uthermöhl 1958). The whole slide was analysed for testate amoebae. The total number of tests counted varied between 23 and 2757 individuals. Living and dead (empty shells) individuals were counted separately.

NO_2 sampling and measurements. Passive samplers (Palmes *et al.* 1976) are calibrated tubes, 7 cm long, with an inside diameter of 1 cm, in which gases move only by molecular diffusion (Gradko International, Winchester, Great Britain). A triethanolamine solution, which was deposited on the grid at one end of the tube, fixed the NO_2. The other end of the tube remained open for diffusion of gases. At 21.1°C and at a pressure of 1 atmosphere, the diffusion coefficient for NO_2 is 0.154 cm²/s, which means that the collection rate for our passive sampler could be calculated at 72 cm³/h. Mean hourly concentration of NO_2 (in µg/m³, hereafter $[NO_2]$) in the air sample was calculated on the basis of the amount of pollutant collected, exposure time, and gas collection rate in the tube. Absorbed NO_2 was measured by spectrophotometry using a variant of the Griess-Saltzman method (Atkins et al. 1986). NO_2 concentrations were expressed in µg/m³, (1 ppb = 1.91 µg/m³ at 21.1°C). In an earlier study, the passive samples were validated on chemiluminescence analysers (Bernard *et al*. 1997). Each passive sampler tube was exposed and allowed an integration of the NO_2 pollution level over a period of 14 days (the recommended exposure time for the model we used was of less than 20 days). For each sampling location, two tubes were placed on April 20th 2001 and removed 14 days later. Passive samplers were set up vertically on freestanding poles at 2 m from the ground and at over 2 m from vertical surfaces. The samples were fixed on wooden blocks, which kept them at a distance of 80 mm from the sides of the support, thus allowing air to circulate freely around them.

RESULTS

Atmospheric NO_2 concentrations and thickness of the green part of the mosses. Atmospheric NO_2 concentrations levels varied between 7 µg/m³ (Marnay, site most distant from the city) and 48 µg/m³ (Besançon, city centre) (mean: 24 ± 12.5 µg/m³) (Fig. 1). The $[NO_2]$ increased from the west to the east, which corresponds to the gradient of increasing urbanization. However, a clear distinction also appears between the sites located within the city zone, with values of 30 and above (mean: 34.8 ± 9.5 µg/m³), and the periphery zone, with values mostly between 10 and 20 (mean: 14.6 ± 4.7 µg/m³). The NO_2 concentrations were significantly different between these two zones (Mann-Whitney-test, p = 0.012). By contrast, the length of the green part of the mosses did not differ between the two zones (Mann-Whitney-test, p = 0.631).

Testate amoebae density. The total density of testate amoebae tests (living + dead) varied between 990 and 26225 ind/g dry weight of moss in the samples (mean: 6767 ± 6279 ind/g). The density of living amoebae was correlated with that of empty tests (0.001<p<0.043 depending on the species). Furthermore, the proportion of tests containing a living cell
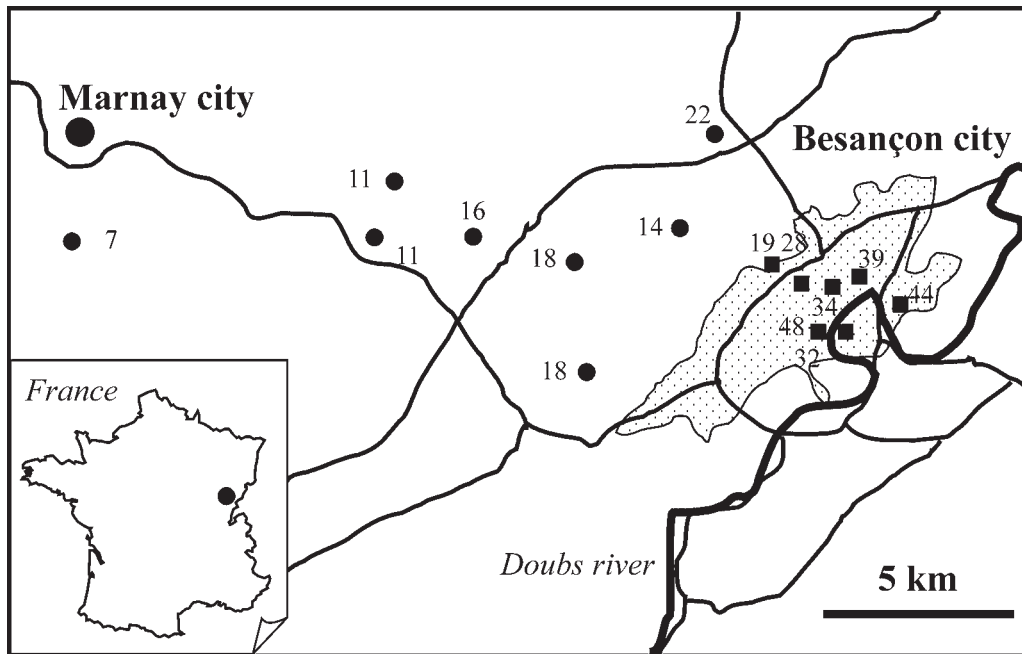
Fig. 1. Location of the study site and average atmospheric NO$_2$ concentrations [μg/m^3] measured at the sampling locations (squares: city; circles: periphery). Stippled area: Besançon agglomeration. Continuous lines: principal roads.

Table 1. Comparison of environmental and testate amoebae variables in the periphery (8 samples) and city (7 samples) zones.

	Periphery			City limits			P-value #
	n	Mean	SE	n	Mean	SE	
NO2 concentration [μg/m^3]	8	14.6	1.7	7	34.8	3.6	**0.001**
Thickness of the living part of mosses [cm]	8	0.6	0.1	7	0.6	0.0	0.631
Total testate amoeba density [ind/g]	8	7218	2115	7	6252	2462	0.487
Testate amoeba species richness	8	6.0	0.53	7	4.71	0.48	**0.016**
Paraquadrula irregularis	7	176	78	0	0	0	**0.017**
Nebela tincta-type	2	3	2	3	121	87	0.270
Euglypha ciliata	8	4260	1447	7	3147	1145	0.487
Difflugia pristis-type	8	255	65	5	132	80	0.487
Assulina muscorum	8	1447	790	6	2527	2361	0.563
Arcella catinus-type	8	327	239	7	183	52	0.728
Trinema complanatum	7	749	468	5	137	51	0.907

Mann Whitney-test between the values for the two zones.

(between 13.2 and 64.2% of the total) was similar in the two studied zones (respectively, 40.3 ± 11.0% and 38.8 ± 13.7% in zones 1 and 2) and was not significantly different between the two zones. Therefore we used the total of dead and living amoebae in all further analyses. The total density of testate amoebae was neither significantly correlated with [NO$_2$], nor with the length of the green part of the mosses, and was only slightly, but not

significantly, lower in the city than in the surrounding areas (Table 1).

Testate amoebae species richness and diversity. A total of nine testate amoebae species were identified in the 15 samples. However, two taxa (*Centropyxis minuta*-type and *Corythion dubium*-type) each occurred in only sample with one and four empty tests. Thus, we decided to remove these two taxa from the data set for further analyses. The species richness of individual samples varied between four and six (mean: 5.4 ± 0.8) and was significantly lower in the city (4.7 ± 0.48) than in the surrounding areas (6.0 ± 0.53; Mann-Whitney test, p = 0.016; Table 1). Furthermore, the species richness was significantly correlated with the NO_2 concentration (n = 15, r = -0.651, p = 0.007).

DISCUSSION

The NO_2 concentrations values measured are similar to those that are regularly published by the service of air pollution monitoring (ASQAB 2001) and are well under the official EC norm (Mean emission limit for 1 h: 200 µg/m³). This indicates an overall good quality of air in the region, at least for NO_2 pollution. Furthermore, this shows that the gradient we have chosen to study is not an extreme case.

The diversity of testate amoebae found in our samples is lower than usually found in mosses. However, our sampling protocol excluded the lower part of the mosses, richer in humus and where conditions are likely more favourable to a higher diversity of testate amoebae and other microorganisms. In the deeper parts of the moss cushion, other environmental factors than atmospheric pollution would be likely to influence the structure of communities and the response of testate amoebae to atmospheric pollutants would most likely be less clear.

In accordance with our hypothesis, the testate amoebae species richness was significantly higher in the peripheral zone where NO_2 concentrations were lower. Furthermore, a significant relationship was found between the testate amoebae data and [NO_2].

One species, *Paraquadrula irregularis*, appeared to be sensible to the atmospheric pollution characterized by NO_2 in the city centre. This species, previously reported in moss ecosystems (Cash and Hopkinson, in Decloitre 1961), has an endogenic calcareous test (Bonnet 1989). The sensitivity of this species might reflect an indirect impact of pollution through an acidification effect causing a leaching of Ca^{2+} through a cation exchange mechanism (H^+ replacing Ca^{2+}) in the mosses. If true, this would mean that *P. irregularis* is rare or totally absent from the city centre of Besançon.

Contrary to our expectations, the density of testate amoebae does not appear to be a good indicator of NO_2 pollution within the range of concentrations covered by this study. The variability of density may be due in great part to other variables, such as the physical characteristics of the sampling location. Indeed the growth of mosses depends on the micro-climatic conditions and the production of amoebae is higher under high humidity conditions (Van Kerckvoorde *et al*. 2000). Our results agree with those of Balik (1991) regarding the relationship between atmospheric pollution and species richness, but not the density.

Despite the well-established relationship between testate amoebae and micro-environmental conditions, no significant correlation emerged between the thickness of the moss cushion and the testate amoebae data. This variable was chosen because it was likely to be correlated to variables related to the structure of their habitat such as the range of moisture, water holding capacity, or temperature fluctuations to which testate amoebae are likely to be sensitive (Lousier 1974a, b; Charman and Warner 1992; Tolonen *et al*. 1994). The absence of such a relationship in our data set does not necessarily contradict these findings but rather suggests that the range of variability of moss cushion thickness (and of other variables related to this variable) present in our samples was not enough to influence significantly the testate amoebae (Mitchell *et al*. 2000a, b).

Our preliminary results suggest testate amoebae have a potential as biomonitors of atmospheric pollution. These results are especially interesting because the pollution level recorded was very low. This tool could prove very useful in two cases: (1) where the temporal resolution of measurements is not a high priority and a more integrative measurement is preferable, (2) where financial resources are not available to undertake continuous direct measurements of air pollution. This second case is certainly common in developing countries, where urban pollution problems are often the most acute.

Further work is needed to assess the full value of testate amoebae as bioindicators of atmospheric pollution before they can be used as a monitoring tool. The correlation between testate amoebae diversity and community structure and [NO_2] does not allow us yet to establish a causal relationship. It is possible that some other variables, such as another atmospheric pollutant, or other environmental variables, were responsible for the

observed differences. Future work should focus on (1) a calibration of the response of testate amoebae along a broader pollution gradient, and under controlled conditions, 2) the effects of other pollutants, such as ozone or heavy metals, and interactions among pollutants and (3) the effects of the urban microclimate on mosses and testate amoebae.

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ACTA
PROTOZOOLOGICA

Ultrastructure and Development of *Ceratomyxa protopsettae* Fujita, 1923 (Myxosporea) in the Gallbladder of Cultured Olive Flounder, *Paralichthys olivaceus*

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Summary. Spore morphology and sporogenesis process of *Ceratomyxa protopsettae* Fujita, 1923 found in the gallbladder of cultured olive flounder, *Paralichthys olivaceus* from southern Korea were studied by light- and transmission electron microscopy. Crescent-shaped mature spores were 11.64 ± 0.95 μm in length, 46.63 ± 5.8 μm in width (in sutural view). Two equal sized polar capsules were spherical, 4.15 ± 0.34 μm in diameter and each contained a polar filament with five to six turns and an opening at the anterior end. A binucleate sporoplasm was distributed unequally in the spore cavity. There were numerous pinocytotic invaginations, and pseudopodia or rhizoid-like projections at the peripheral portion of trophozoites. Plasmodium was proliferated by endo- and exogenous budding. Asynchronous divisions of generative cells without pansporoblast formation give arise to two or more than spores within the trophozoite. Capsulogenic cells in the sporoblast had large amounts of rough endoplasmic reticulum, external tubules, and capsular primordia. Histologically, vacuolization and hyperplasia of the epithelium were seen in sections of the gallbladder of parasitized fishes.

Key words: *Ceratomyxa protopsettae*, Myxosporea, sporogenesis.

INTRODUCTION

The myxosporean parasite *Ceratomyxa protopsettae* was described from the gallbladder of 10 wild flatfish species including olive flounder, *Paralichthys olivaceus*, in Japan (Fujita 1923). However, the original description of *C. protopsettae* lacks adequate data regarding the development, and only line drawings of the spore using light microscopy are available.

Olive flounder is a successfully cultured, commercially valuable species in Korea. Recently, we found a high prevalence of *C. protopsettae* in the gallbladder of cultured olive flounder in Korea. In the present study, we investigated the sporogenesis and histopathology of *C. protopsettae* in the gallbladder of olive flounder using light and transmission electron microscopy (TEM).

MATERIALS AND METHODS

One hundred juvenile olive flounder, *Paralichthys olivaceus* (10-15 cm in body length) were obtained from a commercial farm in southern Korea. From each fish, a drop of bile fluid from the gallblad-

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der was smeared on a slide, air-dried and stained with Diff-Quik (International Reagents Co., Japan). Mature spores were observed under a differential-interference-contrast (DIC) microscope and measured using an ocular micrometer and image analysis software (ImageTool ver 2.0, UTHSCSA, USA) according to Lom and Arthur's (1989) criteria. Mean and standard deviations of each spore dimension were obtained from 150 fresh mature spores. Developmental stages were drawn from Diff-Quik stained specimens using a camera lucida.

For histological study, semithin sections were obtained from small pieces of gallbladder fixed in 2% glutaraldehyde, embedded in Spurr resin and stained with toluidine blue.

For TEM study, a small portion of the gallbladder tissue was fixed in 2% v/v glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4°C overnight and postfixed in 1% w/v cacodylic OsO_4 for 2 h. The specimens were dehydrated, embedded in epoxy resin (Spurr) and ultrathin-sectioned, stained with uranyl acetate and lead citrate, and examined in a JEOL JEM1200 transmission electron microscope (JEOL LTD., Japan).

RESULTS

Spore. Mature spores (Figs 1, 12) were crescent-shaped with round or blunt ends, extremely elongated to the sutural line, and measuring 11.64 ± 0.95 μm in length, 46.63 ± 5.8 μm in width in sutural view. Two smooth valves were highly flexible and unequal in size, adhering together along the sutural line of the spore (Fig. 13). Two polar capsules were spherical and almost equal in size (4.15 ± 0.34 μm in diameter), apposed near the suture line, and each contained a polar filament with 5-6 coils, and an apical opening was present at the anterior end (Fig. 45). A binucleate sporoplasm filled the spore cavity and was generally distributed asymmetrically (Fig. 12). Occasionally, aberrant spores with 3 polar capsules and 3 valves were found (Fig. 14). Immature spores in disporic (Figs 2, 10, 24) or polysporic trophozoite (Fig. 11) were surrounded by remnants of the envelope.

Vegetative form. Trophozoites were freely floating in bile or lodged on the epithelium of the gallbladder. Trophozoites shape varied and included amoeboid or rounded forms (Figs 3-9). They had numerous pseudopodia at their peripheral portion, and a great variety of morphology in their pseudopodia was observed (Figs 5, 6). In attached trophozoites, short or long finger-like projections penetrated between the gallbladder epithelial cells (Figs 3, 4, 48). Enlarged trophozoites had finely granular endoplasm and transparent exoplasm (Figs 9, 18, 19, 23). Plasmodia were proliferated by endogenous or exogenous budding without destroying the integrity of mother plasmodia (Figs 7, 8, 28).
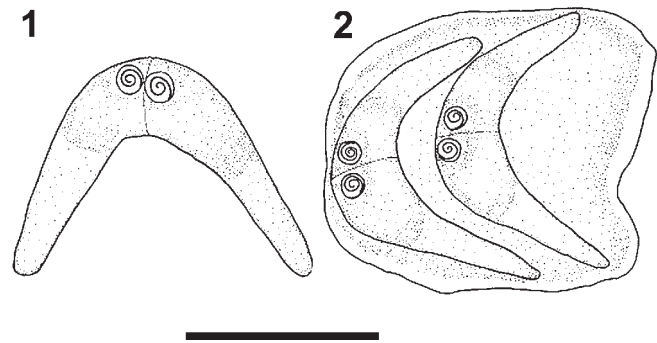
Figs 1, 2. *Ceratomyxa protopsettae* from frontal view. **1 -** mature spore; **2 -** disporic trophozoite. Scale bar 20 μm.

Presporogonic phase. Based on fresh (Figs 9-12), Diff-Quick stained (Figs 15-30) and TEM (Figs 31-46) observations, a hypothetical sporogenesis process was constructed (Fig. 47). The earliest stage found was amoeboid or spherical, with the primary cell containing a nucleus (Figs 31, 47a). The nucleus divided into a vegetative nucleus and a generative one (Figs 15, 34, 47b). The vegetative nucleus was situated freely in the cytoplasm of the primary cell, whereas the generative nucleus was surrounded by its own cytoplasm and appeared as an independent cell (secondary cell). In the ultrastructure, cytoplasmic extensions and long mitochondria were well developed at the peripheral portion of these cells (Figs 36, 38). In later developmental stages, the nucleus and cytoplasm of the secondary cell were enlarged, and the generative cell divided into 2 secondary cells (Figs 16, 17, 47c, d). After a generative cell divided once, the resulting 2 secondary cells developed directly into a di-sporogonic phase (Figs 19-24, 47g-l) or subsequently divided into further generative ones and developed into a poly-sporogonic phase (Figs 25-30, 47g'-j'). As a result of internal cleavage of the secondary cells, one or two tertiary daughter cells were produced in each secondary cell (Figs 39, 40). In TEM observations (Figs 34-36, 39, 40), the cytoplasm of inner generative cells (secondary or tertiary cell) was more electron dense than those in the mother cell (primary cell).

Sporogonic phase. Two or 3 secondary or generative cells were closely associated with each other (Figs 35, 37). These cells were surrounded by a common membrane, without surrounding pericyte or vacuole (Fig. 38). These cell aggregates give rise to sporoblast
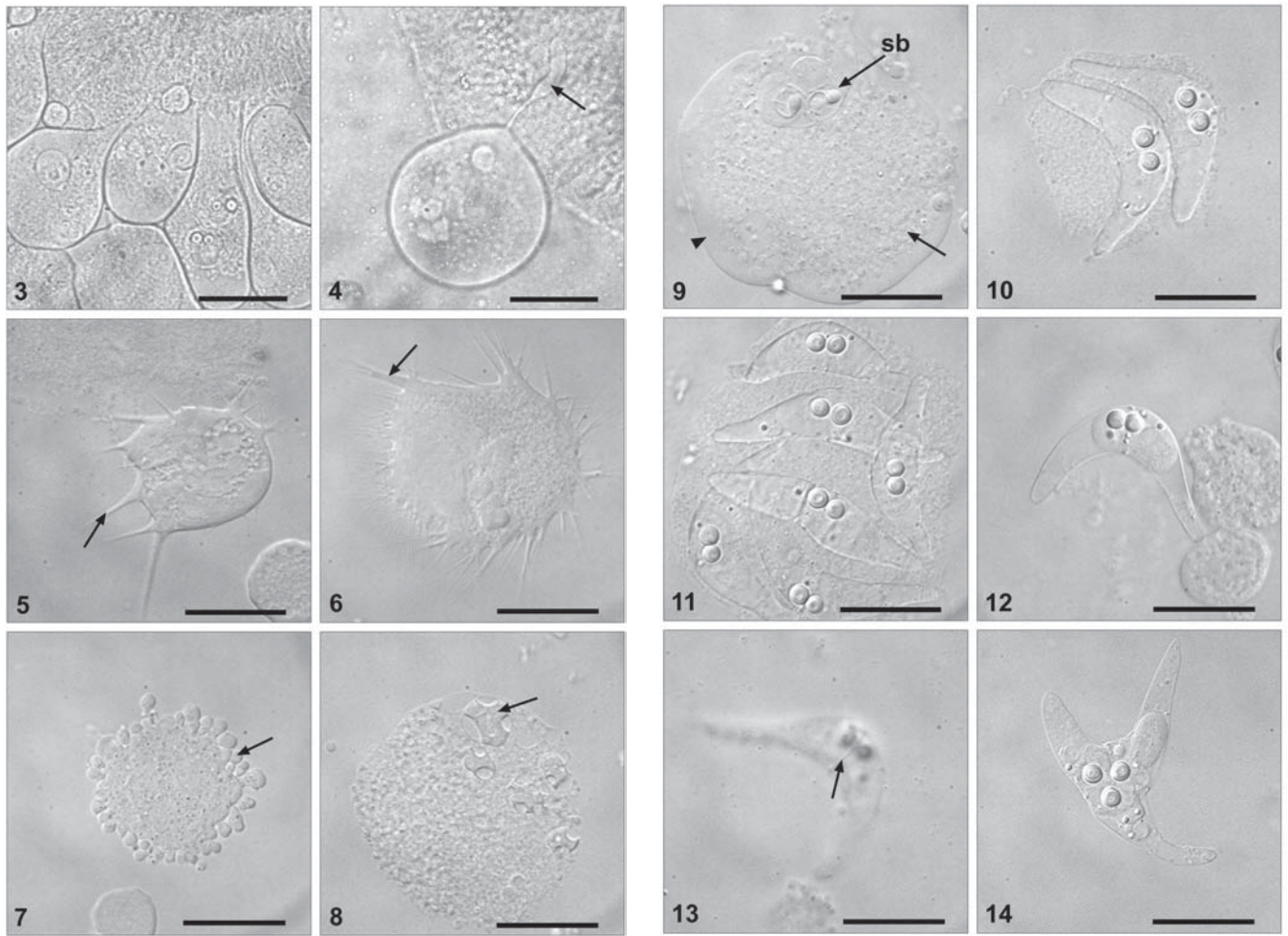
Figs 3-14. Light micrographs of fresh preparations of *Ceratomyxa protopsettae*; **3, 4 -** attached trophozoite on the wall of gall bladder, note pseudopodial projections (arrow); **5, 6 -** irregular form with numerous pseudopodia (arrow); **7 -** exogenous budding (arrow); **8 -** endogenous budding (arrow); **9 -** a sporoblast (sb) in trophozoite (arrow - finely granulated endoplasm, arrowhead - transparent ectoplasm); **10 -** disporic trophozoite; **11 -** polysporic trophozoite; **12, 13 -** mature spore (arrow - suture line); **14 -** abnormal spore with 3 valves and 3 polar capsules. Scale bar 20 µm.

for spore formation (Figs 9, 21-23, 29). Maturation of spores in a primary cell was asynchronous (Fig. 21). In the disporic phase, there are two sporoblasts, each consisting of two valvogenic cells, one binucleate sporoplasmic cell, and two capsulogenic cells (Figs 41-44, 47i-k). The polysporic phase followed the similar pattern to that described for disporic ones (Figs 25, 26, 29, 47g'-j'). The cytoplasm of capsulogenic cells contained a capsular primordium or several external tubules as well as numerous ribosomes and rough ER (Figs 41-43), and often had cytoplasmic invaginations closely associated with sporoplasmic extensions (Fig. 43). Three differential layers in electron density were present in the capsule (Fig. 44). Almost fully matured polar capsules were subspherical and contained 5 to 6 turns of a polar filament with a globular apical opening for filament discharge (Fig. 45). Two valvogenic cells completely enveloped both capsulogenic and sporoplasmic cells and became flattened as the spore matured (Figs 41-44).

Histology. Various developmental stages were found on the epithelium of the gallbladder. In heavily infected fish, various vegetative stages of the parasite covered the entire surface of the wall of the gallbladder, inducing hyperplasia and vacuolization of the base of epithelial cells (Figs 48, 49).

Host: Olive flounder, *Paralichthys olivaceus.*
Locality: Kampo, Kyongsangbuk-Do, South Korea.
Site of infection: Lumen and wall of the gallbladder.
Prevalence: 100% (100 fish infected/100 fish examined).
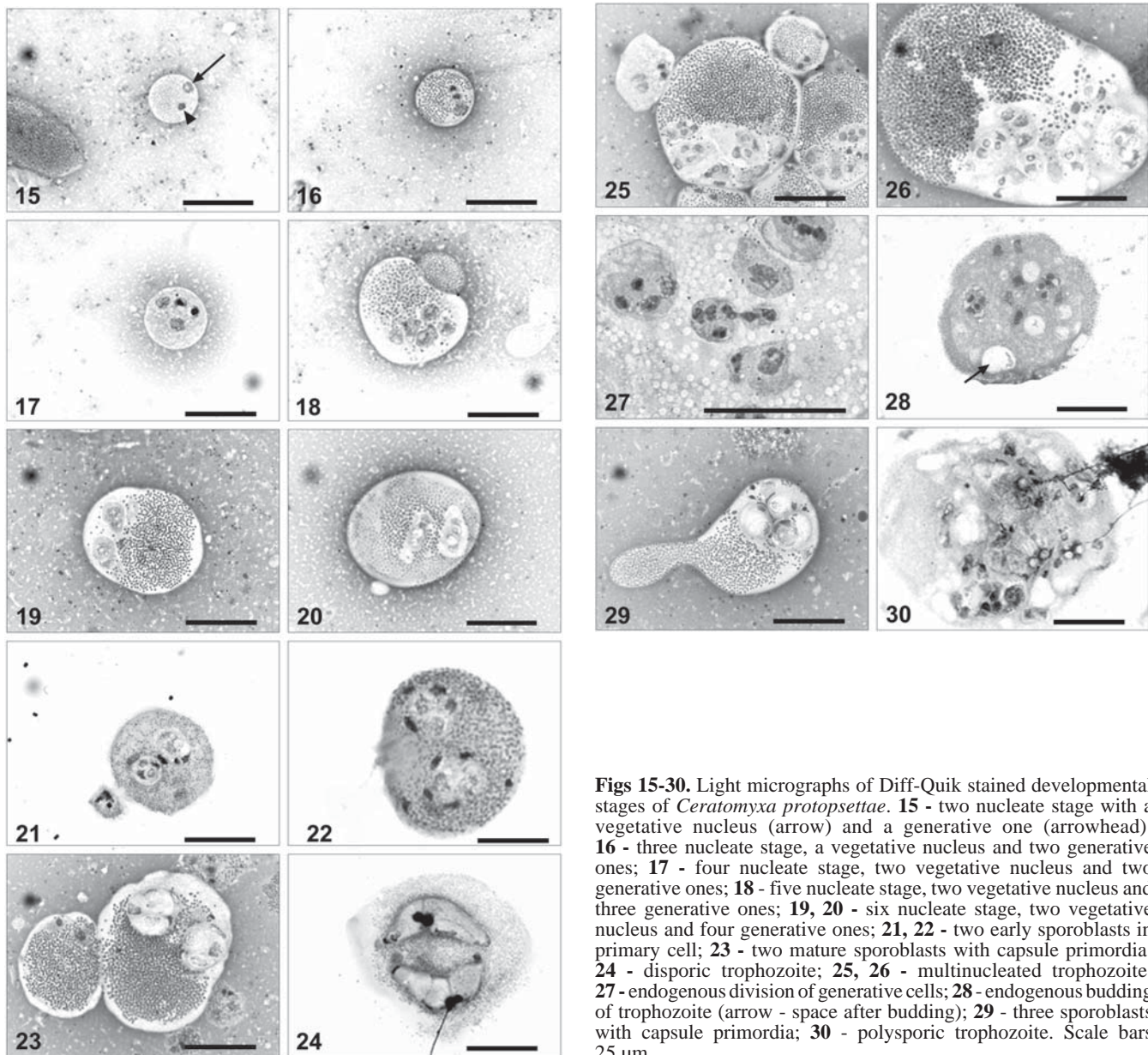
**Figs 15-30.** Light micrographs of Diff-Quik stained developmental stages of *Ceratomyxa protopsettae*. **15 -** two nucleate stage with a vegetative nucleus (arrow) and a generative one (arrowhead); **16 -** three nucleate stage, a vegetative nucleus and two generative ones; **17 -** four nucleate stage, two vegetative nucleus and two generative ones; **18** - five nucleate stage, two vegetative nucleus and three generative ones; **19, 20 -** six nucleate stage, two vegetative nucleus and four generative ones; **21, 22 -** two early sporoblasts in primary cell; **23 -** two mature sporoblasts with capsule primordia; **24 -** disporic trophozoite; **25, 26 -** multinucleated trophozoite; **27 -** endogenous division of generative cells; **28** - endogenous budding of trophozoite (arrow - space after budding); **29** - three sporoblasts with capsule primordia; **30** - polysporic trophozoite. Scale bars 25 µm.

Materials deposited: Diff-Quik stained slides; H&E stained histological sections; 90% alcohol-fixed spores. Laboratory of Fish and Shellfish Parasitology, Department of Aquatic Life Medicine, Pukyong National University, South Korea. Accession number PKNU-Pmy-200212.

DISCUSSION

Although the polar capsule size of the present species was somewhat smaller than that of *Ceratomyxa* *protopsettae* Fujita, 1923 (Table 1), we identified it as *C. protopsettae* on the basis of the same host species and geographical distribution (Lom and Dyková 1992). The general morphology of the *C. protopsettae* spore was typical of the genus *Ceratomyxa*, but aberrant spores with 3 valves and 3 polar capsules were observed occasionally. Sitjà-Bobadilla and Alvarez-Pellitero (1993c) also reported tri-capsular spores of *C. labracis* and *C. diplodae* from wild and cultured sea bass, *Dicentrarchus labrax*. The binucleated sporoplasm has also been reported in other *Ceratomyxa* species - e.g. *Ceratomyxa shasta* (see Yamamoto and Sanders 1979),
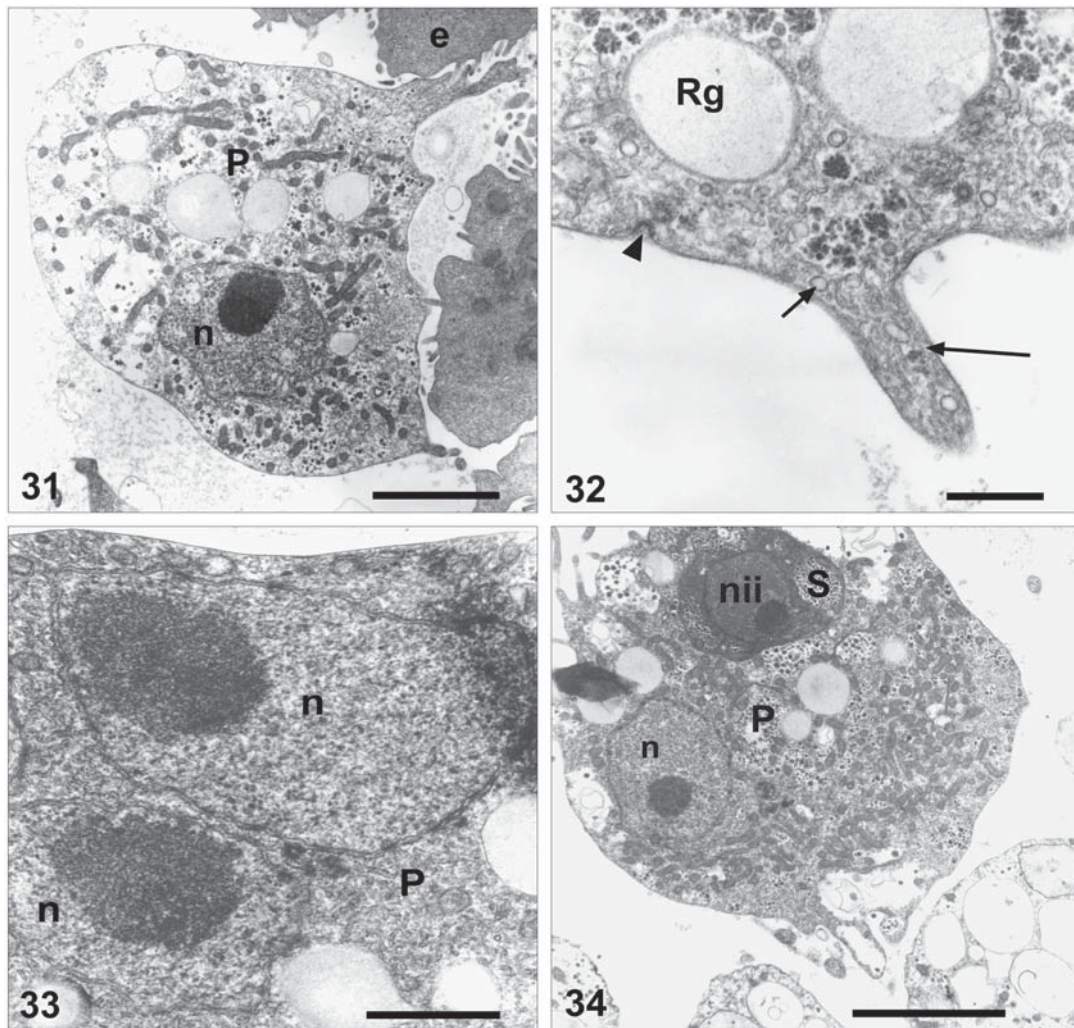
Figs 31-34. Electron-micrographs of early stages of *Ceratomyxa protopsettae*. **31 -** primary cell (P), n - nucleus of primary cell; **32 -** refractive granules (Rg) in endoplasm and, surface projection (long arrow), pinocytotic invagination (arrow head) or pinocytotic vesicle (short arrow) on the surface of trophozoite; **33 -** two vegetative nucleus of primary cell. Note prominent eccentric nucleolus; **34** - secondary cell (S) within primary cell, nii- nucleus of secondary cell. Scale bars 400 nm (32); 750 nm (33); 1 μm (31); 2 μm (34).

Table 1. Comparison of spore characteristics between original description of Fujita (1923) and the present specimens.

	Fujita, 1923	Present
Spore	unequal valve and variation in curvature of the shell	
Length (μm)	10 ~ 12	11.64 ± 0.95
Width (μm)	12 ~ 13	-
Thickness (μm)	50 ~ 65	46.63 ± 5.8
Polar capsule	two large and ovate	two subspherical
Length (μm)	6	4.15 ± 0.34
Breadth (μm)	4	-
Polar filament	-	5 ~ 6 coiled
Sporoplasm	asymmetrically distributed and binucleated	
Host	*Paralichthys olivaceus*	
Organ	gallbladder	
Geographical location	Hokkaido, Japan	East Sea, South Korea
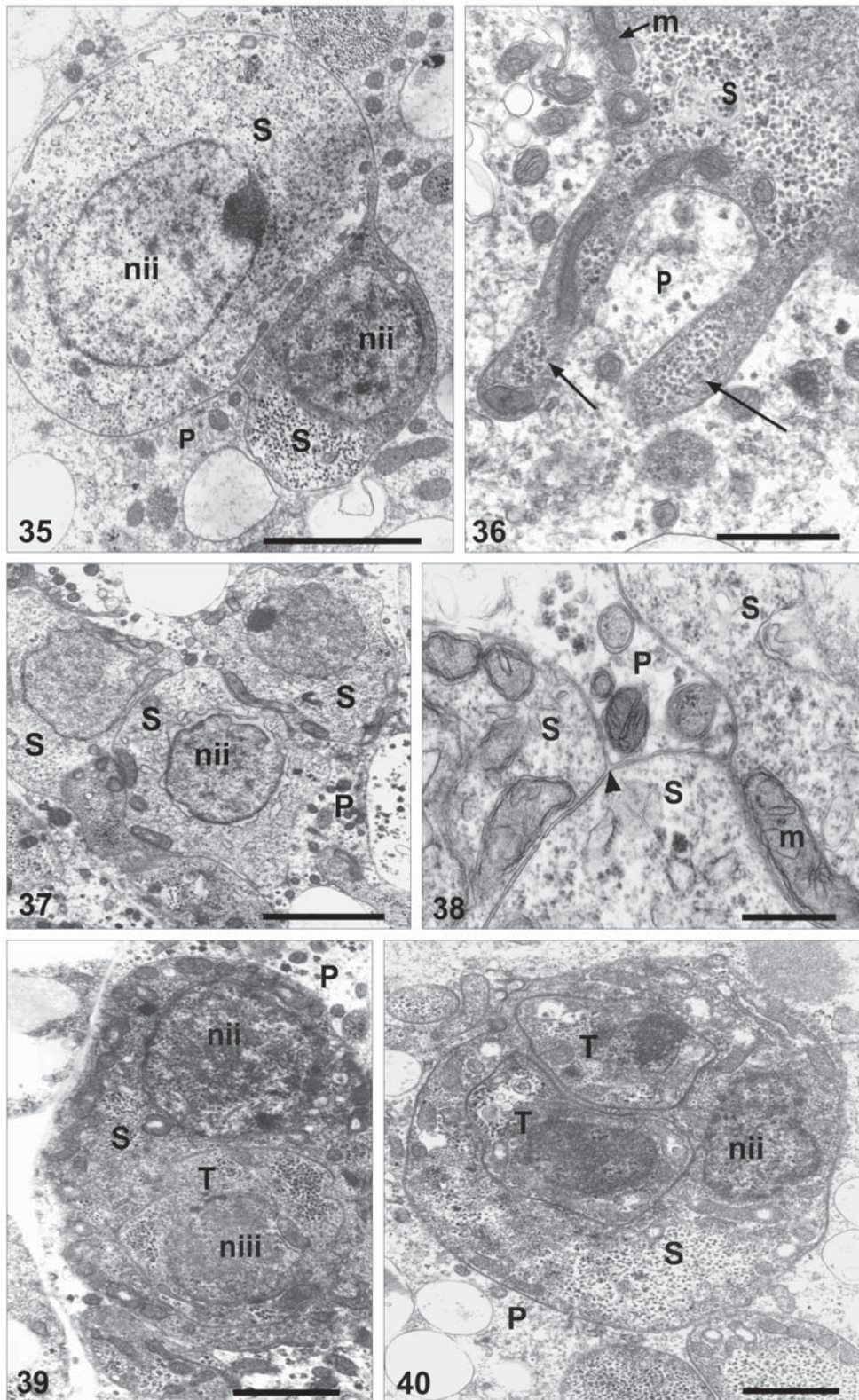
Figs 35-40. Electron-micrographs of presporogonic stages of *Ceratomyxa protopsettae*. **35 -** secondary cell (S) associated with other one, nii - nucleus of secondary cell, P- primary cell or plasmodium; **36** - cytoplasmic extensions (arrow) of secondary cell, m- mitochondria; **37** - association of three secondary cells; **38** - magnification of three secondary cell surrounded by common membrane (arrowhead). There were no surrounding vacuoles around generative cells; **39** - secondary cell containing a tertiary cell (T); **40 -** secondary cell containing 2 tertiary cells. Scale bars 400 nm (38); 500 nm (35); 800 nm (36); 1 μm (39, 40); 2 μm (37).
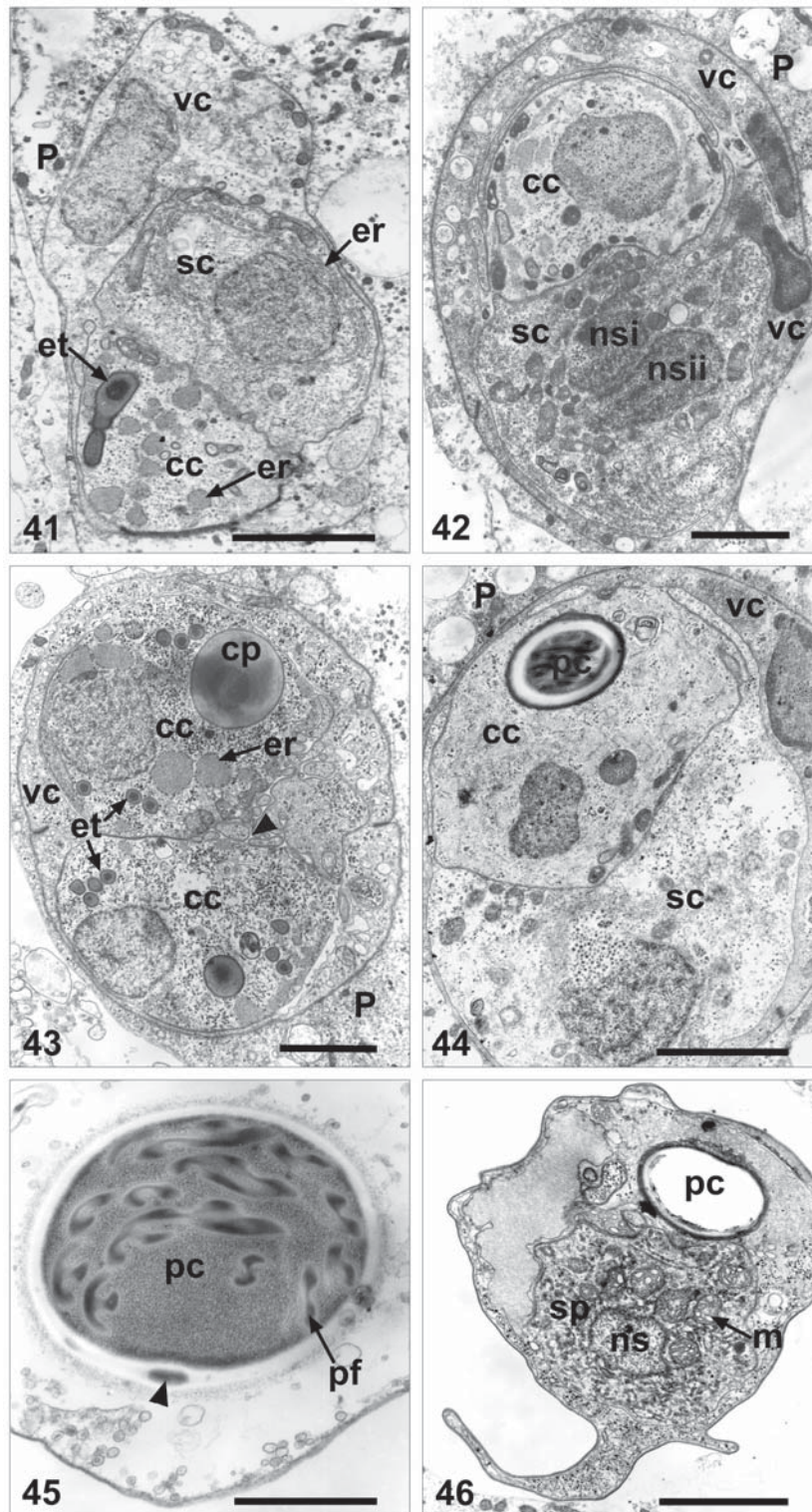
Figs 41-46. Electron-micrographs of sporogonic stages of *Ceratomyxa protopsettae*. **41 -** early sporoblast consists of capsulogenic cell (cc), sporoplasmic cell (sc) and valvogenic cell (vc), et - external tubule, er - endoplasmic reticulum, p - primary cell, sc - sporoplasmic cell, vc - valvogenic cell; **42 -** two valvogenic cell completely surrounding a binucleate sporoplasmic cell and capsulogenic cell. nsi, nsii - nucleus of sporoplasmic cell; **43 -** asynchronous maturation of capsulogenic cells, cp - capsular primordia; **44 -** formation of mature polar capsule (pc) within capsulogenic cell. **45** - apical pore (arrowhead) and polar filaments (pf) of polar capsule of mature spore; **46 -** sporoplasm (sp) of mature spore. Note numerous mitochondria (m) and nucleus (ns). Scale bars 800 nm (45); 1 μm (44); 2 μm, (41-43, 46).
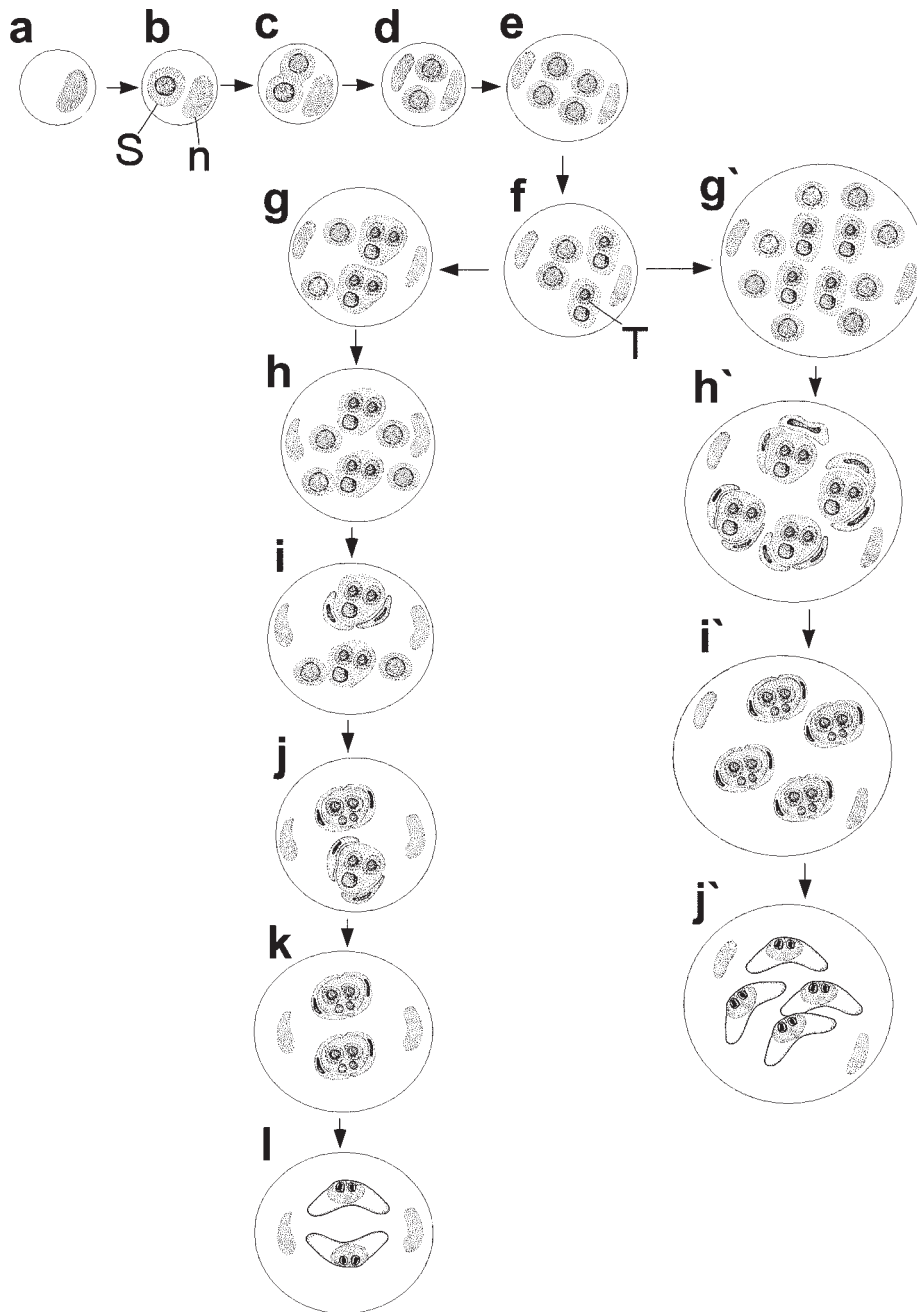
Figs 47a-f. Hypothetical sporogenesis of *Ceratomyxa protopsettae* from gallbladder of olive flounder, *Paralichthys olivaceus*. **a** - earliest stage with a nucleus; **b** - two nucleate stage, S - generative cell or secondary cell, n - vegetative nucleus or nucleus of the primary cell, **c** - three nucleate stage; **d** - four nucleate stage; **e** - six nucleate stage; **f** - eight nucleate stage. Secondary cell harboring inner tertiary cell (T). From subfigure **g** to **l**, each generative cell with inner 2 tertiary cells gives rise to a sporoblast in a primary cell and consequently forming disporic trophozoite (l). In other case (see subfigures **g'-j'**), multiple division of generative cells results in polysporic trophozoite.

C. globulifera (see Desportes and Théodoridès 1982), *C. diplodae* and *C. labracis* (see Sitjà-Bobadilla and Alvarez-Pellitero 1993c).

Free floating trophozoites with long or short needle-like pseudopodia showed sluggish amoeba-like movement, whereas attached trophozoites had rhizoid-like
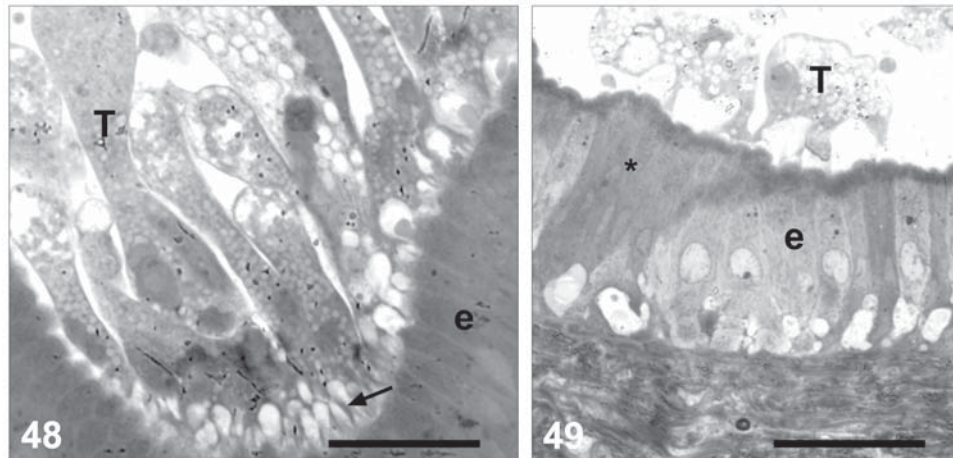
Figs 48-49. Histological sections of gallbladder of olive flounder, *Paralichthys olivaceus* infected by trophozoites of *Ceratomyxa protopsettae*. Semi-thin sectioned, toluidine blue stained. **48** - trophozoites with numerous rhizoid-like pseudopodia (arrow) firmly attached on the epithelial cell; **49** - heavily infected gallbladder wall with trophozoites showing hyperplasia (asterisk) and vacuolization of epithelial cells. T - trophozoite, e - epithelial cell. Scale bars 25 μm.

projections at one side to strengthen attachment to the gallbladder epithelium. These holdfast projections have also been reported in ultrastructural descriptions of other coelozoic species (Lom *et al*. 1986, Paperna *et al*. 1987, Alvarez-Pellitero and Sitjà-Bobadilla 1993a, Sitjà-Bobadilla and Alvarez-Pellitero 1993b, El-Matbouli and Hoffmann 1994). Multiplication of the trophozoite by endo- or exogenous budding have also been described in trophozoites of *Myxidium liberkuhni* by Cohn (1895), *Sinuolinea dimorpha* by Davis (1916), and *Ceratomyxa blennius* by Noble (1941).

Concerning sporogenesis, *C. protopsettae* showed either a disporic or polysporic pattern. Disporous development has been commonly reported from *Ceratomyxa* species (Fujita 1923, Kalavati and MacKenzie 1999, Yokoyama and Fukuda 2001), whereas disporous to polysporous development was reported also in *C. recurvata* by Davis (1917). Unequal division of generative cells and asynchronous spore formation in *C. protopsettae* were in agreement with those of other myxosporeans (Desser *et al*. 1983; Dyková *et al*. 1990; Sitjà-Bobadilla and Alvarez-Pellitero 1992, 1993a).

Myxosporeans that form polysporic trophozoites (i.e. *Myxobolus, Henneguya, Thelohanellus, Sphaeromyxa, Zschokkella, Myxidium*, and *Hoferellus*) produce spores in a pansporoblast. Pansporoblast formations have been frequently reported in light microscopical descriptions of developmental stages of other *Ceratomyxa* species (Averintsev 1908, 1909;

Mavor 1916; Georgévitch 1929; Noble 1941). In the present *C. protopsettae*, internal cleavages of a single generative cell rather than the association of two generative cells gave rise to the sporoblast, indicating no pansporoblast formation. In pansporoblast formation, the membrane of two generative cells persists and the sporogonic cell is enclosed in a tightly fitting vacuole in the pericyte (Lom and Dyková 1992). In the present ultrastructure of generative cells of *C. protopsettae*, reminiscent of a pericyte was not observed. Recently, Sitjà-Bobadilla *et al*. (1995) also reported no pansporoblast formation in *C. sparusaurati*. TEM observations of capsulogenic cells in early sporoblasts of *C. protopsettae* did not reveal the presence of a Golgi apparatus, but high amounts of smooth and rough ER were observed. Therefore, as in previous reports of capsulogenesis (Schubert 1968, Lom 1969), smooth or rough ER seems to be involved in the formation of capsular primordia of *C. protopsettae*.

The parasite induced response in the host was characterized by vacuolization and hyperplastic reaction of epithelial cells. These changes of the epithelial cells resembled that in other gallbladder myxosporean infections (Desportes and Théodoridès 1982; Alvarez-Pellitero and Sitjà-Bobadilla 1993b; Sitjà-Bobadilla and Alvarez-Pellitero 1993b, c).

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ACTA
PROTOZOOLOGICA

Heteroxenous Coccidia (Apicomplexa: Sarcocystidae) in the Populations of Their Final and Intermediate Hosts: European Buzzard and Small Mammals

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Summary. Factors influencing prevalences of heteroxenous coccidia in the populations of small mammals and buzzards (*Buteo buteo*) were studied in the Czech Republic. Seventy one percent of buzzard broods were positive for *Frenkelia*-like sporocysts. Prevalence increased with nestling age and number, and reached 100 % at nest desertion. The prevalences of brain sarcosporidia (*Frenkelia glareoli* and *F. microti*) in rodents were higher in ecotones than in open habitats, in spring than in autumn, in heavier individuals, and on 2nd and 3rd day of trapping. These factors were significant although the overall prevalence was different in different host species (*Clethrionomys glareolus*, *Microtus arvalis*, *Apodemus flavicollis*, *A. sylvaticus*). The prevalences of muscle sarcosporidia in rodents and *Sorex araneus* were also positively influenced by habitat and host weight, while only for *M. arvalis* was the prevalence higher in spring. Host sex, locality and year did not show any effects on the prevalences. Besides two *Frenkelia* species, five *Sarcocystis* species were found (*S. putorii*, *S. cernae*, *S.* cf *sebeki*, and two undescribed species from *C. glareolus* and *Sorex araneus*). Natural infections of *C. glareolus* with *F. microti* and of *A. flavicollis* with *F. glareoli* are reported for the first time. Our study demonstrates that prevalences of brain and muscle sarcosporidians in small mammals are influenced by similar factors (intermediate host habitat and age) in different host-parasite combinations.

Key words: *Apodemus, Buteo*, *Clethrionomys*, *Frenkelia*, life cycles, *Microtus*, *Sarcocystis*, *Sorex,* transmission, wildlife parasites.

INTRODUCTION

Heteroxenous coccidia (Apicomplexa: Sarcocystidae) are dixenous parasites circulating among their vertebrate intermediate hosts and carnivorous final hosts. Many species have been described from raptors and their prey. These parasites were shown to influence their mammalian intermediate hosts by increasing the probability of predation by the definitive host

(Hoogenboom and Dijkstra 1987, Voříšek *et al.* 1998). However, in natural host populations prevalences are generally not known, especially concurrently in both final and intermediate hosts of the parasite.

Frenkelia spp. are dixenous coccidian parasites of buzzards (*Buteo buteo*, *B. jamaicensis*, *B. borealis*) and their rodent prey. In the buzzard final host, parasite development is limited to the intestine, and infective sporocysts are excreted in faeces. In rodents, infective tissue cysts develop in the brain (Rommel and Krampitz 1975, Krampitz and Rommel 1977, Tadros and Laarman 1982, Upton and McKown 1992).

Two *Frenkelia* species are recognized, which differ in the morphology of brain cysts, and intermediate host

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spectrum. The rounded cysts of *F. glareoli* occur in bank voles (*Clethrionomys glareolus*), grey-sided voles (*C. rufocanus*), ruddy voles (*C. rutilus*) and southern water voles (*Arvicola sapidus*) (Erhardová 1955, Zasukhin *et al.* 1958, Doby *et al.* 1965). The lobulated cysts of *F. microti* can develop in a wide range of rodents. Natural infections have been found in field voles (*Microtus agrestis*), meadow voles (*M. pennsylvanicus*), common voles (*M. arvalis*), muskrats (*Ondatra zibethicus*), lemmings (*Lemmus lemmus*), and porcupines (*Erethizon dorsatum*) (Findlay and Middleton 1934, Frenkel 1953, Jírovec *et al.* 1961, Enemar 1962, Karstad 1963, Tadros and Laarman 1982, Kennedy and Frelier 1986). The experimental host spectrum is even wider, and includes the wood mouse (*Apodemus sylvaticus*), yellow-necked mouse (*A. flavicollis*), striped field mouse (*A. agrarius*), golden and common hamster (*Mesocricetus auratus*, *Cricetus cricetus*), rat (*Rattus norvegicus*), house mouse (*Mus musculus*), multimammate rat (*Mastomys natalensis*), chinchilla (*Chinchilla laniger*), and rabbit (*Oryctolagus cuniculus*) (Krampitz and Rommel 1977, Rommel and Krampitz 1978).

The validity of the genus *Frenkelia* was recently questioned by molecular studies, which place it in the genus *Sarcocystis*. To avoid this paraphyly, synonymization of *Frenkelia* with *Sarcocystis* was proposed (Votýpka *et al.* 1998, Modrý *et al.* 2004). In this study, we use *Frenkelia* as a subgeneric name, to distinguish it from muscle sarcosporidians, the latter with final hosts different from buzzards. Besides brain-invading sarcosporidia, rodents can serve as hosts for several species of muscle-cyst forming species, both with bird and mammalian final hosts (Odening 1998).

The aim of this study was to compare the prevalences of *Frenkelia glareoli* and *F. microti* in wild rodents. The influence of intrinsic (host age, sex) and extrinsic (season, year, habitat) factors on parasite prevalence was studied in several localities in the Czech Republic. To determine if the same factors are important for the occurence of brain and muscle sarcosporidia, prevalences of *Sarcocystis* in muscles were also studied. *Frenkelia* prevalence in the common buzzard, the final host, was studied simultaneously.

MATERIALS AND METHODS

Study area. The study was carried out in three localities in the Czech Republic: Locality 1 (50° 23' N, 016° 02' E) is located in the surroundings of the town Česká Skalice (Eastern Bohemia), characteristic by farmland with arable land, meadows and smaller woodland patches and riparian forests (woodland proportion between 10 to 20%) as the main habitat types (Diviš 1990). Habitats in locality 2 (50° 00' N, 016° 13' E), near the town Choceň (Eastern Bohemia), are similar to those in locality 1 with farmland covering nearly 60% and woodland 20% of the area (Voříšek 1995). Mixed forests are typical for woodlands in both localities. Locality 3 (48° 48' N, 016° 38' E) is located in Biosphere Reserve Pálava near the town Mikulov (South-eastern Moravia). The study was carried out in a 22 km^2 oak-hornbeam forest complex (Voříšek 2000).

Population density of buzzards has differed between the study sites: the density was similar in Česká Skalice and Choceň (20-40 breeding pairs per 100 km^2, Diviš 1990, Voříšek 1995) while the density in Mikulov has reached one of the highest values ever known (30-50 breeding pairs per 22 km^2, Voříšek 2000).

Prevalence of *Frenkelia*-like sporocysts in buzzards. The data for final hosts (buzzards) were collected in 1989-1993 in Česká Skalice, 1989-1992 in Choceň, and 1993-1995 in Mikulov. Faecal samples were collected during buzzard nest inspections (late April to early July). Mixed samples from individual nests were stored in 2% $K_2Cr_2O_7$ at 4°C. Before microscopic examination, samples were centrifuged for 10 min at 200 g, the sediment was mixed with 33% $ZnSO_4$, recentrifuged, and flotated for 20 min.

In Choceň and Česká Skalice, one sample per nest was collected. In Mikulov, nests were inspected repeatedly, and faecal samples were collected at each inspection. Age of buzzard's nestlings was estimated using wing length (Voříšek and Lacina 1998), the age of the oldest nestling in a nest was used for further analysis.

Prevalence of sarcosporidia in small mammals. The data for intermediate hosts (rodents and insectivores) were collected in 1990-1994 in Česká Skalice, 1990-1992 in Choceň, and 1993-1995 in Mikulov.

Small mammals were snap-trapped. Snap-trap lines, each containing 50 traps at 3 m intervals, were set in potential buzzards' hunting habitats (open habitats: meadows, alfalfa fields, clear-cuts; ecotones: riparian forests and wood edges) in spring (late March, early April) and autumn (late September, early October). Six trap lines were exposed during three consecutive nights at each trapping session. The lines were checked every morning. Mammals were identified, weighed using Pesola spring scale, and sexed. Correct species identification was difficult in young individuals of wood and yellow-necked mice, thus these individuals were categorized as *Apodemus* sp.

The infection status of snap-trapped small mammals was determined microscopically. Whole brains were checked for the presence of *Frenkelia* cysts in fresh squashed preparations. For the detection of *Sarcocystis*, femoral muscles were homogenized using a tissue grinder, centrifuged (10 min at 200 g), and the sediment was used for smear preparation. Smears were air-dried, fixed with methanol, stained with Giemsa, and checked for 10 min under immersion objective. Cystozoites from positive samples were measured for species determination.

Data analysis. Raw prevalence (in %) was calculated as a proportion of positive samples (broods or individuals). Effects of multiple predictors on infection status (coded 1 = positive, 0 = negative) were analysed by fitting generalized linear mixed models (GLMM) with logit link and binomial error distribution (SAS-based macro Glimmix, Littel *et al.* 1996). Significance of fixed effects was assessed

by the Type III F-test with denominator df estimated using the Satterthwaite method. Restricted maximum-likelihood estimates of model parameters are presented.

Effects of brood age and brood size on the prevalence of *Frenkelia*-like sporocysts in common buzzard nestlings were evaluated by fitting separate models to two partly overlapping data sets: (A) data from all three study localities using only one sample per brood (the last sample in Mikulov data; n = 229 broods/samples); (B) data from only the locality Mikulov using multiple samples per brood (n = 88 broods/271 samples). Random effects included in the models were: locality and year (model A); year, brood and brood × age, the last two nested within year (model B). This means, that both intercept and slope for age effect were allowed to vary among broods in model B.

Prevalence of *Frenkelia* and *Sarcocystis* in small mammals was analysed using two hierarchical subsets of the total data, for which a complete set of predictor variables was available: (i) host species, habitat type, season, trapping day, body weight; (ii) all the above variables with addition of host sex. Only those species with >1 positive individuals for the corresponding response variable were included in the models; all unidentified individuals of field mice were excluded. Body weight was centered within species to obtain a relative value, presumably related to age. Random effects of locality, year and trapping sample (nested within locality × year) were included. Apart from the main effects, all two-way interactions of species with the other effects were also examined.

The smaller data set, including host sex as a predictor variable, was heavily unbalanced (low number of individuals and/or zero prevalence for some combinations of predictors). Hence, the exact logistic regression (LogXact 5; Cytel Software Corporation, 2002) was used to fit simplified models. These included host species, sex, habitat type and season as the categorical predictors, and locality-year combination as the stratification variable. Two-way interactions of host sex with the other effects were examined.

RESULTS

Prevalence of *Frenkelia*-like sporocysts in buzzard nestlings

Overall prevalence of *Frenkelia*-like sporocysts in common buzzard nestlings was 71% (n = 229 broods) and varied from 43 to 89% among locality-year samples (Table 1). Nevertheless, the GLMM model A did not reveal significant random component of variation among localities (approximate z-test, P = 0.32) or years (P = 0.17), and a similar result was obtained with locality and year entered as fixed effects (locality: $F_{2,217} = 1.66$, P = 0.192; year: $F_{6,217} = 1.52$, P = 0.172). Hence, the spatio-temporal variation in prevalence could be accounted for by differences in mean age and size of the sampled broods (Table 1). The two GLMM models, based on different type of data, provided similar results for the direction of fixed effects - prevalence increased with both age and size of broods (number of nestlings),

but the increase with age was steeper in larger broods (significant interaction term; Table 2, Fig. 1). The parameter estimates from the model B (multiple samples per brood) should be considered more realistic, because they were derived from data representing wider range of brood ages (Table 1). In accordance with model A, the random component of variation among years was not significant (approximate z-test, P = 0.19) also in model B, but there was significant (P < 0.001) random variation among individual broods in mean prevalence (intercept) and effect of age (slope).

Prevalence of *F. glareoli* and *F. microti* in rodents

The number of examined individuals and the raw (not adjusted for multiple effects) prevalence is shown in Table 3.

The GLMM model fitted to the larger data set (n = 1316; Table 4, Fig. 2) showed that the prevalence of *Frenkelia* was significantly higher in mammals caught in ecotone than in open habitat, in spring than in autumn, on the 2nd and the 3rd trapping day than on the 1st one (1st *vs.* 2nd: $t_{1302} = -3.6$, P < 0.001; 1st *vs.* 3rd: $t_{1303} = -2.5$, P = 0.013; 2nd *vs.* 3rd: $t_{1296} = 0.6$, P = 0.520), in relatively heavier individuals and in those positive for *Sarcocystis*.

Although the four mammalian host species differed in overall prevalence (P < 0.001 for all pair-wise comparisons, except for yellow-necked mouse *vs.* wood mouse: $t_{1306} = -1.7$, P = 0.097), effects of the above factors were consistent across all host species (nonsignificant interactions with species effect; all P > 0.1). The model suggests significant random component of variation among trapping samples (approximate z-test, P = 0.010), but not among localities or years (both P > 0.9).

The exact logistic regression applied to subset of the larger data set, for which host sex was available as an additional predictor (n = 578), corroborated the direction and significance of the effects of habitat (P = 0.013) and season (P < 0.001), but did not reveal an effect of host sex (slope for males ± SE, logit scale: 0.303 ± 0.361, P = 0.505) or interactions between sex and the other effects.

Prevalence of *Sarcocystis* spp. in rodents and insectivores

Number of examined individuals and the raw prevalence is shown in Table 3.

The GLMM model fitted to the larger data set (n = 888; Table 5, Fig. 3) showed that prevalence of *Sarcocystis* was significantly higher in mammals caught

Table 1. Raw prevalence (% of samples) of *Frenkelia*-like sporocysts in common buzzard nestlings at three localities in different years. Broods in Mikulov were sampled repeatedly throughout the nestling period, hence the multiple samples per brood. The mean age and number of nestlings in the sampled broods is shown.

Locality	Year	Broods (samples)	Prevalence (%)	Brood age (days)		Brood size	
				Mean ± SD	Range	Mean ± SD	Max [c]
Skalice	1989	15	60.0	22.8 ± 4.8	14 - 30	2.4 ± 1.0	4
Skalice	1990	49	65.3	22.0 ± 5.7	7 - 30	2.1 ± 0.9	4
Skalice	1991	16	87.5	23.8 ± 3.4	16 - 30	2.1 ± 0.8	4
Skalice	1992	15	80.0	24.3 ± 3.8	16 - 30	2.1 ± 0.9	4
Skalice	1993	10	60.0	20.5 ± 4.2	14 - 28	2.3 ± 1.3	4
Choceň	1989	8	75.0	17.6 ± 4.0	12 - 25	2.0 ± 0.5	3
Choceň	1990	7	42.9	18.9 ± 4.8	10 - 25	2.4 ± 0.8	3
Choceň	1991	6	66.7	18.4 ± 5.1	8 - 21	1.5 ± 0.5	2
Choceň	1992	15	73.3	15.9 ± 7.3	6 - 30	2.1 ± 0.9	4
Mikulov [a]	1993	34	55.9	25.0 ± 5.7	3 - 36	1.8 ± 0.7	4
Mikulov [a]	1994	35	88.6	29.3 ± 6.4	2 - 36	1.9 ± 0.8	4
Mikulov [a]	1995	19	78.9	24.9 ± 7.4	3 - 33	1.4 ± 0.5	2
Mikulov [b]	1993	120	28.3	16.2 ± 4.5	1 - 36	1.9 ± 0.3	4
Mikulov [b]	1994	100	54.0	18.4 ± 6.4	2 - 36	2.2 ± 0.5	4
Mikulov [b]	1995	51	62.7	18.7 ± 5.0	2 - 33	1.7 ± 0.4	3

[a] One (the last) sample per brood. [b] Multiple samples per brood. [c] Minimum = 1, in all cases.

Table 2. Fixed effect part of the GLMM models (binomial error, logit link) relating prevalence of *Frenkelia*-like sporocysts in common buzzard nestlings (positive = 1, negative = 0) to brood age (days) and brood size (number of nestlings). Model A was fitted to data from all three localities, using one sample per brood (n = 229); locality and year were included as random effects. Model B was fitted only to data from Mikulov, using multiple samples per brood (n = 271 samples and 88 broods); year, individual brood and brood × age were included as random effects.

Fixed effect	Estimate ± SE	Type III test		
		DDF [a]	F	P
Model A				
Intercept	0.370 ± 2.037			
Age	0.007 ± 0.087	218	< 0.1	0.939
Brood size	-2.000 ± 0.999	221	4.0	0.047
Age × brood size	0.105 ± 0.046	221	5.3	0.022
Model B				
Intercept	-5.473 ± 0.981			
Age	0.212 ± 0.039	242	29.4	0.001
Brood size	-0.413 ± 0.350	221	1.4	0.240
Age × brood size	0.070 ± 0.019	240	13.6	0.001

[a] Denominator df estimated by the Satterthwaite method; numerator df = 1 in all cases.
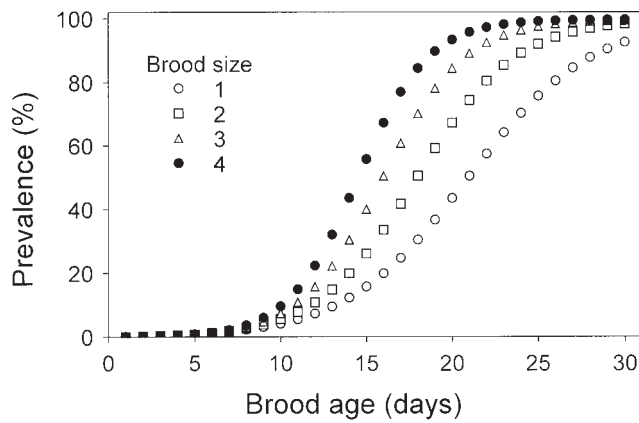
Fig. 1. Prevalence of *Frenkelia* sp. in common buzzard nestlings in Mikulov, predicted by the GLMM model (Table 2: model B) for broods of different age and size (number of nestlings).
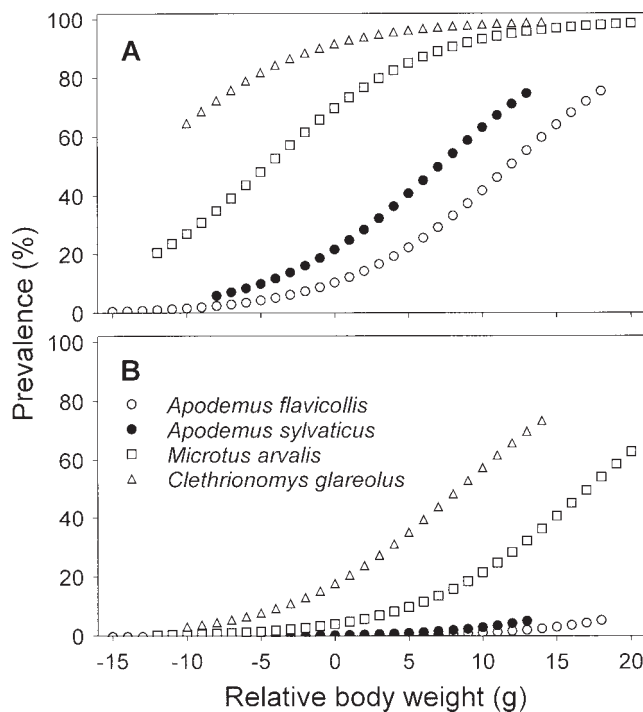

Fig. 2. Prevalence of *Frenkelia microti* (*Apodemus flavicollis*, *A. sylvaticus*, *Microtus arvalis*) or *F. glareoli* (*Clethrionomys glareolus*), predicted by the GLMM model (Table 4) for either the edge habitat in spring (**A**), or the open habitat in autumn (**B**). Values of the other predictors were held constant (the 2nd trapping day, positive for *Sarcocystis*). Body weight was centered within species.

Fig. 3. Prevalence of *Sarcocystis* spp. predicted by the GLMM model (Table 5) for either the edge habitat in spring (**A**), or the open habitat in autumn (**B**). Values of the other predictors were held constant (the 2nd trapping day, positive for *Frenkelia*). Body weight was centered within species.

in ecotone than in open habitat, in relatively heavier individuals and in those positive for *Frenkelia*; which effects were consistent across all host species (nonsig-
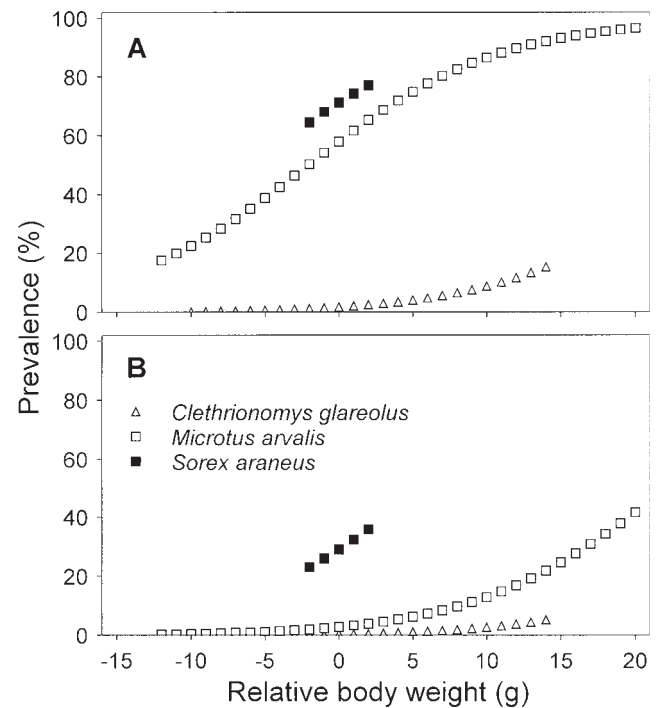
nificant interactions with species effect; all $P > 0.1$). The effect of season differed among host species (significant interaction, Table 5): prevalence was significantly higher in spring than in autumn only in common vole (2.59 ± 0.60, $t_{29} = 4.3$, $P < 0.001$) but not in common shrew (0.63 ± 0.68, $t_{17} = 0.9$, $P = 0.374$) and bank vole (-0.03 ± 0.73, $t_{38} = -0.05$, $P > 0.9$). The overall prevalence differed among the three mammal host species ($P < 0.001$ for all pair-wise comparisons); no significant effect of trapping day was found. The model did not reveal significant random component of variation among localities (approximate z-test, $P = 0.32$), years ($P = 0.24$) or trapping samples ($P = 0.17$).

The exact logistic regression applied to a subset of the larger data set, for which host sex was available as an additional predictor ($n = 365$), failed to reveal significant effects of habitat ($P = 0.9$), season ($P = 0.09$), host sex (slope for males: 0.274 ± 0.714, $P > 0.9$) or interactions between sex and the other effects.

Parasite and host species

The species identification revealed that the main host of *F. glareoli* is the bank vole, and that of *F. microti* is

Table 3. Raw prevalence [% of individuals (n)] of *Frenkelia microti* (apofla, aposyl, aposp, micarv), *F. glareoli* (clegla) and Sarcocystis spp. in different mammalian host species. Prevalence is shown for the total sample and separately for the three localities, three trapping days, two habitat types, two parts of year and host sex.

Sample	*Frenkelia*					*Sarcocystis*		
	apofla	aposyl	aposp	micarv	clegla	sorara	micarv	clegla
Skalice	4.7 (107)	0.9 (115)	1.2 (82)	5.9 (256)	42.0 (293)[aa]	20.0 (50)	3.5 (257)	1.4 (293)
Choceň	0.0 (15)	5.3 (94)	--	5.9 (170)	35.4 (65)[a]	8.3 (36)	2.9 (70)	1.7 (58)
Mikulov	0.8 (128)	2.6 (77)	0.0 (36)	3.1 (194)	32.0 (128)	33.3 (9)	2.1 (194)	1.6 (128)
1st day	0.9 (109)	2.0 (149)	0.0 (58)	5.4 (223)	35.7 (224)[aa]	14.9 (47)	2.7 (186)	2.3 (221)
2nd day	3.6 (84)	3.3 (90)	2.9 (35)	5.8 (225)	40.5 (158)[a]	20.0 (25)	2.6 (192)	0.6 (155)
3rd day	3.5 (57)	4.3 (47)	0.0 (25)	3.5 (172)	41.4 (104)	17.4 (23)	3.5 (143)	1.0 (103)
Ecotone	3.1 (194)	3.7 (218)	1.9 (53)	12.9 (170)	41.8 (373)[aaa]	21.9 (73)	2.3 (173)	1.9 (365)
Open	0.0 (56)	0.0 (68)	0.0 (27)	2.5 (363)	14.3 (63)	0.0 (22)	3.8 (261)	0.0 (64)
Spring	7.3 (55)	8.7 (46)	6.7 (15)	13.0 (46)	63.0 (100)[a]	21.7 (23)	15.2 (46)	3.0 (99)
Autumn	1.0 (195)	1.7 (240)	0.0 (103)	4.4 (574)	32.1 (386)[aa]	15.3 (72)	1.7 (475)	1.1 (380)
Male	0.0 (66)	0.0 (43)	0.0 (12)	4.5 (112)	29.7 (74)	50.0 (4)	1.8 (114)	2.7 (74)
Female	2.6 (77)	0.0 (40)	3.8 (26)	3.1 (127)	32.8 (64)	16.7 (6)	1.6 (129)	1.5 (65)
Total	2.4 (250)	2.8 (286)	0.8 (118)	5.0 (620)	38.5 (486)[aaa]	16.8 (95)	2.9 (521)	1.5 (479)

[a] Cases of *F. microti* ([a] one case, [aa] two cases, [aaa] three cases). Host species: apofla = *Apodemus flavicollis*, aposyl = *A. sylvaticus*, aposp = *Apodemus* sp., micarv = *Microtus arvalis*, clegla = *Clethrionomys glareolus*, sorara = *Sorex araneus*.

the common vole. However, three individuals of bank vole were infected with *F. microti*, and in one case, *F. glareoli* cysts were found in the brain of yellow-necked mouse.

Several species of *Sarcocystis* occured in small mammals, which differed in the length and morphology of their cystozoites. In common voles, *S. cernae* and *S. putorii* was found. Species from bank vole and common shrew are probably undescribed species. One yellow-necked mouse had sarcocysts in muscles, which probably belonged to *S. sebeki*.

DISCUSSION

This study describes for the first time prevalences of heteroxenous coccidians in populations of both their final and intermediate hosts.

The prevalence of *Frenkelia*-like sporocysts in buzzard faecal samples increased with the age of nestling, and reached 100% at the time of nest desertion. During

the first week of life, chicks did not shed sporocysts due to the prepatent period of 7-9 days in both *Frenkelia* species (Rommel and Krampitz 1975, Krampitz and Rommel 1977). First positive samples are therefore found at the 2nd week of nestling life. The increase is not linear, because the daily amount of food consumed by chicks increases with age.

The prevalence increases with the size of brood, and the increase is steeper in bigger broods. The absolute amount of consumed prey is higher in bigger broods at a certain age, and the probability of finding sporocysts in a mixed faecal sample is higher for a bigger brood at a certain time point.

Although differences between study localities (habitats, buzzard's breeding density) are relatively large, no significant effect of locality or year on prevalence was found. The results show that the age of nestlings and the number of nestlings in the nest explain variation in prevalence in buzzards.

Species identification of *Sarcocystis* based on sporocyst morphology is not possible. The numbers of sporo-

Table 4. Fixed effect part of the GLMM model (binomial error, logit link) relating prevalence (positive = 1, negative = 0) of *Frenkelia* to effect of host species (*Apodemus flavicollis*, *A. sylvaticus*, *Clethrionomys glareolus*, *Microtus arvalis*), habitat type (ecotone *vs.* open), season (spring *vs.* autumn), relative body weight (centered within species), trapping day (three days) and infection by *Sarcocystis* (positive *vs.* negative). Model predictions shown in Fig. 2.

Fixed effect (modelled level)	Estimate ± SE	Type III test			
		NDF	DDF[a]	F	P
Intercept	-3.097 ± 0.653				
Species (apofla)	-2.980 ± 0.476	3	1266	59.9	0.001[b]
Species (aposyl)	-2.113 ± 0.420				
Species (clegla)	1.586 ± 0.234				
Habitat (ecotone)	2.251 ± 0.489	1	33	21.2	0.001
Season (spring)	1.553 ± 0.436	1	22	12.7	0.002
Body weight	0.182 ± 0.021	1	1262	73.5	0.001
Day (1st)	-0.530 ± 0.213	2	1300	6.9	0.001[c]
Day (2nd)	0.137 ± 0.213				
Sarcocystis (+)	1.509 ± 0.516	1	1302	8.6	0.004

[a] Denominator df estimated by the Satterthwaite method. Total n = 1316. [b] Test of species effect. [c] Test of trapping day effect.

Table 5. Fixed effect part of the GLMM model (binomial error, logit link) relating prevalence (positive = 1, negative = 0) of *Sarcocystis* to effect of host species (*Clethrionomys glareolus*, *Microtus arvalis*, *Sorex araneus*), habitat type (ecotone *vs.* open), season (spring *vs.* autumn), relative body weight (centered within species), trapping day (three days) and infection by *Frenkelia* (positive *vs.* negative). Model predictions shown in Fig. 3.

Fixed effect (modelled level)	Estimate ± SE	Type III test			
		NDF	DDF [a]	F	P
Intercept	-0.316 ± 0.893				
Species (clegla)	-4.112 ± 0.616	2	356	26.8	0.001[b]
Species (micarv)	-2.552 ± 0.460				
Habitat (ecotone)	1.174 ± 0.517	1	17	5.2	0.037
Season (spring)	0.627 ± 0.686	1	8	5.6	0.045
Body weight	0.155 ± 0.037	1	598	18.0	0.001
Day (1st)	-0.420 ± 0.362	2	854	1.1	0.321[c]
Day (2nd)	-0.565 ± 0.390				
Frenkelia (+)	1.864 ± 0.467	1	678	15.9	0.001
Species × season (clegla)	-0.660 ± 0.889	2	152	5.2	0.006[d]
Species × season (micarv)	1.966 ± 0.851				

[a] Denominator df estimated by the Satterthwaite method. Total n = 888. [b] Test of species effect. [c] Test of trapping day effect. [d] Test of species × season interaction.

cysts in the samples were usually low, probably due to the small amounts which are shed at the beginning of the patent period. Sporocysts found in the faeces of buzzard might belong to other *Sarcocystis* species infecting raptors. However, to our knowledge, only *S. citellibuteonis* is known to infect buzzards (Pak *et al.* 1989), and its intermediate host, the yellow suslik (*Spermophilus fulvus*), is absent in the study area.

Muscle sarcocysts were found in the examined rodents, but in considerably lower prevalences than brain cysts; moreover, only a part of those were species with bird final host [e. g., *S. cernae* of kestrel (*Falco tinnunculus*) in common vole]. Of the *Sarcocystis* species with avian final host, only *S. dispersa* from owls was described from two host genera (Černá *et al.* 1978). *Sarcocystis* species with small mammals as intermediate hosts are

generally more specific for their final host than those with large mammals as hosts (Cawthorn and Speer 1990). Therefore, we suppose that sporocysts in buzzards belonged to the subgenus *Frenkelia*.

The prevalence of *F. glareoli* in wild populations reported in other studies ranges from 2% to 30-50%, and may be as high as 85% (Jírovec *et al.* 1961, Skofitsch 1980, Enemar 1962, Kepka and Krampitz 1969). However, comparison among studies is difficult as the data were obtained in different habitats and seasons. In our study, prevalence of *F. glareoli* in bank vole was 32% in autumn, 63% in spring; 42% in ecotones and 14% in open habitats (Table 3).

Mammals are more frequently infected in ecotones than in open habitats, and the prevalence is higher for both *Frenkelia* and *Sarcocystis* spp. Buzzards and other potential final hosts use wood edges and riparian forests as perches and for roosting, and faeces may concentrate at these sites, resulting in higher probability of intermediate host contact with infective sporocysts. Small mammals differ in their habitat preferences. The common vole lives in open habitats, namely fields and meadows, while the bank vole is a sylvatic species, typical for deciduous and mixed forests with undergrowth. The wood mouse is opportunistic but mainly lives in open habitats near wood edges or in bushes, yellow-necked mouse prefers deciduous or mixed forests (Anděra and Horáček 1982). As a result, mammalian species are exposed to different risks of infection, and differ in their potential to serve as intermediate hosts. Other studies have also found habitat differences in prevalence. In Germany, infected bank voles were found mostly near "forests along the rivers" (Kepka and Skofitsch 1979), in Udmurt (Russia) in "broad-leaved" and "dark coniferous", in comparison with "small-leaved" tree forests; in pine forests parasites were absent (Kalyakin *et al.* 1973). Intermediate host habitat is probably one of the most important factors in sarcosporidia transmission.

Prevalence of both *Frenkelia* and *Sarcocystis* is higher in heavier individuals. Body weight correlates with the age of small rodents and shrews (Šebek 1959, Pucek 1970, Zejda 1971), therefore, heavier individuals are, on average, older. Since the host remains infected till the end of his life, this result is not surprising. Double infections with *Frenkelia* and *Sarcocystis* are found significantly more frequently than would be expected by chance. This suggests that at least some factors influencing the probability of infection are similar; indeed, in both parasites, the habitat and host body weight significantly influence the prevalences in the same direction.

Frenkelia prevalences were higher in spring than in autumn in all host species, while in the case of *Sarcocystis*, only the common vole was more often infected in spring. Higher prevalences in spring than in autumn are probably attributable to the higher age of overwintering animals. Higher prevalences of *Frenkelia* in spring have been reported also in other areas, e. g. in Russia and Germany (Kalyakin *et al.* 1973, Skofitsch 1980).

Prevalence of *Frenkelia* in snap-trapped rodents is lower the first day of trapping than in the consecutive days. This suggests that host behaviour may be influenced by the parasites. Changes in host behaviour that enhance transmission have been reported in several host-parasite combinations including heteroxenous coccidia. Rodents naturally infected with sarcosporidians were found more frequently in the prey of the final hosts than in snap traps (Hoogenboom and Dijkstra 1987, Voříšek *et al.* 1998), and predation experiment using *S. dispersa* in mice and long-eared owl (*Asio otus*) as a predator confirmed that the results were not biased by the snap-traps preferentially trapping uninfected rodents (Voříšek *et al.* 1998). Differences in the host social status or neophilia could explain our result. However, trapping day has no effect on muscle *Sarcocystis* prevalences in small mammals. This suggests that brain and muscle dwelling sarcosporidians differ in their effects on host behaviour.

In rodents, higher prevalence of *F. glareoli* than that of *F. microti* seems surprising. Buzzards in Central Europe prey mostly on common voles, while bank voles are only occasionaly found in the prey (Haberl 1995, Voříšek *et al.* 1997). During winter, bank vole represents only 1% of prey (Ševčík 1981). In Poland, common vole represented 36% of prey during breeding, while bank vole only 14% (Goszczyński and Piłatowski 1986). In Česká Skalice, the proportion of bank vole does not exceed 10% in the buzzard prey but is probably lower (Diviš, pers. comm.). Moreover, *F. microti* is able to infect several rodent genera including the bank vole.

Although *F. microti* has a wide host spectrum, its prevalence is however higher in common voles than in bank voles, even in individuals from the ecotones, where we could expect the same risk of infection. However, differences in host food could cause different exposure to sporocysts. Common voles feed on green plant parts, while bank voles have a diverse food which includes seeds and fruits, green plant parts, fungi, and insects

(Holišová 1959, Obrtel and Holišová 1974). Insects may be very important in the transmission, as they may serve as transporting or paratenic hosts (Smith and Frenkel 1978, Markus 1980). The bank vole diet therefore does not explain the lower prevalence of *F. microti*. Rather, the infectivity of *F. microti* sporocysts is different for those rodents. In the original studies on host spectrum, sporocysts of *F. microti* were not infective to bank voles (Krampitz and Rommel 1977, Rommel and Krampitz 1978). In fact, in this paper we report for the first time natural infection of bank vole with *F. microti*. Our results suggest that in Central Europe, common voles are the main host for *F. microti*, while infections in bank vole are rather occasional. The same is probably true for yellow-necked mouse infected with *F. glareoli*, which was reported only once from a genus different than *Clethrionomys* (Doby *et al.* 1965), and in our study we report it for the first time from the genus *Apodemus*.

Relatively few studies have been done on protozoan parasites of small mammals in Central Europe, and most of them only described the parasite species spectrum (e. g., Šebek 1975a, b). Few studies report factors influencing parasite prevalences, and the results differ depending on parasite species studied. Apicomplexan infections (*Babesia*, *Hepatozoon*) were more prevalent in adult rodents, while trypanosomes in younger ones (Wiger 1979, Healing 1981). On the other hand, in a study of bank vole haemoparasites in Poland, temporal and seasonal variation was detected in prevalences, while age and sex were not important (Bajer *et al.* 2001). In our case, host sex did not influence prevalences of sarcosporidians, which is consistent with most other studies on bacterial, protozoan, and helminth parasites of rodents (Turner 1986, Healing 1981, Bajer *et al.* 2001, Behnke *et al.* 2001). Increased *S. muris* infection intensity was demonstrated for male house mouse (*Mus domesticus*) and male hybrids with *M. musculus* (Derothe *et al.* 2001), but the animals were kept under laboratory conditions. In nature, more males were found infected with *Babesia microti* than females (Krampitz and Baumler 1978).

Our study demonstrates that prevalences of brain and muscle sarcosporidians in small mammals are influenced by similar factors in different host-parasite combinations; these intrinsic factors include intermediate host habitat and age. Locality and year did not show any effect on prevalences, as well as host sex. Although the prevalence of *F. glareoli* is significantly higher than prevalence of *F. microti* in their respective intermediate hosts, both parasites successfully cycle in their final and intermediate host populations. The efficiency of transmission is proven by the prevalence of sporocysts in buzzard, the final host, which reaches 100% already at the time of nest desertion.

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ACTA
PROTOZOOLOGICA

Ultrastructural Description of *Microsporidium brevirostris* sp. n., Parasite of the Teleostean *Brachyhypopomus brevirostris* (Hypopomidae) from the Amazon River

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Summary. *Brachyhypopomus brevirostris* sp. n. (family Hypopomidae), a fish from the estuarine region of the Amazon River, collected near the city of Belém, Brazil, is parasitized by numerous microsporidian spores that form xenomas. These xenomas are found in the skeletal muscle adjacent to the abdominal cavity. The xenoma wall, with an irregular surface, consists of concentric laminated layers of compressed cells, possibly fibroblasts. Developing cells are in direct contact with host cell cytoplasm. Among the mature spores, small groups of juxtaposed immature spores are observed. The spores are ellipsoidal and uninucleate, measuring ~ 2.95 × 1.68 μm (n = 50). The isofilar filament (~ 55 nm diameter), consists of a regular coil in a single layer with 9-10 (or rarely 8) turns surrounding the posterior vacuole of the spore. The vacuole occupies about half of the total volume of the spore. The angle of tilt of the turns is ~ 37°. The spore wall is in direct contact with the cytoplasm of the host cells. A few larger grouped spores, measuring ~ 6.9 × 2.5 μm (n = 20), were observed mainly at the periphery of the most xenomas. The filament consists of two or three irregular layers of coils with 27-28 turns surrounding the posterior vacuole. The ultrastructural morphology of the spores and host specificity suggest that they may be included in the collective group of new *Microsporidium* species and named *Microsporidium brevirostris*. The taxonomic affinities and morphological comparisons with other similar species of some genera were discussed.

Key words: fish parasite, microsporidian, *Microsporidium brevirostris* sp. n., spore, ultrastructure.

INTRODUCTION

Microsporidia are common parasites of fish from different geographical areas (Canning and Lom 1986,

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Lom and Dyková 1992, Sprague *et al.* 1992, Dyková 1995, Larsson 1999, Shaw and Kent 1999, Lom and Nilsen 2003). Several microsporidian species parasitizing fish have been assigned: *Glugea, Heterosporis, Ichthyosporidium, Kabatana, Loma, Microfilum, Microgemma, Neonosemoides, Nosemoides, Nucleospora, Ovipleistophora, Pleistophora, Spraguea, Tetramicra and Amazonspora* (Vinckier 1975, Matthews and Matthews 1980, Ralphs and Matthews 1986, Lom

and Dyková 1992, Sprague *et al.* 1992, Faye *et al.* 1996, Larsson 1999, Shaw and Kent 1999, Sprague and Becnel 1999, Lom 2002, Azevedo and Matos 2003, Lom and Nilsen 2003). Among them, however, only few are xenoma-forming genera of Microsporidia.

Although there is considerable information on the species of Microsporidia (Lom and Dyková 1992, Sprague *et al.* 1992, Lom 2002, Lom and Nilsen 2003), little is known about those from South America, and particularly those from the Amazon River, where a diverse assemblage of several hundred species of fish live. Light microscopicy and ultrastructural data are available for only two Amazonian species, *Loma myrophis* (Azevedo and Matos 2001) and *Amazonspora hassar* (Azevedo and Matos 2003). Here, a detailed ultrastructural study is presented of the xenoma, the spores and the host cell interaction of a parasite of *Brachyhypopomus brevirostris* (family Hypopomidae), a fish from the estuarine region of the Amazon River. Based on the ultrastructural features and host specificity observed, we propose the creation of a new microsporidian species.

MATERIALS AND METHODS

Forty specimens of the teleost *Brachyhypopomus brevirostris* (Steindachner, 1868) (family Hypopomidae) (common Brazilian name, "itui rajado"), were collected in the estuarine region of the Amazon River (01° 11´ 30´´ S / 47° 18´ 54´´ W) near the city of Belém (Pará), Brazil. Infection was determined by the presence of xenomas located in the skeletal muscle of the abdominal cavity, recognizable by the naked eye. Measurements of xenomas and fresh spores were made in wet mount preparations with an eye-piece micrometer at ×1,000. After crushing the xenoma, the spores were identified by differential interference contrast microscopy (DIC). For transmission electron microscopy (TEM), the xenoma and surrounding tissues were fixed in 3% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2) at 4°C for 20-24 h, rinsed overnight in the same buffer at 4°C and post-fixed in 2% OsO_4 in the same buffer at 4°C for 4 h. After dehydration in an ascending ethanol series (70, 80, 90, 95 and 100% (2 h in each change) and in propylene oxide (two changes of 3 h each), the infected tissues were embedded in Epon (10-12 h in each change). Semithin sections were stained with 1% methylene blue, 1% Azur II (v/v) and observed by DIC optics. The ultrathin sections were double stained with uranyl acetate and lead citrate (Reynolds 1963) and observed with a JEOL 100CXII TEM operated at 60 kV.

RESULTS

Irregular whitish xenomas, were macroscopically observed only in the internal muscular tissue of the abdominal cavity of the fish, *Brachyhypopomus brevirostris* (family Hypopomidae). Eighteen of 40 specimens were infected (45%). The xenomas, measuring 85 to 465 μm (n=15), were photographed by DIC (Fig. 1). Most of the xenoma was filled with numerous spores (Fig. 2). Among the spores (microspores) contained in the xenoma, small groups of immature spores were also observed (Figs 2, 10). After rupture of the xenomas, numerous ellipsoidal spores were identified as belonging to the phylum Microsporidia. Unfixed spores observed in DIC optics were 2.95 ± 0.32 μm long and 1.68 ± 0.18 μm wide (n=50) (Fig. 3).

The xenoma wall has an irregular surface and is formed of concentric laminated structures, spaced by electron-lucent layers intermingled with layers of the compressed cell coat (possibly fibroblasts), which forms an electron-dense substance (Fig. 4). Some fibroblasts were observed external to the xenoma (Fig. 4). Small groups of juxtaposed immature spores were observed among the mature spores (Fig. 5). The spores were in direct contact with the host cell cytoplasmic matrix, where no cytoplasmic structure was observed other than some vesicular structures and granular cytoplasmic debris (Figs 4, 6). The walls of mature spores (~ 37 nm thick) are composed of an electron-dense exospore (~ 15 nm thick) and an electronlucent endospore (~ 22 nm thick) (Figs 7-9). The anchoring disk was eccentric and the polar tube had an oblique position in relation to the longitudinal axis of the spore (Figs 6, 7). The manubrium constituted a straight anterior part of the polar filament measuring ~ 125 nm diameter at its midpoint (Fig. 8).

The isofilar polar filament (~ 55 nm diameter) consisted of a regular coil in a single layer with 9-10 turns (or rarely 8 turns) surrounding the posterior vacuole (Figs 7, 9) that occupied about half of the spore length (Fig. 7). In a favourable series of longitudinal ultrathin sections, it was possible to measure the angle of tilt as being about 37° (35-41°) (n=15) (Fig. 7).

The lamellate polaroplast occupied the apical position around the anterior portion of the polar filament and consisted of two membranous system. The anterior region contained closely packer and arranged lamellae, while the posterior was more widely spaced lamellae (Figs 7, 8).

The nucleus occupies a position between the polaroplast and the posterior vacuole, and some helically arranged aggregate polyribosomes could be observed surrounding the nucleus.

In some sections, it was possible to observe the presence of a few larger uninucleate spores, measuring
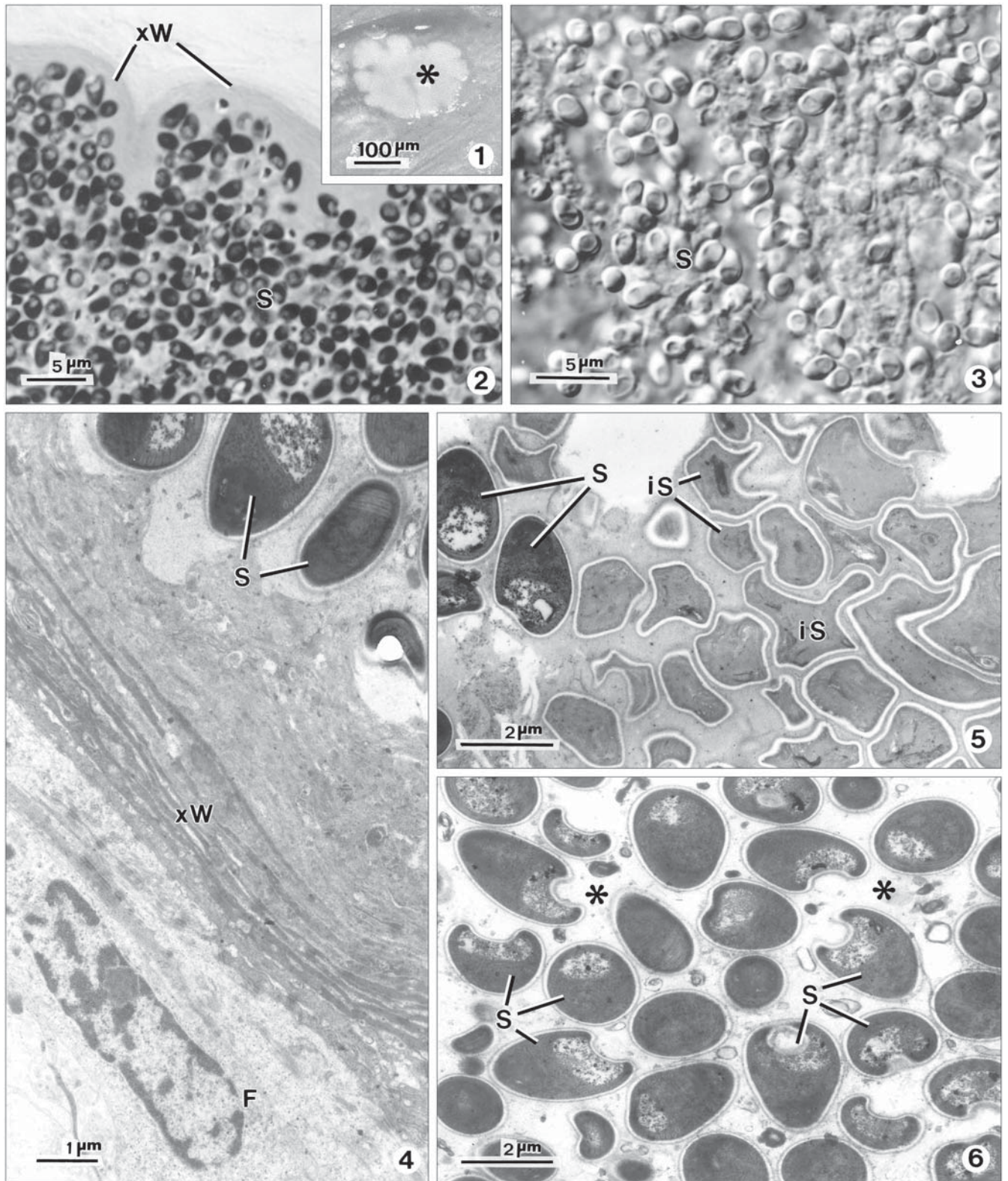
Figs 1-6. *Microsporidium brevirostris* sp. n. **1** - single xenoma (*) observed in the internal abdominal wall of the fish; **2** - semithin section of the xenoma periphery showing the xenoma wall surrounding numerous ellipsoidal spores; **3** - fresh spores released from a xenoma observed in DIC optics. Electron micrographs showing: **4 -** fibroblast and possibly other compressed fibroblasts located at the periphery of the xenoma wall. Internally some spores are present; **5 -** small groups of immature spores next to mature spores; **6 -** several spores without a surrounding membrane, sectioned in different planes and located in an amorphous matrix (*) of host cytoplasm. F - fibroblast, iS - immature spores, S - spores, xW - xenoma wall.
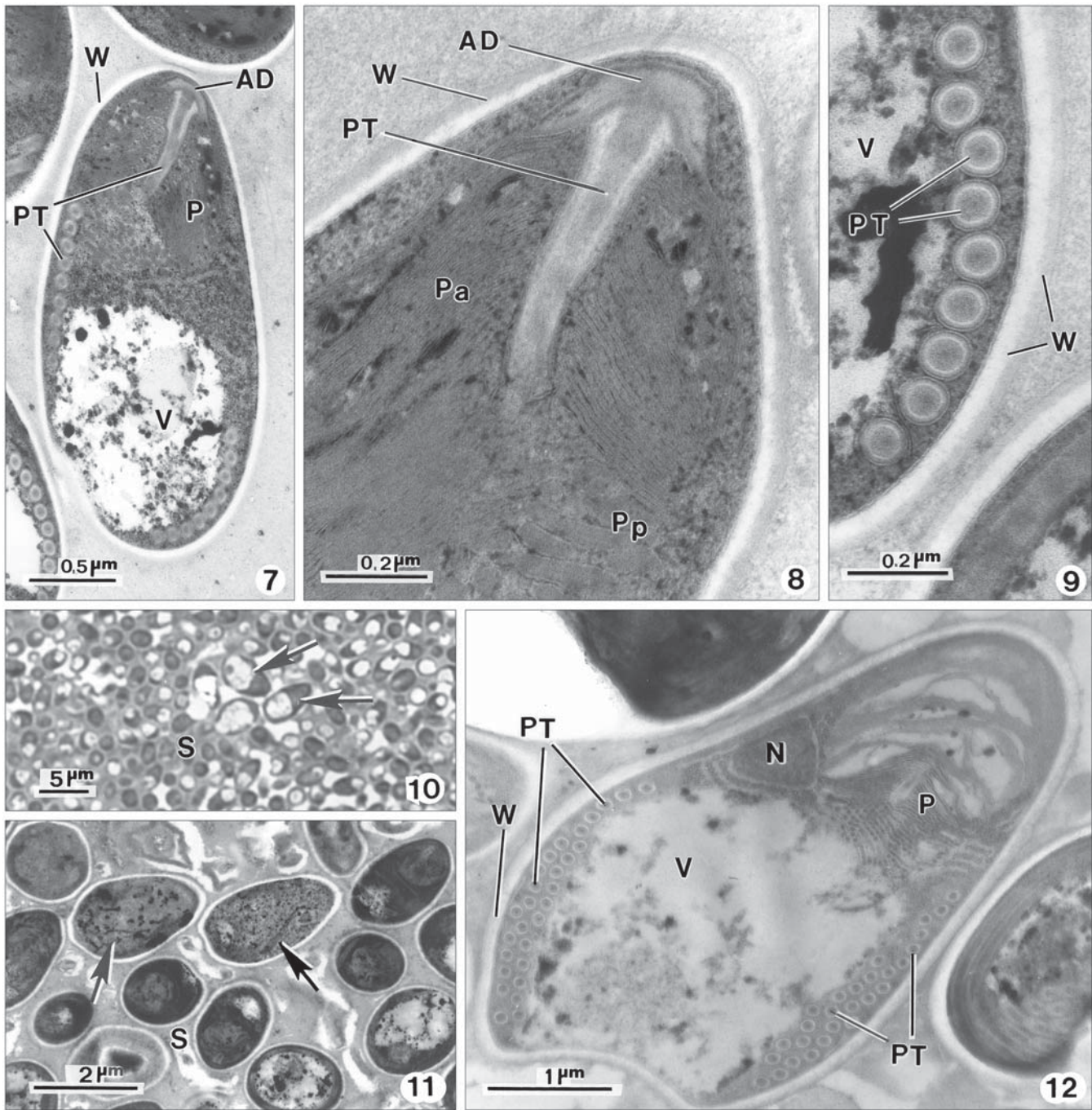
Figs 7-12. *Microsporidium brevirostris* sp. n. electron micrographs of: **7** - mature spore sectioned longitudinally showing the different typical structures of a microsporidian spore. The nucleus occupied a central position, was not observed in this section; **8** - details of the apical region of a spore, showing the spore wall, anchoring disk, the initial portion of the polar tube (manubrium) and the organization of the polaroplast; **9 -** of transverse sections of the polar tube located between the posterior vacuole and spore wall. The vacuole contains dense masses; **10** - semithin section of the xenoma showing some macrospores (arrows) among numerous microspores; **11** - some macrospores (arrows) among microspores; **12 -** macrospores sectioned longitudinally showing the spore wall, the polar tube with its 27-28 turns, the vacuole, the nucleus and the polaroplast. AD - anchoring disk, N - nucleus, P - polaroplast, Pa - anterior region, Pp - posterior region, PT - polar tube, S - microspores, V –vacuole, W - spore wall.

~ 6.9 (6.4-7.2) μm long and × 2.5 (2.0-2.8) μm wide (n=20) (Fig.10), containing polar filaments with two or three irregular coils of 27-28 turns surrounding the posterior vacuole (Figs 11, 12). These spores appeared in grouped, containing from 4-10 in number among the microspores (Fig. 10).
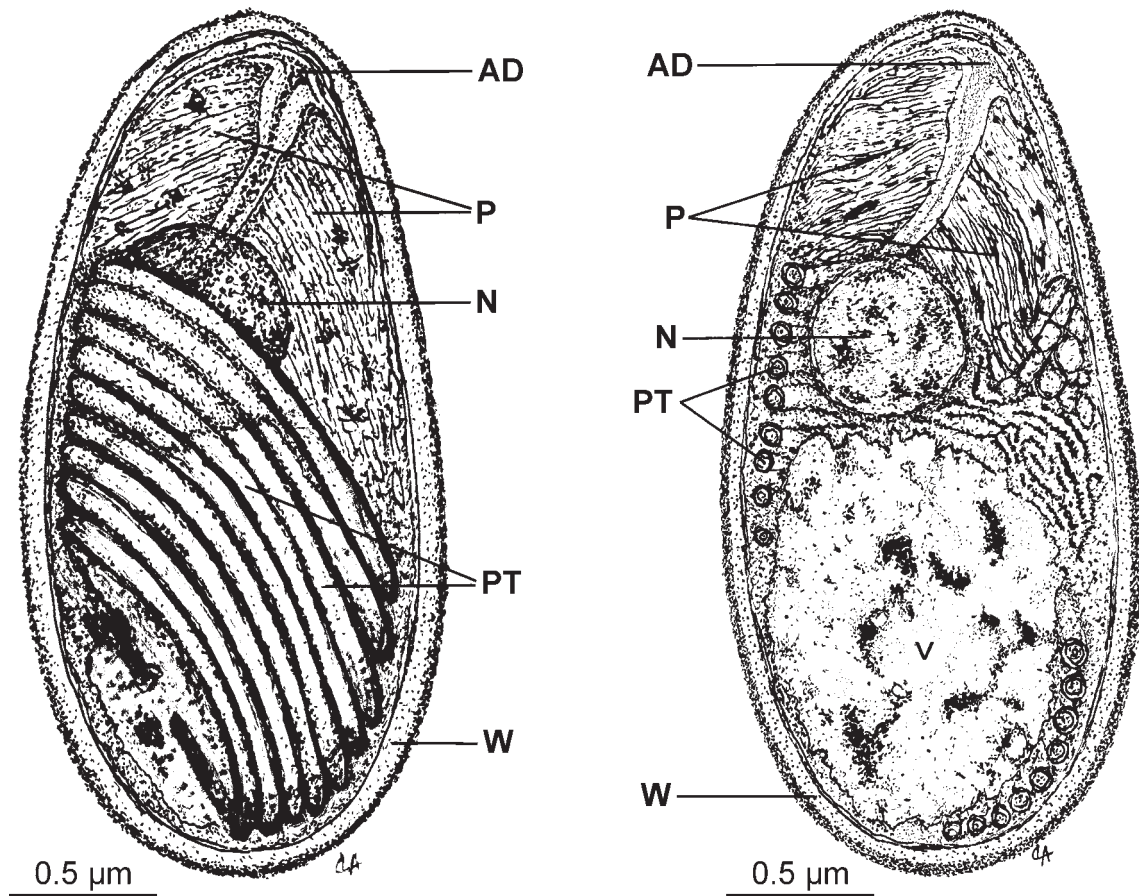
Fig. 13. Two semischematic drawings summarizing the tridimensional morphology (left) of the spore of *Microsporidium brevirostris* sp. n., and the ultrastructural morphology in longitudinal section (right), as described in the text and illustrated in the micrographs. AD - anchoring disc, N - nucleus, P - polaroplast, PT - polar tube, V - posterior vacuole, W- spore wall.

Schematic drawings of the spore morphology (Fig. 13) were made from serial ultrathin sections.

Taxonomy summary of *Microsporidium brevirostris* sp. n.

Type host: *Brachyhypopomus brevirostris* (Steindachner, 1868) (family Hypopomidae).

Site of infection: Skeletal muscle of the abdominal cavity.

Type locality: Estuarine region of the Amazon River, near city of Belém, Brazil.

(Latitude: 01° 11' 30" S Longitude: 47° 18' 54" W)

Diagnosis: Host cells form macroscopic xenomas filled with spores. Ellipsoidal uninucleate spores in direct contact with the host cell cytoplasm, measured ~ 2.95 × 1.68 μm. Spore wall measured ~ 37 nm thick, were composed of electron-dense exospore (~ 15 nm thick) and electronlucent endospore (~ 22 nm thick). Isofilar polar filament with 9-10 (rarely 8) turns. Angle of tilt was ~ 37°. Polaroplast of the anterior region consisted of closely packer arranged lamellae and the posterior region more spaced lamellae. Larges spores measured ~ 6.9 × 2.5 mm. Polar filament with 27-28 turns in two or three irregular layers.

Type specimens: 2 slides containing semithin sections of the xenomas with spores of the holotypes were deposited in the International Protozoan Type Slide Collection at Smithsonian Institution, Washington D. C. 20560, USA with USNM no. 1025353.

Prevalence of infection: 18/40 (45%).

Etymology: The specific epithet, "*brevirostris*", is derived from the specific epithet of the host species.

DISCUSSION

The more conspicuous characteristics of the spores - the shape, wall, polaroplast, polar filament and posterior vacuole - are used to distinguish microsporidia from other taxonomic groups (Sprague *et al.* 1992). The results of our study demonstrate that the ultrastructure of the spore found in xenomas of *Brachyhypopomus brevirostris* (family Hypopomidae) corresponds to that of the phylum Microsporidia (Vávra and Larsson 1999).

In a recent paper it was stated that the 156 fish microsporidian species recorded are distributed among 14 genera (Lom and Nilsen 2003). Some of these produce xenomas: *Glugea* Thélohan, 1891; *Ichthyosporidium* Caullery et Mesnil, 1905; *Loma* Morrison et Sprague, 1981; *Microfilum* Faye, Toguebaye et Bouix, 1991; *Microgemma* Ralphs et Matthews, 1986; *Nosemoides* Vinckier, 1975; *Spraguea* Vávra et Sprague, 1976; and *Tetramicra* Matthews et Matthews, 1980. More recently, a new genus *Amazonspora* was added to these (Azevedo and Matos 2003).

The recently created new genus and species *Pseudoloma neurophilia,* which is found in the central nervous system of the zebrafish (*Danio rerio*), differs from the latter genera because the spores are segregated into clusters of up to 16 spores and appear to develop within a true sporophorous vesicle (Matthews *et al.* 2001). The ultrastructure of the xenoma described in *Pseudoloma* is not typical of must xenomas (Lom 2002). Spores of *Tetramicra* found in xenoma from skeletal muscle have a conspicuous inclusion in the sporoplasm and posterior vacuole, is unique among fish-infecting microsporidian (Lom and Dyková 1992). Such an inclusion was never observed in our study. The xenomas of *Amazonspora,* which consist of a single hypertrophic host cell and a xenoma wall composed of up to 22 juxtaposed crossed layers of collagen fibres (Azevedo and Matos 2003), are very different to the xenomas we describe here. The distinction between the genera *Glugea* and *Loma* is not clear (Cali and Takvorian 1999, Lom and Pekkarinen 1999). However, in the present study using ultrathin sections, the developmental stages, the sporogonial plasmodium dividing into sporoblast mother-cells which gives rise to two sporoblasts (Canning *et al.* 1982), the mature spores and the xenoma wall, all more closely resemble features of the genus *Glugea* (Canning *et al.* 1982).

Compared with these previously described genera, our results show that this parasite has differences in the morphology of the spores and the ultrastructure of the

developing cells, xenoma and the xenoma wall. *Ichthyosporidium* sp. and *Kabatana* sp. differ from our results with respect to the developing cells that are in contact with host cells (Lom *et al.* 2000, Lom 2002) and the absence of xenoma formation in *Kabatana* sp. (Lom *et al.* 1999, 2000). In *Microgemma* sp. (Ralphs and Matthews 1986) and *Microfilum* sp. (Faye *et al.* 1991), the life cycles give rise to the formation of xenomas with a microvillous surface, which does not occur in the microsporidia described here. The genus *Ovipleistophora* has both micro- and macrospores, as in *Microsporidium brevirostris*. However these two kind of spores are specific parasite of oocytes (Pekkarinen *et al.* 2002). No microsporidia have been observed or described with comparable spore morphology and picture of infection from freshwater fishes living in the same geographic area. Considering these data and the host specificity, we believe that this microorganism represents a new species that should be included in the collective genus *Microsporidium* Balbiani, 1884, and we propose the name *Microsporidium brevirostris.* However, more detailed studies, particularly, on life cycle stages and host specificity are need to identify the appropriate existing or new genus to include the parasite.

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ACTA
PROTOZOOLOGICA

First Record of *Trichodinella epizootica* (Raabe, 1950) Šramek-Hušek, 1953, with Description of *Trichodina notopteridae* sp. n. (Ciliophora: Peritrichida) from Freshwater Fishes of India

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Summary. Very few studies of ectoparasitic trichodinid ciliophorans occurring on edible fish in India have been done. During surveys of the trichodinid parasites in the Churni River, India, one each species of the genus *Trichodina* and *Trichodinella* from the gills of freshwater fishes were investigated and morphologically studied. Of these, one is *Trichodinella epizootica* (Raabe, 1950) Šramek-Hušek, 1953 found for the first time in India from the gills of a minor carp *Puntius gelius* (Hamilton-Buchanan) and the other is described as new: *Trichodina notopteridae* sp.n. from the gills of *Notopterus notopterus* (Pallas). Taxonomic descriptions of these trichodinids based on the wet silver nitrate impregnated specimens are presented. For the new species comparisons with closely related species are provided.

Key words: Ciliophora, freshwater fish, India, taxonomy, *Trichodina notopteridae* sp. n., *Trichodinella epizootica*.

INTRODUCTION

In India, studies on the trichodinid ciliophorans, although not very comprehensive, is getting momentum in many sectors. As a result, 10 species of trichodinid ciliophorans representing the genera *Trichodina* Ehrenberg, 1838; *Paratrichodina* Lom, 1963 and

Tripartiella Lom, 1959 were identified from different freshwater and estuarine Indian fishes (Hagargi and Amoji 1979; Mukherjee and Haldar 1982; Das and Haldar 1987; Das *et al*. 1987; Mishra and Das 1993; Saha *et al*. 1995a, b; Saha and Haldar 1996, 1997; Asmat and Haldar 1998; Basu and Haldar 1998; Mitra and Haldar 2003). Surveys of the trichodinid ciliophorans in the edible fishes of the Churni River, revealed the occurrence of *Trichodinella epizootica* (Raabe, 1950) Šramek-Hušek, 1953 from the gills of a minor carp *Puntius gelius* (Hamilton-Buchanan) and a new species from the gills of *Notopterus notopterus* (Pallas). In this paper taxonomic descriptions of both the species are

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provided with *T. epizootica* recorded for the first time from India.

MATERIALS AND METHODS

Fishermen were employed to collect host fishes from the Churni River and adjoining beels and kharis, situated in the district of Nadia (Latitude 23°N and Longitude 88.5°E), West Bengal, India. Host fishes were brought to the laboratory alive condition and gill and skin smears were made on grease free slides. Slides containing trichodinid ciliophorans were impregnated using Klein's dry silver impregnation technique (Klein 1958). Examinations of preparations were made under an Olympus phase contrast microscope at objective ×100 with an oil immersion lens and photographs were taken with an Olympus camera. All measurements are in micrometers and follow the uniform specific characteristics proposed by Lom (1958), Wellborn (1967) and Arthur and Lom (1984). In each case minimum and maximum values are given, followed in parentheses by arithmetic mean and standard deviation. In the case of denticles and radial pins, the mode is given instead of the arithmetic mean. The span of the denticle is measured from the tip of the blade to the tip of the ray. Body diameter is measured as the adhesive disc plus border membrane. The description of denticle elements follows the guidelines proposed by Van As and Basson (1989). Sequence and method of the description of denticle elements follows the recommendations of Van As and Basson (1992).

RESULTS AND DISCUSSION

Two species of trichodinids were identified from collected fish. These are *Trichodinella epizootica* (Raabe, 1950) Šramek-Hušek, 1953 and *Trichodina notopteridae* sp. n. Descriptions of these is provided below.

Trichodinella epizootica (Raabe, 1950) Šramek-Hušek, 1953 (Figs 1, 2, 7; Table 1)

Body of ciliophoran small and has shape of a vaulted disc. Adhesive disc concave. Border membrane narrow, but distinct with fine striations. Blade elongated with almost parallel margins and tapering to region of anterior projection. Distal margin closely adjoining border membrane truncated or slightly curved with blunt tangent point which forms small line with y-1- axis, situated lower than distal margin (Fig. 7). Anterior margin runs at slight angle to y-axes. Anterior projection slender, but prominent and fits well into notch between central part and blade of preceding denticle, directed obliquely in a distal direction in some specimens. Posterior projection not well developed. Central part rounded and pronounced touches and extends slightly beyond y axes.

(Fig. 7). Ray forms a short delicate hook curved along central part, not easy to distinguish in live specimens and rather difficult to impregnate. Macronucleus horseshoe shaped, but micronucleus could not be detected. Adoral ciliary spiral takes a turn of 180°.

Taxonomic summary

Host: *Puntius gelius* (Hamilton-Buchanan)
Locality: River Churni, West Bengal, India (Lat. 23°N and Lon. 88.5°E)
Location: Gills
Reference material: PG/3/11-2001 in the collection of authors.
Remarks: Raabe (1950) was the first to describe *Trichodinella epizootica* from the gills of various host fishes by using Klein's silver impregnation technique. During the present investigation, a moderate infestation of trichodinid in minor carp, *Puntius gelius* (Hamilton-Buchanan) was observed. This ciliophoran was identified as belonging to the genus *Trichodinella* based on Lom (1963) specifically as T. *epizootica*. *T. epizootica* obtained in the present study is morphometrically compared with those of other authors in Table 1.
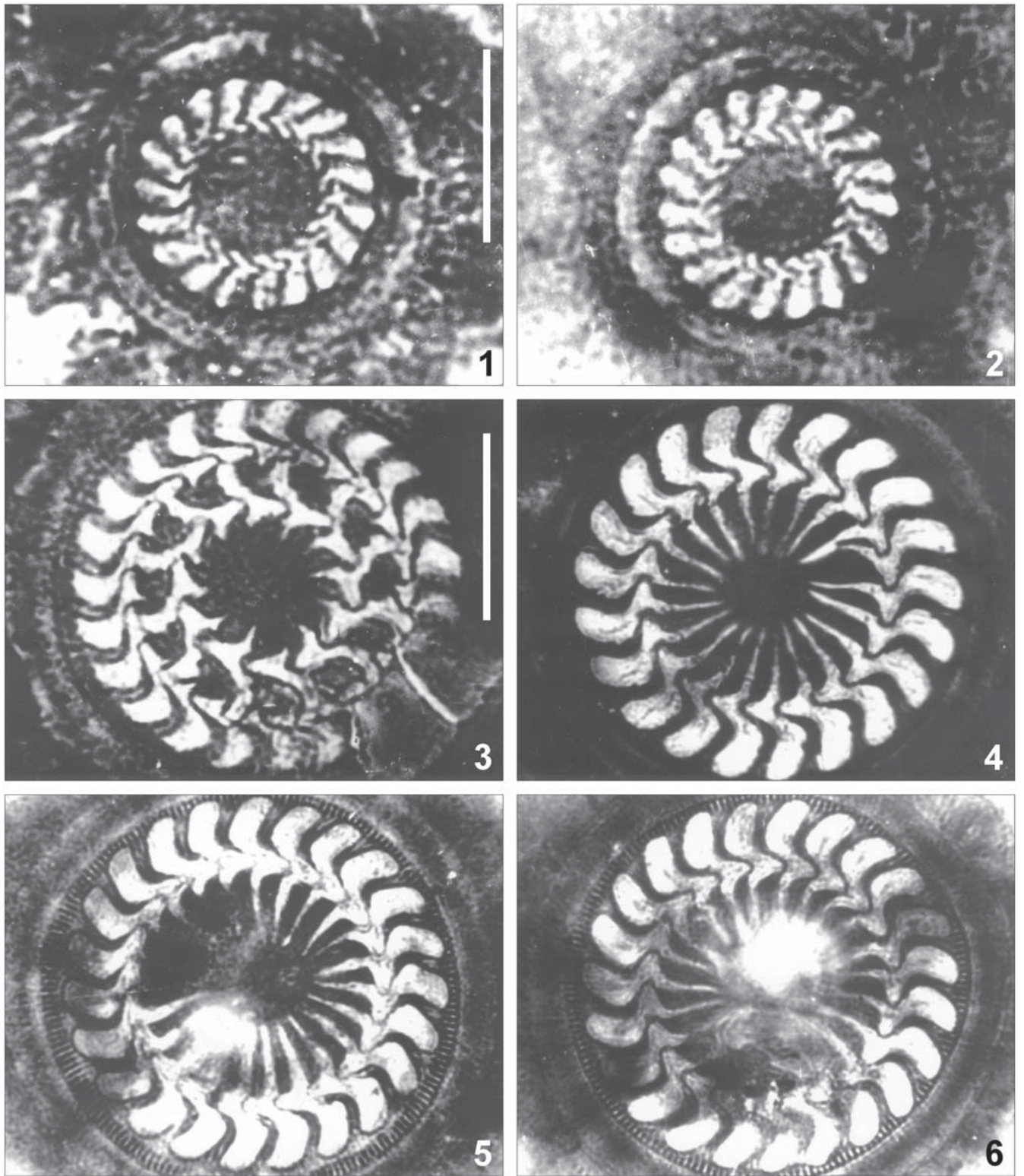
Trichodina notopteridae sp. n. (Figs 3-6, 8A; Table 2)

Falls in range of medium sized ciliophorans. Body disc shaped. Concave adhesive disc surrounded by relatively broad border membrane. Blade almost rectangular in shape in majority of specimens. Distal margin of blade flat, runs parallel to border membrane, situated at higher level than tangent point. Tangent point rounded. Blade broad, fills most of spaces between y-axes (Fig. 8A). Anterior margin of blade almost touches y+1 axis (Fig. 8A). Apex prominent. Blade apophysis distinct. Posterior margin of blade runs parallel to anterior margin and forms a shallow semilunar curve, at same level of apex. Moderately sized, triangular central part ends in blunt rounded tip and extends up to halfway to y-1 axis (Fig. 8A), fitting tightly into preceding denticle. Sections of central part above and below x-axis similar. Ray connection delicate with ray apophysis situated high and directed distally. Ray of equal thickness along its length, ending in rounded tip. Direction of rays towards y+1 axis.

Taxonomic summary

Type host: *Notopterus notopterus* (Pallas)
Locality: Churni River, West Bengal, India (Lat. 23°N and Lon. 88.5°E)

Figs 1-6. Photomicrographs of silver nitrate impregnated adhesive discs of trichodinid ciliophorans. **1, 2** - *Trichodinella epizootica* (Raabe, 1950) Šramek-Hušek, 1953 from gills of *Puntius gelius*. **3-6 -** *Trichodina notopteridae* sp. n. from gills of *Notopterus notopterus* (Pallas). Scale bars 10 μm (1, 2); 20 μm (3-6).

Table 1. Morphometric comparison of *Trichodinella epizootica* (Raabe, 1950) Šramek-Hušek, 1953 obtained in the present study with those of other authors.

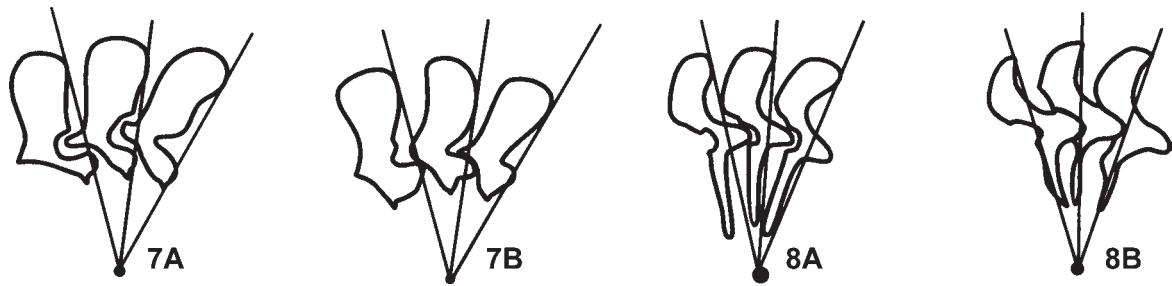| Species	*T. epizootica*	*T. epizootica*	*T. epizootica*	*T. epizootica*
Host	*Puntius gelius*	*P. fluviatilis*	*Cyprins carpio*	*Cyprins carpio*
Locality	Ranaghat, India	Czechoslovakia	South Africa	Philippines
Location	Gills	Gills	Gills	Gills
References	present study	Lom and Haldar (1977)	Basson *et al*. (1983)	Albaladejo and Arthur (1989)
Diameter of				
body	17.3-23.4 (19.4 ± 2.0, 20)	23-50	18.2-26.5 (22.2 ± 2.2)	19.0-26.0 (22.5 ± 1.8)
adhesive disc	13.3-19.4 (15.8 ± 1.9, 20)	30	14.4-22.5 (18.4 ± 2.0)	15.0-21.9 (18.5 ± 1.6)
Dimension of body				
denticulate ring	6.1-9.2 (7.6 ± 0.9, 20)	27	7.4-13.2 (10.3 ± 1.4)	9.0-12.5 (11.2 ± 1.1)
central area	-	-	-	-
Width of the border membrane	1.3-2.2 (1.8 ± 0.3, 20)	1.4-3.3	1.5-2.3 (2.1 ± 0.2)	-
Number of denticles	17-24 (19, 20)	16-28	20-25 (23)	19-24 (21.2 ± 1.0)
radial pins/denticle	3.0-6.0 (4, 20)	4-6	5-6 (5)	5-6
Dimension of denticle				
span	3.0-5.1 (4.1 ± 0.5, 20)	-	-	4.0-6.0 (5.1 ± 0.6)
length	1.5-2.5 (2.1 ± 0.3, 20)	-	1.8-2.9 (2.3 ± 0.3)	1.6-2.7 (2.2 ± 0.3)
Dimension of denticle components				
length of the ray	-	-	-	-
length of the blade	2.1-4.1 (3.3 ± 0.5, 20)	2.2-3.6	1.7-3.8 (2.7 ± 0.4)	2.5-4.0 (3.2 ± 0.4)
width of the central part	0.5-1.0 (0.9 ± 0.2, 20)	0.6-2.2	0.7-1.2 (1.0 ± 0.2)	1.1-2.8 (2.0 ± 0.5)
Adoral ciliary spiral	180-190°	180°	180°	-

Type specimen: *Trichodina notopteridae* sp. n.

Reference material: Holotype, slide NNX-1, and paratype, slide NNX-12 in the collection of the Protozoology Laboratory, Department of Zoology, University of Kalyani, Kalyani 741235, West Bengal, India and slide NNX-2 bearing some paratype materials in the collection of Harold W. Manter Laboratory of Parasitology, Lincoln, Nebraska USA (HWML 16744).

Remarks: The present trichodinid species, in having a rectangular blade, a slightly curved distal margin, a moderately sized and triangular central part; a straight and strong ray of even thickness with rounded tip, differs significantly from other known trichodinid species and only shows some resemblance with *Trichodina luciopercae* Lom, 1970.

Trichodina luciopercae was described by Lom (1970) from gills of *Stizostedian lucioperca* in Tisza River near Kotelek at Szolovok, Hungary. The distal surface of the blade is flat in the case of *Trichodina notopteridae* and runs parallel with the border membrane (Fig. 8A), but truncated (Fig. 8B) in *T. luciopercae*. Trichodinid ciliophorans obtained from *Notopterus notopterus* have robust blades with the anterior and posterior margins running parallel (Fig. 8A). But in case

Figs 7A, B. Diagrammatic drawings of the denticles of two specimens of *Trichodinella epizootica* (Raabe, 1950) Šramek -Hušek, 1953 obtained from the gills of *Puntius gelius* (Hamilton-Buchanan).
Figs 8A, B. Diagrammatic drawings of the denticles of two *Trichodina* Ehrenberg, 1838 species. **A** - *Trichodina notopteridae* sp. n. obtained from the gills of *Notopterus notopterus* (Pallas); **B** - *Trichodina luciopercae* Lom, 1970; redrawn from Lom (1970).

Table 2. Morphometric comparison of *Trichodina notopteridae* sp. n. with *Trichodina luciopercae* Lom, 1970.

Species	*Trichodina notopteridae* sp. n.	*T. luciopercae*
Host	*Notopterus notopterus*	*Stizostedion lucioperca*
Locality	Churni River, West Bengal, India	Tisza River, Hungary
Location	gills	gills
References	present study	Lom (1970)
Diameter of		
body	43.0-55.7 (50.7 ± 3.2, 20)	41-55 (45-52)
adhesive disc	35.7-45.9 (41.9 ± 3.1, 20)	33-44 (37-40)
Dimension of body		
denticulate ring	20.9-29.6 (25.0 ± 2.3, 20)	19-26 (21-33)
central area	4.1-9.2 (6.5 ± 1.7, 20)	-
clear area	-	-
Width of the border membrane	3.6-5.1 (4.4 ± 0.5, 20)	3.5-5.0
Number of denticles	21-24 (22, 20)	20-23 (20)
radial pins/denticle	7-10 (9, 20)	7-9
Dimension of denticle		
span	11.5-17.2 (14.6 ± 1.6, 20)	-
length	5.3-8.1 (6.8 ± 0.7, 20)	8-9
Dimension of denticle components		
length of the ray	4.1-8.1 (6.5 ± 1.1, 20)	5-5.5
length of the blade	5.1-7.1 (6.1 ± 0.6, 20)	4.5-5.0
width of the central part	1.2-2.3 (1.9 ± 0.2, 20)	2.1-3.0
Adoral ciliary spiral	310-400°	-

of *T. luciopercae* the anterior and posterior margins are not parallel. In case of *T. notopteridae* the posterior tip of the central part extends almost halfway to y-1 axis (Fig. 8A), but in case of *T. luciopercae* it almost touches y-1 axis (Fig. 8B). The rays of *T. notopteridae* are of equal length along entire their length with almost rounded tips, while the rays of *T. luciopercae* end with pointed tips. However, the morphometric data do not vary significantly (Table 2). We propose this species as a distinct one and designate it in this paper as *Trichodina notopteridae* sp. n. Morphometric comparison of the *Trichodina notopteridae* and *Trichodina luciopercae* Lom, 1970 is provided in Table 2.

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ACTA
PROTOZOOLOGICA

Descriptions of Two New Species of Acephaline Gregarines (Protozoa: Apicomplexa: Eugregarinida), *Apolocystis chotonagpurensis* sp. n. and *Stomatophora janovyi* sp. n. from Earthworms (Annelida: Oligochaeta) of India

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Summary. During surveys of the endoparasitic acephaline gregarines in the Chotonagpur district of Bihar, seminal vesicles of earthworm *Amynthas roubsta* were found to be infested with a new species of the genus *Apolocystis* Cognetti de Martiis, 1923, *A. chotonagpurensis* sp. n. Trophozoites of the new species are rounded or ovoidal in shape and measure 42-83 μm in diameter. Nucleus of the trophozoite is ovoid and measures $12\text{-}17 \times 8\text{-}16$ μm. Gametocysts are ovoidal, enclose two unequal gamonts and measure $71\text{-}96 \times 46\text{-}83$ μm. Oocysts are biconical measuring $6.5\text{-}7 \times 3.5\text{-}4$ μm. A different species of earthworm *Amynthas hawayanus* collected from the hill region of Darjeeling district of West Bengal revealed the existence of a new species of the genus *Stomatophora* Drzhevetskii, 1907. *Stomatophora janovyi* sp. n. is petaloid shaped and measure 80-85 μm in diameter. Gametocysts are ovoid and measure $52\text{-}66 \times 33\text{-}39$ μm and oocysts are navicular shaped with sharply pointed ends and measure $10\text{-}12 \times 6\text{-}7$ μm.

Key words: Acephaline gregarines, *Apolocystis chotonagpurensis* sp. n., earthworm, seminal vesicles, *Stomatophora janovyi* sp. n.

INTRODUCTION

Aseptate gregarine fauna have been reported from various parts of the world including India. But especially in India the search is far from complete. While investigating acephaline gregarines in the oligochaete worms, seminal vesicles of earthworm *Amynthas robusta*

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Perrier, 1872 collected from Chotonagpur district of Bihar were found to harbour an undescribed species of *Apolocystis* Cognetti de Martiis, 1923. A separate species of earthworm, *Amynthas hawayanus* Rosa, 1891 obtained from the hill region of Darjeeling district of West Bengal was infected with an undescribed species of *Stomatophora* Drzhevetskii, 1907, in their seminal vesicles. Little work has been done in India on the representatives of the genus *Apolocystis* and *Stomatophora* parasitizing the oligochaete worms. Only five species of the genus *Apolocystis* (Bhatia and Setna 1926, Pradhan and Dasgupta 1983) and six species of

the genus *Stomatophora* (Hesse 1909, Pradhan and Dasgupta 1980, Roychowdhury and Haldar 1984, Bandyopadhyay *et al*. 2001) have so far been described from India. In this paper taxonomic descriptions of two new species of acephaline gregarines of the genera *Apolocystis* and *Stomatophora,* as well as comparisons with previously species, are provided.

MATERIALS AND METHODS

Earthworms were collected and taken to the laboratory. They were dissected while alive and their seminal vesicles were carefully removed. These were placed on clean glass with a drop of 0.5 % NaCl solution. A thin film of the seminal fluid was drawn out on a slide covered with a cover slip for examination of live protozoans under a phase contrast microscope. The content of seminal vesicles was semidried and fixed in Schaudin's fluid (20 min). The fixed smears were stored in 70 % ethyl alcohol for removal of mercuric chloride. The slides were then passed through a descending series of alcohols (5 min each) and stored in distilled water. These were transferred to a 3% iron alum solution and stained with Heidenhain's haematoxylin solution (20 min). Differentiation (over night) was done with 1 % iron alum solution. The slides were then washed thoroughly, dehydrated in an ascending series of alcohol, cleared in xylene and mounted in Canada balsam.

TAXONOMY

Apolocystis chotonagpurensis sp. n. (Figs 1-4, Table 1)

Phylum: Apicomplexa Levine, 1977
Order: Eugregarinida Leger, 1900
Family: Monocystidae Bütschli, 1882
Subfamily: Monocystinae Bhatia, 1930
With the characters of genus *Apolocystis* Cognetti de Martiis, 1923, as given by Levine (1988); gamonts spherical, solitary, oocysts biconical. Trophozoite rounded or ovoidal in shape without polar differentiation. Diameter ranges from 42.0-83.0 (57.0 ± 19.0). Ectosarc very thin. Episarc fine with very fine rows of cytoplasm, which is the most characteristic feature in the species of *Apolocystis* arranged in concentric fashion. Nucleus large, elongated with deeply stained, round karyosome; lengths range from 12.0-17.0 (14.0 ± 2.0), widths from 8.0-15.0 (11.0 ± 3.0). Nucleus rather oval in young stages may also be spherical in immature stages. Gametocysts egg shaped; two unequal gametes present lengths range from 71.0-99.0 (77.0 ± 9.0), widths from 46.0-83.0 (62.0 ± 15.0). Endosarc homogeneously granu-

lar; nucleus nearly spherical but endosome not. Oocysts biconical; lengths range from 6.5-7.0 (6.0 ± 1.0) widths from 3.5-4.0 (3.0 ± 0.1).

Taxonomic summary

Type material: *Apolocystis chotonagpurensis* sp. n.
Host: *Amynthus robusta* Perrier, 1892
Type locality: India, Bihar, Chotonagpur (22° N; 84° E).
Symbiotype: Host AR-11/12/2002 deposited in the museum of the Department of Zoology, University of Kalyani, Kalyani 741235, West Bengal, India.
Site of infection: Seminal vesicles.
Prevalence: 6 of 12 (50%).
Elevation: 700 m above mean sea level.
Type material: The syntype no. AC/2-2002 deposited in the Zoological Survey of India (ZSI)), Calcutta - 700016 (Catalogue No. 2407).
Etymology: The species name has been derived from the collection locality, Chotonagpur.
Remarks: Two species of *Apolocystis* have so far been described from the earthworm *Amynthus robusta*. These two species namely *A. akaryoseminiferus* and *A. monokaryoseminiferus* are known from India (Pradhan and Dasgupta 1983). Table 1 summarizes differences in morphometric characters of the three species of *Apolocystis*. Trophozoites of the present species are considerably smaller than the two previously described species. The gametocysts are ovoidal in the new species described, but gametocysts shape was not reported in the previously described species. Oocysts of *A. chotonagpurensis* differ from those of previously described species in having sharply pointed end instead of slightly flattened ends. The oocysts are biconical in the present species. The *Apolocystis* species treated in the present study is therefore considered new. The comparisons with previously described species are provided in Table 1.

Stomatophora janovyi sp. n. (Figs 5-12, Table 2)

Phylum: Apicomplexa Levine, 1977
Order: Eugregarinida Leger, 1900
Family: Monocystinae Bütschli, 1882
Subfamily: Monocystinae Bhatia, 1930
With the characters of genus *Stomatophora* Drzhevetskii, 1907, as given by Levine (1988); gamonts spherical or ovoid; sucker petaloid, with radiating sides, oocysts biconical with a flattened button at each end, attached to each other end to end in long chains inside
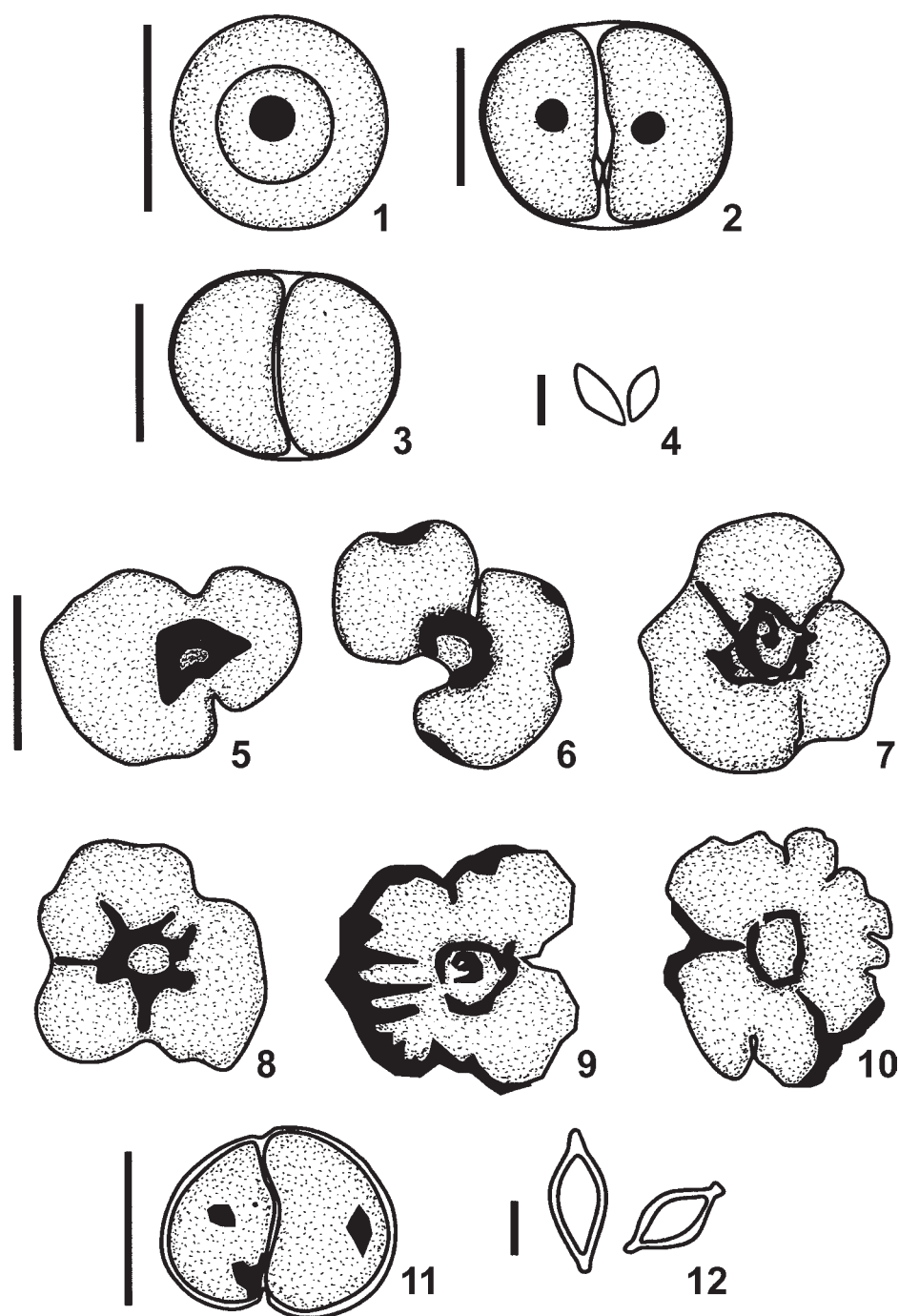
Figs 1-4. Camera lucida drawings of different stages of *Apolocystis chotonagpurensis* sp. n. **1** - mature trophozoite; **2** - early gametocyst; **3** - late gametocyst; **4** - oocysts. Scale bars 5 μm (4); 50 μm (1-3).
Figs 5-12. Camera lucida drawings of different stages of *Stomatophora janovyi* sp. n. **5**-**8** - young trophozoites; **9**-**10** - mature trophozoites; **11** - gametocyst; **12** - oocyst. Scale bars 5 μm (12); 50 μm (5-11).

Table 1. Comparison of Indian species of *Apolocystis* from the seminal vesicles of the earthworm host *Amynthas robusta*. ni - not indicated. All measurements in microns (μm)

Parasite species/Character	*A. akaryoseminiferus*	*A. monokaryoseminiferus*	*A. chotonagpurensis* sp. n.
Trophozoite shape	spherical, solitary	spherical, solitary	spheroidal or ovoid
Trophozoite size	54-100	61-115	42-83
Gametocyst shape	ni	ni	ovoidal
Gametocyst size	ni	ni	71-99 × 46-83
Oocyst shape	biconical, ends slightly flattened	biconical, ends slightly flattened	biconical, ends sharply pointed
Oocyst size	ni	ni	6.5-7 × 3.5-4
Reference	Pradhan and Dasgupta (1983)	Pradhan and Dasgupta (1983)	present paper

Table 2. Comparison of Indian species of *Stomatophora* from the seminal vesicles of the earthworm hosts. ni - not indicated. All measurements in microns (μm)

Parasite species/Character	*S. bahli*	*S. globa*	*S. pedongensis*	*S. majumdari*	*S. janovyi* sp. n.
Trophozoite shape	discoidal; flattened between poles	spheroidal; flattened and compressed between poles	discoidal; flattened between poles	spheroidal; flattened and compressed between poles	discoidal; flattened and flower like
Trophozoite size	43-82	31-97	50-94	40-63	80-85
Gametocyst shape	ni	ni	spheroidal	ovoidal	ovoidal
Gametocyst size	ni	ni	90-119	90-130 × 90-115	52-66 × 33-39
Oocyst shape	ni	ni	navicular	navicular	navicular
Oocyst size	ni	ni	in	8 × 5	10-12 × 6-7
Host	*Amynthas diffringens*	*Pheretima alexendri*	*Amynthas diffringens*	*Metaphire posthuma*	*Amynthas hawayanus*
Soil type	podzol	podzol	podzol	alluvial	podzol
Elevation (m)	ni	ni	ni	ni	3323
References	Pradahan and Dasgupata (1980)	Pradahan and Dasgupata (1980)	Pradahan and Dasgupata (1980)	Bandyopadhyay *et al*. (2001)	present paper

gametocyst. Trophozoites solitary, flattened, flower-like and petaloid in appearance; diameter range from 80-85 (82.0 ± 2.0). Pellicle thin. Mucron is ring-like, centrally located, with some vacuolated areas range in size from 17-23 (20 ± 2.0). Nucleus round to slightly ellipsoidal; diameters from 9.0-14.0 (11.0 ± 3.0) typically located close to mucron; Cytoplasm densely granulated; epicyteal striations not distinct but in mature stages observed to extend from periphery towards mucron. Ectosarc very thin, without external processes. Gametocysts ovoidal; lengths range from 52.0-66.0 (60.0 ± 4); widths from

33.0-39 (37.0 ± 3.0). Each gametocyst encloses two gametes. Oocysts shape navicular, bluntly rounded; lengths range from 10.0-12.0 (12.0 ± 0.5); widths from 6.0-7.0 (6.0 ± 0.6).

Taxonomic summary

Type material: *Stomatophora janovyi* sp. n
Type host: *Amynthas hawayanus* Rosa, 1891
Symbiotype: Host AH - 03/22/2002 deposited in the Museum of the Department of Zoology, University of Kalyani, Kalyani 741235, West Bengal, India.

Site of infection: Seminal vesicles.

Type locality: Singamari, Darjeeling, W. Bengal, (Lat. 27°N, Lon. 88 °E).

Elevation: 3323 m above mean sea level.

Prevalance: 6 out of 22 (27%).

Type material: Syntypes on a single slide no. SJ/12 /02 deposited in the Zoological Survey of India (ZSI), Calcutta - 700016 (Catalogue No. 2408).

Etymology: The specific epithet "*janovyi*" is given after the name of Prof. John Janovy, Jr., of University of Nebraska, Lincoln, USA, for his outstanding contribution in the field of Apicomplexan biology.

Remarks: A new species of *Stomatophora* is described from an earthworm collected at high altitudes in podzol soil in Singamari. Pradhan and Dasgupta (1980) described three gregarine species, namely *S. bhali, S. pedogensis* in *Amynthas diffringens* and *S. globa* in *Metaphire alexandri* from the same locality, elevation and habitat; Singamari, Darjeeling, West Bengal, India. Bandyopadhyay and co-workers (2001) later reported *S. majumdari* from *M. posthuma* from an alluvial soil. Three of the five earthworm species belong to the genus that typically lives in podzol soil. The remaining two belong to the genus *Metaphire*, species of which live in either podzol soils (hilly areas) or alluvial soils (plains). Table 2 summarizes the characteristic features that distinguish the present form from the other mentioned species. Measurements of the trophozoites of *S. janovyi* are distinctly different from the four previously described species. The mucron of *S. janovyi* is larger in diameter in comparison with the smaller mucron in *S. bahli.* The diameter of the nucleus in *S. janovyi* is smaller than in *S. bahli, S. globa* and *S. pedongensis* but larger than the nucleus of *S. majumdari* and it contains some vacuolated areas. In *S. janovyi* the gametocyst is ovoid and smaller than in *S. pedongensis* and *S. majumdari.* Oocyst is similar in shape in all five species but the measurements are distinctly longer than in both *S. pedongensis* and *S. majumdari.* Based on distinct differences in comparison with species previously reported from the Indian oligocheates, a new species, *S. janovyi* is designated and named after the name of Prof. John Janovy Jr. of Nebraska University of U.S.A. The comparisons with previously described species are provided in Table 2.

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ACTA
PROTOZOOLOGICA

Redescription of *Difflugia tuberspinifera* Hu, Shen, Gu *et* Gong, 1997 (Protozoa: Rhizopoda: Arcellinida: Difflugiidae) from China

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Summary. The freshwater testate amoeba *Difflugia tuberspinifera* Hu *et al*. 1997 collected from pond and lake in China, is investigated by light and scanning electron microscopy. This little known taxon is redescribed and its morphology, biometry and ecology are supplied. After carefully comparison with other six similar species including *Difflugia bartoši* Štěpánek, *D. corona* Wallich, *D. corona cashi* Deflandre, *D. corona tuberculata* Vucetich, *D. muriformis* Gauthier-Liévre *et* Thomas and *Netzelia tuberculata* (Wallich) Netzal we believe that the sub-spherical to spherical shell, the mulberry-shaped appearance, the 7-10 apertural tooth-like structures, the short collar and the conical spines numbering from 4 to 8 at the upper equatorial region in *D. tuberspinifera* set it apart from other species. Besides, statistical analysis indicates that *D. tuberspinifera* is a size-monomorphic species characterized by a main-size class and a small size range and the shell height is significant correlated with other morphometric characters at $p < 0.05$ excepting the number of aperture tooth-like structures and the number of spines. Moreover, *D. tuberspinifera* inhabits not only lotic but also lentic environment.

Key words: biometry, *Difflugia tuberspinifera*, ecology, morphology, Testacea.

INTRODUCTION

The testate amoeba genus *Difflugia* established by Leclerc in 1815 is the most extensive one regarding the number of taxa (Cash and Hopkinson 1909, Bartoš 1954, Bovee 1985, Meisterfeld 2000). The taxonomy of this genus is based mainly on differences in shape and size of their shells. As the shell is often opaque, cytoplasmic characters are rarely used. Small differences in shell size, shape, or composition have been sufficient for many authors to describe more than 300

species and about 200 subspecies, varieties, or forms with little regard to the value of the characters used, the previous literature, or the rules of nomenclature. Many of these descriptions are inadequate by modern standards and therefore the determination to species level is extremely difficult, even for the specialist (Meisterfeld 2000). *Difflugia tuberspinifera* Hu, Shen, Gu *et* Gong, 1997 is one of poorly studied species of the genus *Difflugia*.

Difflugia tuberspinifera was firstly observed and described in Wujiang River, Guizhou province, China (Hu *et al*. 1997). The empty shells were observed only. According to the original description, "The shell is spherical, with 5 spines at the equatorial region of the body. Aperture: round, petal shape, with 8 dentate lobes. Along

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the margin of the aperture, small sand granules were arranged in a ring regularly. There is a short neck between the aperture and the body of shell. The surface of shell is not smooth, and having many regular blunt protuberances. The sizes of the shell are: diameter of test 115-120 μm, diameter of aperture 57.6 μm, length of spine 38 μm, length of neck 9.6 μm". Unfortunately, no data about the detailed morphometrical characterization and the pseudopodia was presented. During our investigation on the testate amoebae of the Changjiang Valley we have observed an abundant material of living specimens of *D. tuberspinifera* with a high population density. This allows us to make more detailed studies on their morphology, on the variation of shell sizes and on the pseudopodia. The results of our studies are the subject of the present paper.

MATERIALS AND METHODS

Difflugia tuberspinifera was collected from the pond in Xinzhou, Hubei province, China in August 2002 and Mulan Lake (oligotrophic lake, area 105 km², average depth 18 m, surface water temperature 34°C, and pH 6.0), Hubei province, China in July 2003. Both Xinzhou and Mulan Lake are located in the same climate region (the semitropical humid monsoon climate). The annual mean air temperature of both Xinzhou and Mulan Lake is 16.0°C. The annual mean precipitation is 1250 mm in Xinzhou and is 1100 mm in Mulan Lake. The materials were obtained from the surface water by horizontal hauls of a plankton net made of No. 25 silk bolting cloth (mesh 64 μm in diameter) for about 10 min. Next, they were put in plastic bottles. Examination was immediately made using Leitz optical microscope. Observations on the outline and fine structure of the body were made from living specimens. Some photomicrographs were taken under bright field illumination. After observation of the specimens, they were fixed with Bouin's fluids.

For scanning electron microscopy specimens were first cleaned individually by transference through distilled water using a single-hair brush. Next, they were placed on a cover slip previously cleaned with lint-free tissue. The shells were exposed in air at room temperature until they dried completely. Then the cover slip was mounted on an aluminium-stub using a double-sided adhesive tape and coated with a thin layer gold in Eiko IB-3 Ion Coater before observing. The photographs were obtained from a Scanning Electron Microscopy (X-650 HITACHI, Japan) operating at 20kV.

Nine morphometric characters were measured in our study, namely shell height (character 1 in Fig. 2); shell diameter (character 2 in Fig. 1); aperture diameter (character 3 in Fig. 1); spine length (character 4 in Fig. 2); collar height (character 5 in Fig. 2); rear end length (character 6 in Fig. 2), that is, the distance between the base of conical spine and the shell end; foreside length (character 7 in Fig. 2), that is, the distance between the base of conical spine and the collar; number of aperture tooth-like structures (character 8); number of conical spines (character 9). All measurements were made at middle magnifi-

cation (320×) using an ocular micrometer. Statistics were performed using the computer program STATISTICA, version 6.0.

RESULTS

Morphology

The shell has a sub-spherical to spherical form, composed of fine sand granules, flattish pieces of quartz and muddy particles (Figs 3-14). In apertural view, the shell is circular, furnished with a variable number of conical spines, varying from 4 to 8, usually 5-6. The aperture is terminal, circular, its border denticulated to crenulated with a variable number of small, but perfectly regular tooth-like structures, numbering from 7 to 10, usually 8-9, without any accompaniment of larger quartz grains (Figs 3, 9, 11, 13). In lateral view, the aperture shows a short collar, and the position of the conical spines at the upper equatorial region (Figs 5, 6, 14).

The surface of shell is not smooth and has many regular blunt protuberances. In other words, the shell has a mulberry-shaped appearance (Figs 3-5, 13, 14). However, the shell walls are even in thickness. Accordingly, internal walls of the shell are sunken (Figs 6, 7). The protuberance is composed of small sand granules and flattish pieces of quartz (Fig. 8). No cement structures were recognizable in the scanning electron microscope.

The shell is yellowish to brown, the pseudopodia long, colourless and rather thin, generally 3 to 7 (Figs 10, 14). As the shell is opaque, cytoplasmic characters were not observed.

Biometry

Table 1 shows the morphometric characterization of *Difflugia tuberspinifera* according to our studies. The values are represented together with those reported in the original description (Table 2). Despite the fact that shell measurements of aperture diameter, spine length, collar height, rear end length and foreside length have high variability (CV between 7.41 and 22.10), shell height and shell diameter are fairly constant and have low variability (CV between 4.47 and 5.36) (Table 1). Numbers of aperture tooth-like structures and conical spines both have low standard error of the mean (0.07-0.11), so does collar height (0.21-0.25) (Table 1).

Size frequency distribution analysis indicates that *D. tuberspinifera* has a main-size class and a small size range. All measured individuals have a shell height
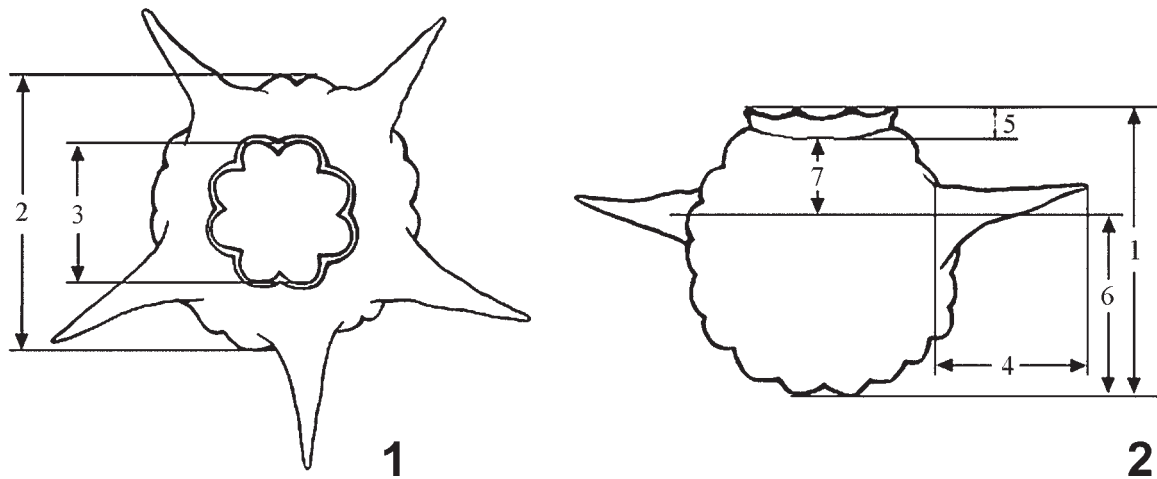
Figs 1, 2. Shell outline and position of measured axis used in this study. 1 - shell height; 2 - shell diameter; 3 - aperture diameter; 4 - spine length; 5 - collar height; 6 - rear end length; 7 - foreside length.

Table 1. Morphometric characteristics of *Difflugia tuberspinifera* from pond of Xinzhou (first line for each character) and Mulan Lake (second line for each character).

| Characters[1]	$\overline{X}$	M	SD	SE	CV	Min	Max	n
Shell height (1)	111.4	112.0	5.71	0.81	5.13	94.0	129.0	50
	114.8	115.0	6.15	0.61	5.36	100.0	128.0	102
Shell diameter (2)	109.4	110.5	5.33	0.75	4.88	94.0	118.0	50
	109.5	110.0	4.90	0.49	4.47	97.0	119.0	102
Aperture diameter (3)	49.8	49.0	5.02	0.71	10.09	39.0	64.0	50
	53.0	53.0	3.93	0.39	7.41	45.0	63.0	102
Spine length (4)	28.7	28.0	6.35	0.90	22.10	15.0	42.0	50
	48.2	47.0	8.79	0.87	18.24	23.0	77.0	102
Collar height (5)	10.6	11.0	1.74	0.25	16.31	8.0	15.0	50
	11.6	11.5	2.15	0.21	18.51	6.0	18.0	102
Rear end length (6)	-	-	-	-	-	-	-	-
	71.1	72	6.38	0.63	8.98	53.0	86.0	102
Foreside length (7)	-	-	-	-	-	-	-	-
	32.0	33.0	6.79	0.67	21.20	18.0	57.0	102
Number of aperture tooth-like structures (8)	8.4	8.0	0.78	0.11	9.32	7	10	50
	8.5	8.0	0.74	0.07	8.72	7	10	102
Number of conical spines (9)	5.6	6.0	0.61	0.09	10.82	4	7	50
	5.7	6.0	0.68	0.07	12.00	4	8	102

[1]Numbers 1-9 in parenthesis designate features as shown in Figs1 and 2. Data based on randomly selected and character 4 is from only a spine length randomly selected in each shell. Measurements in μm. CV - coefficient of variation in %; M - median; Max - maximum; Min - minimum; n - number of individuals investigated; SD - standard deviation; SE- standard error of mean; $\overline{X}$ - arithmetic mean.

94-129 μm and more than half of them (57%) are within the limits of 111-120 μm. The frequency analysis of the other morphometric characterization (shell diameter, aperture diameter, spine length, collar height, rear end length and foreside length) shows almost the same results. The number of aperture tooth-like structures varies from 7 to 10. In 84% of the measured shells, this number is restricted to 8-9. All measured individuals have conical spines numbering between 4 and 8, but 53% of them are within the limits of 6 and 91% within the ranges of 5-6.

The information in Table 3 illustrates that SH (shell height) is well positively correlated with SD (shell diameter), AD (aperture diameter), SL (spine length),
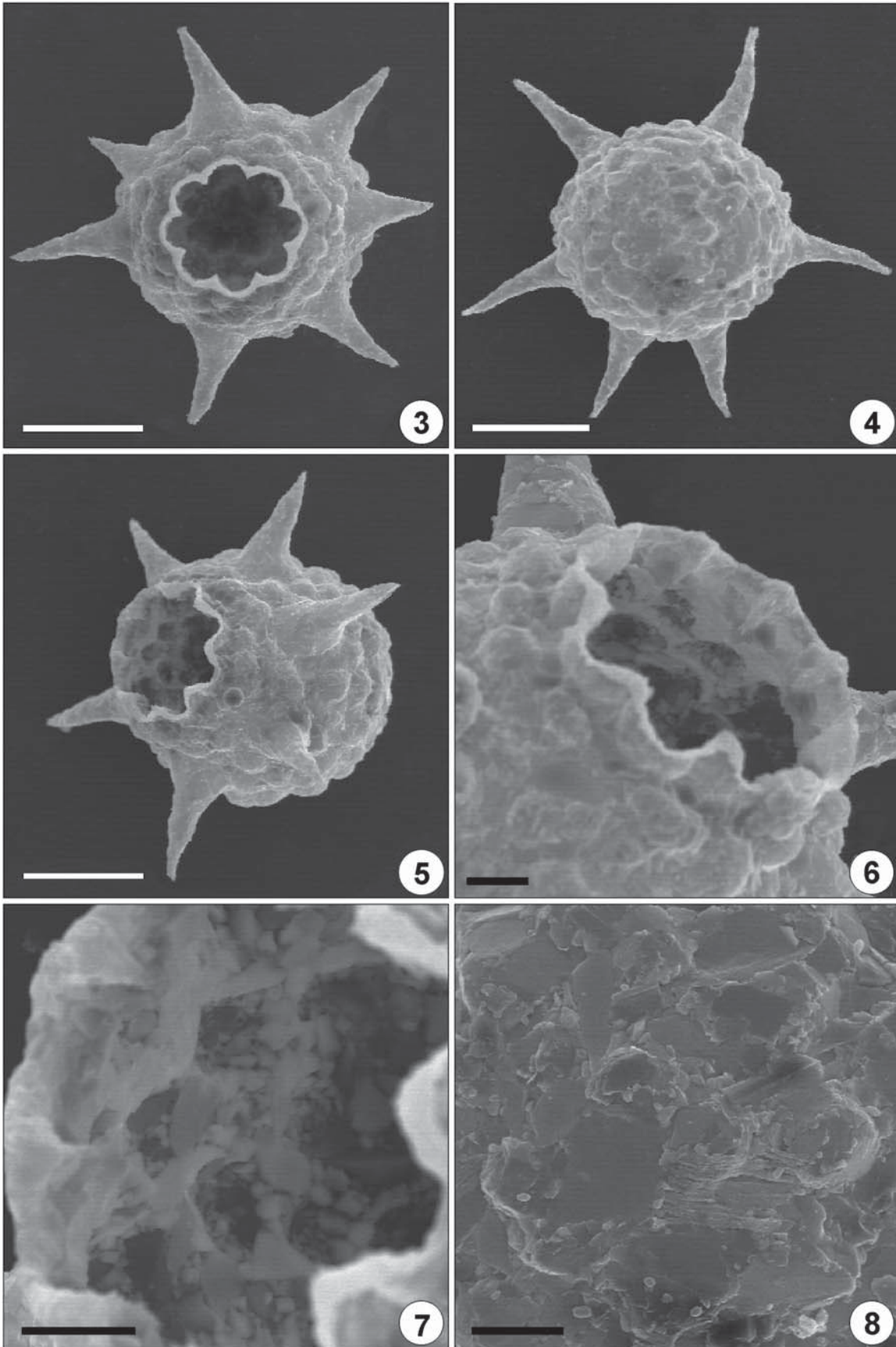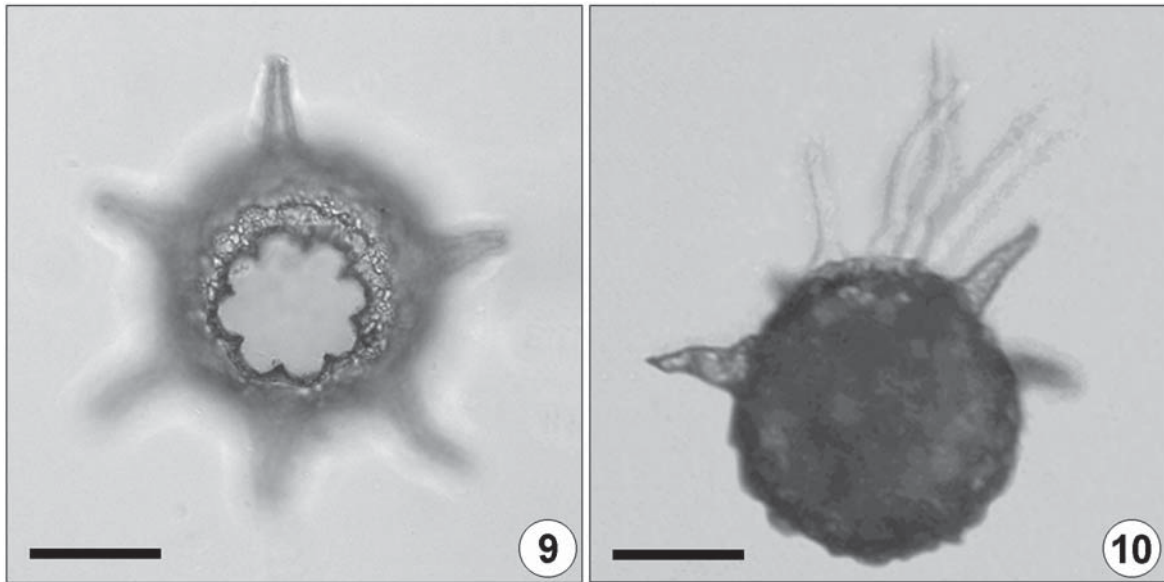
Figs 9, 10. LM photographs of *Difflugia tuberspinifera*. **9** - apertural view, showing shell, aperture and spine shape; **10** - lateral view, showing pseudopodia. Scale bars 50 μm.

CH (collar height), RL (rear end length) and FL (foreside length), they are significant correlation at $p < 0.001$, $p < 0.05$, $p < 0.001$, $p < 0.001$, $p < 0.001$ and $p < 0.001$ respectively. The AD (aperture diameter) is positive correlation with SL (spine length) at $p < 0.01$ (Table 3). The given Table 3 also shows that RL (rear end length) is positively correlated with SD (shell diameter) and CH (collar height) at $p < 0.001$ and $p < 0.05$ respectively, but highly negatively correlated with FL (foreside length) at $p < 0.001$.

DISCUSSION

Morphology, biometry and ecology

The ideal individual of *Difflugia tuberspinifera* from China is constructed from median values of all characters in Figs 13 and 14. In terms of its general appearance, especially the shape and size of shell with a denticular collar and tooth-like structures, conical spines and blunt protuberances, the pond of Xinzhou and Mulan Lake populations both largely correspond with the original description (Figs 11, 12). However, in the original description, only a few empty shells (no observation of pseudopodia) with 8 aperture tooth-like structures and 5 spines were investigated. Furthermore, the variation of the aperture tooth-like structures and the number of conical spines was not mentioned at all (Hu *et al*. 1997). By contrast, in both populations of the pond of Xinzhou and Mulan Lake, there are variable numbers of aperture tooth-like structures going from 7 to 10, and of the conical spines varying from 4 to 8 (Table 1). At the same time, more detailed characters are supplied: the shell is yellowish to brown, opaque; the pseudopodia colourless, long and rather thin, generally 3 to 7.

The variability of shell size in some testate amoeba is high and the biggest individuals can be as twice as large as the smallest in the same taxon (Foissner and Korganova 1995). All morphometric characters in the population from Mulan Lake are a little larger than those in the population from the pond of Xinzhou, especially regarding the aperture diameter and spine length (Tables 1, 2). However, shell size of *D. tuberspinifera* is relatively constant. The regularity of shell height in *D. tuberspinifera* is such that over 93% of all measured individuals (n=152) fall within a range of ±10% of the average value (114 μm). In addition, the shell height is

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Figs 3-8. SEM photographs of *Difflugia tuberspinifera*. **3** - apertural view, showing shell, aperture and spine shape; **4** - bottom view, showing shell, spine and protuberances shape; **5** - lateral view, showing shell, collar and spine shape; **6** - showing aperture, collar and protuberances; **7** - showing sunken internal walls; **8** - showing the protuberances. Scale bars 50 μm (3-5); 10 μm (6-8).

Table 2. Morphometric[1] comparisons of different populations of *Difflugia tuberspinifera*.

References	Shell height (1)	Shell diameter (2)	Aperture diameter (3)	Spine length (4)	Collar height (5)	Number of aperture tooth-like structures	Number of conical spines
Hu *et al*. 1997 (n=?)	115-120	115-120	57.6	38	9.6	8	5
Present data, Xinzhou pond (n=50)	94-129	94-118	39-64	15-42	8-15	7-10	4-7
Present data, Mulan Lake (n=102)	100-128	97-119	45-63	23-77	6-18	7-10	4-8

[1]Numbers 1-5 in parenthesis designate features as shown in Figs 1 and 2. Measurements in μm.

Table 3. Correlation coefficient between morphometric characteristics in *Difflugia tuberspinifera*. SH - shell height; SD - shell diameter; AD - aperture diameter; SL - spine length; CH - collar height; RL - rear end length; FL - foreside length; NT - number of aperture tooth-like structures; NS - number of conical spines (see Figs 1 and 2).

	SH	SD	AD	SL	CH	RL	FL	NT	NS
SH	-								
SD	0.5312***	-							
AD	0.1676*	0.0290	-						
SL	0.3026***	0.1245	0.2614**	-					
CH	0.3841***	0.1556	0.1466	0.0971	-				
RL	0.4189***	0.3629***	0.0141	0.1146	0.2190*	-			
FL	0.3902***	0.1758	-0.0026	-0.0562	-0.1739	-0.6298***	-		
NT	0.0031	0.0569	0.0544	0.0606	-0.1121	0.0460	-0.1720	-	
NS	0.0488	0.1227	-0.0697	0.0577	-0.0611	0.0525	-0.0479	0.0078	-

Significant relationship *p < 0.05, **p < 0.01, ***p < 0.001

very important because it is significant correlated with other morphometric characters at p < 0.05 with the exception of the number of aperture tooth-like structures and the number of spines (Table 3). According to Hu *et al*. (1997), the shell is furnished with 5 spines at the equatorial region and the number of aperture tooth-like structures is 8. However, in the populations from pond of Xinzhou and Mulan Lake, they both have a variable number of conical spines varying from 4 to 7 (8) and 91% within the ranges of 5-6. Furthermore, the statistical analysis indicates that the spines are not situated in the equatorial region of the shell but in the upper equatorial region. Similarly, the populations from the pond of Xinzhou and the Mulan Lake have a variable number of the aperture tooth-like structures ranging from 7 to 10 with 84% inside the limits of 8-9. Nevertheless, the number of aperture tooth-like structures and the

number of conical spines are both quite constant, because they do not follow the change in shell size (for example: shell height), but vary randomly in a limited range (Tables 1, 3). These results have led us to a conclusion that *D. tuberspinifera* is a size-monomorphic species characterized by a main-size class and a small size range.

Since the firstly reported of *D. tuberspinifera* there have been more data about the ecology of this species. Hu *et al*. (1997) pointed out that this species occurred in Wujiang River of Guizhou, China, with water temperature 16°C and pH 6.7. It is evident that it exists in lotic environment. However, our investigation shows that *D. tuberspinifera* inhabits also lentic environments, for example the pond of Xinzhou (neither water temperature nor pH detected) and Mulan Lake (water temperature 34°C and pH 6.0). In these both habitats the quite high

Table 4. Comparisons of *Difflugia tuberspinifera* with six similar Testacea species. All measurements in µm. MS - mulberry-shaped; SQ - sand granules and quartz particles; TS - tooth-like structures; ? - data not available; Ab - character absent

Character	*Difflugia tuberspinifera*	*D. bartoši*	*D. corona*	*D. corona*	*D. corona cashi*	*D. corona tuberculata*	*D. muriformis*	*Netzelia (Difflugia) tuberculata*
Shell height	94-129	245-385	126-190	216-225	180-230	120-160	115-150	91-150
Shell diameter	94-118	192-297	126-177	240-260	160-180	100-140	110-138	85-132
Aperture diameter	39-64	70	51-86	160-173	80-90	50-68	31-50	30-40
Collar	6-18	Ab	present	present	present	Present	3-7	>7
Neck	Ab	35-70 × 75	Ab	Ab	Ab	Ab	Ab	Ab
Shell shape	sub-spherical to spherical	spherical	spherical or ovoid	spherical	sub-spherical	spherical	sub-spherical to spherical	sub-spherical
Shell color	yellow to brown and opaque	opaque	brown and opaque	opaque	opaque	?	yellow to brown and transparent	brown and transparent
Shell wall	MS	irregular SQ	smooth SQ	irregular SQ	irregular SQ	MS	MS	MS
Aperture	circular	circular	circular	circular	circular	circular	3-5 lobes	hexagonal
Number of lobes	7-10	Ab	10-20	?	12-20	?	3-5	6-7
Collar or neck shape	TS	circular	TS	TS	TS	TS	TS	hexagonal with sinuous lobes
Number of spines	4-8	4-8	2-8	4-6	variable	variable	Ab	Ab
Spine position	regularly at the upper equatorial region	regularly around mid-body	randomly at the aboral region	regularly behind mid-body	irregularly behind mid-body	at the aboral region	Ab	Ab
Biometry	monomorphic	?	monomorphic	monomorphic	monomorphic	monomorphic	monomorphic	monomorphic
Building material	?	exogenesis	exogenesis	exogenesis	exogenesis	?	exogenesis	endogenesis
Sample location	China	Slovakia	Yugoslavia and G. Britain	Venezuela	G. Britain and Venezuela	Argentina	Africa	Africa
Reference	present study	Bartoš 1954	Ogden and Hedley 1980, Ogden and Živković 1983	Deflandre 1926	Cash and Hopkinson 1909, Deflandre 1926	Vucetich 1973	Gauthier-Liévre and Thomas 1958	Gauthier-Liévre and Thomas 1958, Ogden and Meisterfeld 1989
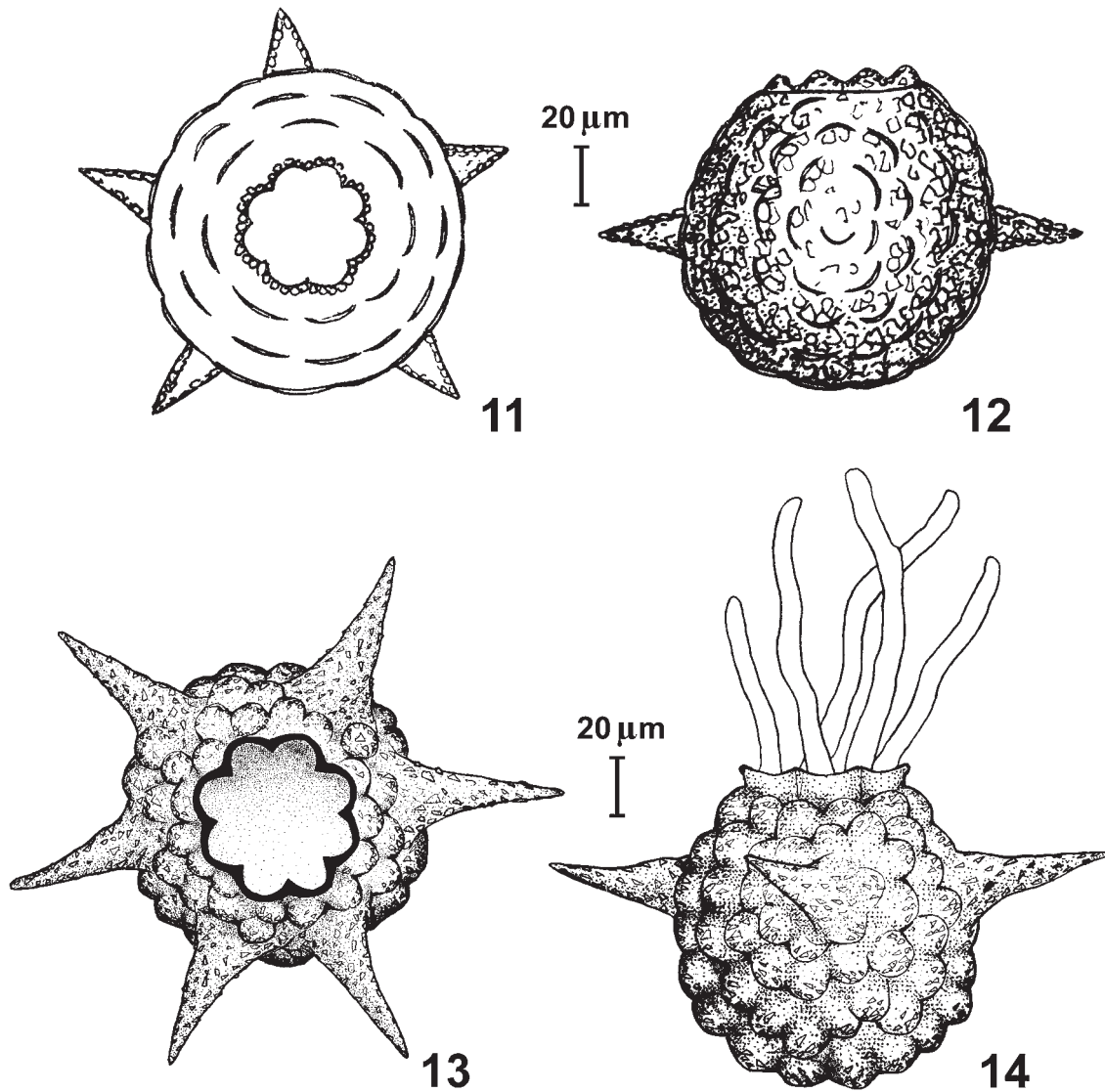
Figs 11, 12. Figures of *Difflugia tuberspinifera* copied from the original literature (Hu *et al*. 1997).
Figs 13, 14. Ideal individual of *Difflugia tuberspinifera*, constructed from median values of all specimens.

population densities have been observed. Furthermore, *D. tuberspinifera* was a dominant species in many of the investigated samples and its density was by a long way higher than those of other testate amoebae. Probably *D. tuberspinifera* is also a widespread freshwater testate amoeba in the Changjiang Valley, as well as the majority of the known species of the genus *Difflugia*.

Comparison with similar species

Considering the morphology, there are some taxa similar to *Difflugia tuberspinifera*. At least six testate amoebae species, namely *D. bartoši* Štěpánek, 1952 (Bartoš 1954); *D. corona* Wallich, 1864 (Deflandre

1926, Ogden and Hedley 1980, Ogden and Živković 1983); *D. corona* var. *cashi* Deflandre, 1926 (Cash and Hopkinson 1909, Deflandre 1926); *D. corona* f. *tuberculata* Vucetich (Vucetich 1973); *D. muriformis* Gauthier-Lievre *et* Thomas, 1958 (Gauthier-Liévre and Thomas 1958) and *Netzelia (Difflugia) tuberculata* (Wallich) Netzel, 1983 (Gauthier-Liévre and Thomas 1958, Netzel 1983, Ogden and Meisterfeld 1989) should be compared with *D. tuberspinifera* (Table 4). None of these six species have the 7-10 apertural tooth-like structures and the conical spines numbering from 4 to 8 at the upper equatorial region. *D. tuberculata* was transferred to *Netzelia* by Netzel (1983) because it

differs from other *Difflugia* species in its ability to endogenously synthesize the building material. *D. bartoši* can be distinguished from other species by its long neck with a round aperture without dentate lobes and conical spines of 1 or 2 cycles regularly around the body. Further, the shell size in *D. bartoši* is much larger than any other six taxa. *D. corona* differs from other species in its 10-20 apertural tooth-like structures and a variable number of conical spines behind mid-body. *D. corona cashi* resembles *D. corona*, but mainly differs in dimension, the sub-spherical shell (shell height > shell diameter) and the irregularly arranged spines. *D. corona tuberculata* is perhaps the most similar organism to the *D. tuberspinifera*. Nevertheless, the spines in *D. corona tuberculata* are situated in the aboral region and they are short in length. In view of the shell wall appearance, *D. muriformis* and *N. tuberculata* are both close to *D. tuberspinifera* in having mulberry-shaped protuberances sometimes masked by the sand-grains of irregular size. However, *D. muriformis* and *N. tuberculata* both lack the spine. Further, aperture has 3-5 lobes in *D. muriformis* and is hexagonal with sinuous lobes in *N. tuberculata*. To summarize: the sub-spherical to spherical shell outline, the mulberry-shaped appearance, the 7-10 apertural tooth-like structures, the short collar, the conical spines numbering from 4 to 8 at the upper equatorial region set *D. tuberspinifera* apart from all other *Difflugia* species.

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