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

Sabine AGATHA
University of Salzburg, Institute for Zoology, Salzburg, Austria

Summary. Currently, gene sequence genealogies of the Oligotrichida 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 Oligotrichida, Halteria, Oligotrichia, Oligotrichida, and Choreotrichida. The main synapomorphies of the Oligotrichida 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 Oligotrichida is suggested, including all sufficiently known families and genera.

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

INTRODUCTION

Since the Oligotrichida 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 Oligotrichida on suprafamilial level, using morphologic and ontogenetic features. According to their genealogy and revised classification, the Halteriia are an adelphaxon 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 strombidids (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
RESULTS AND DISCUSSION

Characters, character states, and convergences considered

The Oligotrichia share several features with the Hypotrichia: 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 Hypotrichia, 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, Rufolo 1976, Petz and Foissner 1992); even the cirri of some stichotrichs show a dikinetidal composition (Wimsberger-Aeschl et al. 1989). In contrast to the Hypotrichia and Halteria, most Oligotrichida have only a single longitudinal kinety,
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),

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

<table>
<thead>
<tr>
<th>Character states</th>
<th>Apomorphy</th>
<th>Plesiomorphy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paroral lacks (coded 1), paroral and endoral lack (coded 2)</td>
<td></td>
<td>Endoral and paroral (coded 0)</td>
</tr>
<tr>
<td>Membranellar zone apical (coded 1)</td>
<td>Membranellar zone ventral (coded 0)</td>
<td></td>
</tr>
<tr>
<td>Membranellar zone closed (coded 1)</td>
<td>Membranellar zone C-shaped (coded 0)</td>
<td></td>
</tr>
<tr>
<td>Without ventral membranelles (coded 1)</td>
<td>Ventral membranelles (coded 0)</td>
<td></td>
</tr>
<tr>
<td>Cyrtos-like pharyngeal fibres (coded 1)</td>
<td>Common pharyngeal fibres (coded 0)</td>
<td></td>
</tr>
<tr>
<td>Reduction of somatic ciliature to ≤2 kineties (coded 1)</td>
<td>Comprehensive somatic ciliature (coded 0)</td>
<td></td>
</tr>
<tr>
<td>Dextral spiral of somatic kineties (coded 1)</td>
<td>Longitudinal somatic kineties (coded 0)</td>
<td></td>
</tr>
<tr>
<td>Ventr al kinety longitudinal (coded 1)</td>
<td>Ventr al kinety dextrally spiralled (coded 0)</td>
<td></td>
</tr>
<tr>
<td>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)</td>
<td>Girdle kinety dextrally spiralled (coded 0)</td>
<td></td>
</tr>
<tr>
<td>Somatic cilia clavate (coded 1)</td>
<td>Somatic cilia rod-shaped or fusiform (coded 0)</td>
<td></td>
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<tr>
<td>Somatic kinetids bare (coded 1)</td>
<td>Somatic kinetids ciliated (coded 0)</td>
<td></td>
</tr>
<tr>
<td>Somatic cilia arranged in bristle complexes (coded 1)</td>
<td>Somatic cilia arranged in ordinary rows (coded 0)</td>
<td></td>
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<tr>
<td>Kinetodesmal fibre of somatic kinetids lacking or transient (coded 1)</td>
<td>Kinetodesmal fibres of somatic kinetids permanent (coded 0)</td>
<td></td>
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<tr>
<td>Polysaccharidic cortical platelets (coded 1)</td>
<td>No or other cortical platelets (coded 0)</td>
<td></td>
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<tr>
<td>Perilemma (coded 1)</td>
<td>Without perilemma (coded 0)</td>
<td></td>
</tr>
<tr>
<td>Oligotrichid extrusomes (trichites; coded 1)</td>
<td>No or other extrusomes (coded 0)</td>
<td></td>
</tr>
<tr>
<td>Tail (coded 1)</td>
<td>Without tail (coded 0)</td>
<td></td>
</tr>
<tr>
<td>Enantiotropy (coded 1)</td>
<td>Homocotropy (coded 0)</td>
<td></td>
</tr>
<tr>
<td>Stomatogenesis hypoapokinetal in temporary tube (coded 1) or pouch (coded 2) or permanent neoformation organelle (coded 3)</td>
<td>Stomatogenesis epiapokinetal (coded 0)</td>
<td></td>
</tr>
<tr>
<td>Posterior end of oral primordium performs clockwise rotation (coded 1)</td>
<td>Anterior end of oral primordium performs anticlockwise rotation (coded 0)</td>
<td></td>
</tr>
<tr>
<td>Undulating membranes originate de novo (coded 1)</td>
<td>Undulating membranes originate from oral primordium or cirral anlagen (coded 0)</td>
<td></td>
</tr>
<tr>
<td>Entire somatic ciliature originates de novo (coded 1)</td>
<td>At least parts of somatic ciliature originate by intrakinetal proliferation (coded 0)</td>
<td></td>
</tr>
<tr>
<td>Extensive reorganization of somatic ciliature (coded 1)</td>
<td>No or indistinct (intrakinetal) reorganization of somatic ciliature (coded 0)</td>
<td></td>
</tr>
</tbody>
</table>
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 Hypotrichhea (hypotrichs and stichotrichs) and Oligotrichhea 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 Hypotrichhea. In the Halteriida 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 Hypotrichhea, Halteriida, 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 Oligotrichida 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 Hypotrichhea and Oligotrichida is sup-
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 Hypotrichida, Halteriida, 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 Oligotrichida.

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**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 *Prosostrombidium* (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.
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.

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 strobilata 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 Oligotrichidea and Stichotrichida.

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

Figs 3a-g. Evolution of the ciliary patterns in the Oligotrichida (from Agatha 2004). a - ancestor with many longitudinal somatic kineties, whose dikinetids bear a distinct cilium only at each anterior basal body (see detail); b - reduction in kinety number to two. The clockwise torsion of the proximal end of the membranelar zone and the cell proper caused the dextrally spiralled pattern of the girdle and ventral kinety; c - the ventral kinety orientated longitudinally; d - the right portion of the girdle kinety migrated posteriorly; both kinety ends are thus close to the cell’s posterior on ventral side; e - the posterior portion of the girdle kinety curved anteriorly and is thus inversely orientated to the parallel ventral kinety; f - the left portion of the dextrally spiralled girdle kinety migrated anteriorly, causing a horizontal orientation; g - the right portion of the horizontal girdle kinety spiralled sinistrally to the rear end. The number of whorls performed by the girdle kinety is possibly positively correlated with the cell size because Tontonia turbinata with a length of 50-80 µm after protargol impregnation has ~ 1.5 whorls, while T. grandis with a size of up to 180 µm has 3-3.5 whorls (Song and Bradbury 1998, Suzuki and Han 2000, Agatha et al. 2004). Arrows indicate orientation of kineties. GK - girdle kinety, VK - ventral kinety.
tintinnids, and strobilidiids (own observ.; Laval-Peuto and Barria de Cao 1987, Wimsberger 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 Oligotrichia, 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 Halteria and Oligotrichia (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),...
S. Agatha

Table 3. Revised classification of the Oligotrichia (for further explanations, see “Classification of the Oligotrichia and diagnosis of some taxa”).

<table>
<thead>
<tr>
<th>Superclass</th>
<th>Class Hypotrichia Stein, 1859</th>
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<tbody>
<tr>
<td></td>
<td>Order Hypotrictichida Stein, 1859</td>
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<tr>
<td></td>
<td>Order Stichotrichida Fauré-Fremiet, 1961</td>
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<tr>
<td>Class Oligotrichia Bütschli, 1889</td>
<td></td>
</tr>
<tr>
<td>Subclass Halteriia Petz and Foissner, 1992</td>
<td></td>
</tr>
<tr>
<td>Order Halteriida Claparède and Lachmann, 1859</td>
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</tr>
<tr>
<td>Family Halteriidae Claparède and Lachmann, 1859</td>
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<tr>
<td>Genus Halteria Dujardin, 1841</td>
<td></td>
</tr>
<tr>
<td>Genus Pelagohalteria Foissner, Skogstad and Pratt, 1988</td>
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<tr>
<td>Genus Meseres Schewiakoff, 1892</td>
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<tr>
<td>Order Oligotrichia Bütschli, 1889</td>
<td></td>
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<tr>
<td>Suborder Strobilidiida Kahl in Doflein and Reichenow, 1929</td>
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<tr>
<td>Genus Strobilidium Schewiakoff, 1892</td>
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<tr>
<td>Genus Rumostrombidium Jankowski, 1978</td>
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<tr>
<td>Genus Pelagostrombidium Petz, Song and Wilbert, 1995</td>
<td></td>
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<tr>
<td>Genus Strombidinopsidium Small and Lynn, 1885</td>
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<tr>
<td>Genus Strombidinopsis Kent, 1881</td>
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<tr>
<td>Family Leegaardiellidae Lynn and Montagnes, 1988</td>
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<td>Genus Leegaardiella Lynn and Montagnes, 1988</td>
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<td>Suborder Tintinnina Kofoid and Campbell, 1929</td>
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<td>Order Oligotrichia Bütschli, 1889</td>
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<td>Genus Parallelostrombidium Agatha, 2004</td>
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<td>Genus Omegastrombidium Agatha, 2004</td>
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<td>Genus Novistrombidium Song and Bradbury, 1998</td>
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<td>Genus Laboea Lohmann, 1908</td>
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<tr>
<td>Genus Strombidium Claparède and Lachmann, 1859</td>
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1Again established by Small and Lynn (1985). 2The 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), Pelagostrombidium (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; Yagi 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 (Noiriot-Timothée 1960); transitions to a subsurface development of the oral primordium are also found in some Stichotrichida (Foissner 1983). The hypoapokinetal stomatogenesis is therefore...
regarded as derived state and developed probably convergently in the taxa mentioned above, as other arguments 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 Halteria, 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 Entodiniomorpha) 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
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(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 Halteria and Oligotrichia is similar at first glance, there is a difference, supporting a closer affiliation of the former with the Hypotrichia, 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 Halteria and the outgroup Hypotrichia. Accordingly, the distinct clockwise torsion is assumed to be apomorphic.

**Character 21: Origin of undulating membranes.** The undulating membranes of the outgroup Hypotrichia 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 Oligotrichia (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 Hypotrichia 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* (Wimsberger-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 Halteria.

**Character 23: Reorganization of somatic ciliature.** The somatic ciliature is usually not distinctly reconstructed during ontogenesis (Foissner 1996); thus, the extensive reorganization in the Hypotrichia is regarded as apomorphy, and the reorganization of the entire somatic ciliature in the Halteria as a convergence (Petz and Foissner 1992, Song 1993). This explanation is more parsimonious than to assume a common ancestor of the Hypotrichia and Halteria, which would require the assumption of several convergences in the Halteria 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...
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 Oligotrichida bases on the macronuclear replication band; the apokinetid stomatogenesis is a newly introduced strong synapomorphy. Since the perilemma is apparently absent in the Hypotrichida, it is not a synapomorphy of the Hypotrichia and Oligotrichia, as suggested by Petz and Foissner (1992), but possibly developed convergently in the Stichotrichida and Oligotrichia. Otherwise, it is a synapomorphy of the Oligotrichia and Stichotrichida, and the cirri are either a convergence in the Hypotrichida and Stichotrichida or a symplesiomorphy which was lost in the Oligotrichia. However, there are no morphologic or ontogenetic data that support these two latter explanations. The Oligotrichia 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 Halteria 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, 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 Pelagostrombiididae and the oral structures of the Cyrtostrombiididae (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 Hypotrichia, Oligotrichia, Halteria, 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 Halteria and Oligotrichia. The classical and PAUP tree reveal a monophyly of the Tontoniidae and Pelagostrombiididae 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 Oligotrichia 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., Diophys (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 Oligotrichia, 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 Strombididae 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 Oligotrichia 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 evolutional 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 evolutional 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 Oligotrichia. 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 Oligotrichia Bütschli, 1889


Comparison with related taxa: The members of the class Hypotrichia, i.e., the hypotrichs and stichotrichs, are mainly dorsoventrally flattened benthic organisms, which can be distinguished from the Oligotrichia 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 Oligotrichia is not necessary.

Subclass Halteriia Petz and Foissner, 1992

Improved diagnosis: Oligotrichia with endoral and minute paroral. Somatic ciliature comprises more than three kinetics 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 Halteriida differ from the Hypotricha 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 kinetics vs. bristle complexes).

Subclass Oligotrichia Bützchli, 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 kinetics.

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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), *Epimastigum ecaudatum* (Poland), *Eudiplodinium maggi* (France and Poland), *Isotricha intestinalis* (Poland), *I. prostoma* (France and Slovakia), *Metadinium medium* (Poland), *Ophryopsis caudatus* (Poland), *Ophe. purkynei* (Poland), *Ostracodinium gracile* (Poland), *Oste. dentatum* (Poland), *Polyplostra multivesiculatum* (France and Poland). For study of protozoal variability *Ophe. 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 protease K (Merck, Germany) was then added to the reaction mixture to a final concentration of 20 µg/ml. After protease 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 deoxynucleoside triphosphate, 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’-AAATGGTTGATCCGTCCAGT-3’) and EuKRev (5’-TGACCTCTCTGAGTTCACTTAC-3’). Quality and quantity of amplified DNA was determined by electrophoresis in 1% agarose gel (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 *BanI* and *CfoI* restriction endonucleases, substantial variability was observed after the cleavage by *AccI* (four different profiles) or *AseI* endonuclease (five different profiles). The highest discriminatory power was observed for *MspI* and *BsrUI* 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 *AvaiII* cleavage and by using six selected endonucleases any from 20 protozoal species be can unambiguously identified (Table 1).
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 *Ophryoscolex caudatus f. tricoronatus* (Figs 2A, B). No differences were found in morphologically different *Diplodocus dentatum* strains (data not shown) and similarly no differences were found in several

**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 *BstUI* (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 *BstUI* restriction endonuclease. Lane M - 100 bp DNA ladder (Gibco BRL, California USA), standard of molecular weight. The arrow indicates marker band of 600 bp.
Table 1. Key for RFLP analysis of rumen protozoa.

<table>
<thead>
<tr>
<th>Digestion</th>
<th>Cut Site</th>
<th>Species/Genus</th>
</tr>
</thead>
<tbody>
<tr>
<td>AvaII</td>
<td>(fragments 1080, 550)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>(fragments 1080, 460, 90)</td>
<td>10</td>
</tr>
<tr>
<td>NruI</td>
<td>(no cleavage, fragment 1650)</td>
<td>Entodinium sp. 3</td>
</tr>
<tr>
<td></td>
<td>(fragments 1100, 450)</td>
<td>7</td>
</tr>
<tr>
<td>MspI</td>
<td>(fragments 1530, 100)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>(fragments 920, 610, 100)</td>
<td>5</td>
</tr>
<tr>
<td>AceI</td>
<td>(fragments 910, 620)</td>
<td>Ent. nannellum</td>
</tr>
<tr>
<td></td>
<td>(no cleavage, fragment 1650)</td>
<td>Ent. bursa</td>
</tr>
<tr>
<td>EcoRV</td>
<td>(fragments 910, 620)</td>
<td>Ent. caudatum 6</td>
</tr>
<tr>
<td>MspI</td>
<td>(fragments 1330, 200, 100)</td>
<td>Ophr. purkynjei</td>
</tr>
<tr>
<td></td>
<td>(fragments 920, 610, 100)</td>
<td>Ophr. caudatum 9</td>
</tr>
<tr>
<td>NruI</td>
<td>(fragments 800, 530, 300)</td>
<td>Epid. caudatum 11</td>
</tr>
<tr>
<td></td>
<td>(fragments 800, 530, 150)</td>
<td>Epid. ecaudatum 12</td>
</tr>
<tr>
<td>AceI</td>
<td>(fragments 750, 600, 300)</td>
<td>Enoploplastron triloricatum</td>
</tr>
<tr>
<td></td>
<td>(no cleavage, fragment 1650)</td>
<td>Isotricha intestinalis</td>
</tr>
<tr>
<td>MspI</td>
<td>(fragments 1350, 300)</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>(fragments 1000, 650)</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>(fragments 3350, 300)</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>(no cleavage, fragment 1650)</td>
<td>Dasytricha ruminantium</td>
</tr>
<tr>
<td>NruI</td>
<td>(fragments 1350, 300)</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>(fragments 1100, 450)</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>(no cleavage, fragment 1650)</td>
<td>Ostracodinium dentatum</td>
</tr>
<tr>
<td>NruI</td>
<td>(fragments 840, 800)</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>(no cleavage, fragment 1650)</td>
<td>Eudiplodinium maggii</td>
</tr>
<tr>
<td>MspI</td>
<td>(fragments 1350, 300)</td>
<td>Ostracodinium gracile</td>
</tr>
<tr>
<td></td>
<td>(fragments 1100, 450)</td>
<td>Diplodinium dentatum</td>
</tr>
</tbody>
</table>
Rumen protozoa identification key

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 intra-species variability of studied protozoa. These data are in correlation with a previous report on very limited intra-species 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.

**Acknowledgements.** This investigation has been supported by the ERCULE project (European Rumen Ciliate Collection QLRT-CT-2000-01455).

**REFERENCES**


Dehority B. A. (1994) Rumen ciliate protozoa of the blue duiker (*Cephalophus monticola*), with observations on morphological variation lines within the species *Entodinium dubardi*. *J. Eukaryot. Microbiol.* **41**: 103-111


Wright A. D. G. (1999) Analysis of intraspecific sequence variation among eight isolates of the rumen symbiont, *Isotricha prostoma* (Del Rio et al. 2003), with observations on morphological diversity presented in the present work. 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.

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.

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(Ciliophora), from two continents. J. Eukaryot. Microbiol. 46: 445-446


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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 hippoboscid 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 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 Noyv 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 (*Corvus 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 World 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 secundiforme*), 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*).
Phylogenetic relationship of *T. corvi* 227

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.

<table>
<thead>
<tr>
<th>Species</th>
<th>Acc. No.</th>
<th>Strain</th>
<th>Host species</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. &quot;avium&quot;</em></td>
<td>AF416559</td>
<td>AAOU/5K97/APO1</td>
<td>lesser-spotted eagle</td>
<td><em>Aquila pomarina</em></td>
</tr>
<tr>
<td><em>T. &quot;avium&quot;</em></td>
<td>AF416563</td>
<td>IEUS/CZ99/SIM3</td>
<td>black fly</td>
<td><em>Eusimulium securiforme</em></td>
</tr>
<tr>
<td><em>T. &quot;avium&quot;</em></td>
<td>AJ009140</td>
<td>LSHTMT144B</td>
<td>chaffinch</td>
<td><em>Fringilla coelebs</em></td>
</tr>
<tr>
<td><em>T. &quot;avium&quot;</em></td>
<td>U39578</td>
<td>A1412</td>
<td>raven</td>
<td><em>Padda oryzoidea</em></td>
</tr>
<tr>
<td><em>T. corvi</em></td>
<td>AF416565</td>
<td>ITMAP 180795</td>
<td>raven</td>
<td><em>Corvus frugilegus</em></td>
</tr>
<tr>
<td><em>Trypanosoma sp.</em></td>
<td>AF416561</td>
<td>ICUL/CZ/98/CUL1</td>
<td>mosquito</td>
<td><em>Culex pipiens</em></td>
</tr>
<tr>
<td><em>T. bennetti</em></td>
<td>AJ223562</td>
<td>KT-2 (ATCC 50102)</td>
<td>American kestrel</td>
<td><em>Falco sparverius</em></td>
</tr>
</tbody>
</table>
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. pestanae* and Trypanosoma sp. “wombat” (Fig. 2) 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. cruci, 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. bennettii* 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.
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
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 hippoboscids, 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 hippoboscids. 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 hippoboscids 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-Ameri-
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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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, Para quadrula 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 re-
corded by non-continuous pollution monitoring. The sen-
sitivity 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 com-
monly used are mosses (Palmieri et al. 1997, Pearson et
al. 2000), and lichens (Hamada and Miyawaki 1998,
Conti and Cecchetti 2001). Farmer et al. (1992) per-
formed fumigation experiments with bryophytes and
lichens and criticized the utilization of unrealistically high
concentration of NO₂ used in many experiments. The
effects of a realistic NO₂ concentration (122.2 µg/m³ =
64 ppb - using for NO₂ 1 ppb = 1.91 µg/m³ at 21.1°C) on
the endohydric moss Polytrichum formosum were var-
ed: 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 experi-
ment, Morgan et al. (1992) showed that, at about half that
concentration (35 ppb = 66.9 µg/m³), NO₂ 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 Pleurocium schreberi and Rhytidiadelphus
triquetra (Bharali 1999).

By comparison to bryophytes and lichen, only few
studies have focused on microorganisms. The rare ex-
ceptions 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

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 (Shan-
non 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 mea-
sured: 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 NO₂ 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 NO₂
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) NO₂ 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 NO₂ 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°C to 11.4°C and the maximal from 7.2°C 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 200x and 400x 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.

NO₂ 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 NO₂. 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 NO₂ is 0.154 cm²/s, which means that the collection rate for our passive sampler could be calculated at 72 cm²/h. Mean hourly concentration of NO₂ (in µg/m³, hereafter [NO₂]) 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 NO₂ was measured by spectrophotometry using a variant of the Griess-Saltzman method (Atkins et al. 1986). NO₂ 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 NO₂ 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 NO₂ concentrations and thickness of the green part of the mosses. Atmospheric NO₂ 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 [NO₂] 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). The NO₂ 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
(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 [NO₂], nor with the length of the green part of the mosses, and was only slightly, but not significantly, correlated with thickness of the living part of mosses.

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

<table>
<thead>
<tr>
<th></th>
<th>Periphery</th>
<th>City limits</th>
<th>P-value #</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean ± SE</td>
<td>n</td>
</tr>
<tr>
<td>NO₂ concentration [µg/m³]</td>
<td></td>
<td>8 14.6 ± 1.7</td>
<td>7 34.8 ± 3.6</td>
</tr>
<tr>
<td>Thickness of the living part of mosses [cm]</td>
<td>8 0.6 ± 0.1</td>
<td>7 0.6 ± 0.0</td>
<td>0.631</td>
</tr>
<tr>
<td>Total testate amoeba density [ind/g]</td>
<td>8 7218 ± 2115</td>
<td>7 6252 ± 2462</td>
<td>0.487</td>
</tr>
<tr>
<td>Testate amoeba species richness</td>
<td>8 6.0 ± 0.53</td>
<td>7 4.7 ± 0.48</td>
<td>0.016</td>
</tr>
<tr>
<td><em>Paraquadra irregularis</em></td>
<td>7 176 ± 78</td>
<td>0 0 ± 0</td>
<td>0.017</td>
</tr>
<tr>
<td><em>Nebela tincta</em>-type</td>
<td>2 3 ± 2</td>
<td>3 121 ± 87</td>
<td>0.270</td>
</tr>
<tr>
<td><em>Euglypha ciliata</em></td>
<td>8 4260 ± 1447</td>
<td>7 3147 ± 1145</td>
<td>0.487</td>
</tr>
<tr>
<td><em>Difflugia pristis</em>-type</td>
<td>8 255 ± 65</td>
<td>5 132 ± 80</td>
<td>0.487</td>
</tr>
<tr>
<td><em>Assulina muscorum</em></td>
<td>8 1447 ± 790</td>
<td>6 2527 ± 2361</td>
<td>0.563</td>
</tr>
<tr>
<td><em>Arcella cutinata</em>-type</td>
<td>8 327 ± 239</td>
<td>7 183 ± 52</td>
<td>0.728</td>
</tr>
<tr>
<td><em>Trinema complanatum</em></td>
<td>7 749 ± 468</td>
<td>5 137 ± 51</td>
<td>0.907</td>
</tr>
</tbody>
</table>

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

![Location of the study site and average atmospheric NO₂ concentrations [µg/m³] measured at the sampling locations (squares: city; circles: periphery). Stippled area: Besançon agglomeration. Continuous lines: principal roads.](image)
Atmospheric pollution and testate amoebae

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/m$^3$). This indicates an overall good quality of air pollution monitoring (ASQAB 2001) and are well under those that are regularly published by the service of air 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 biomonitor 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$_3$] 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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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-
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 OsO₄ 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).

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-I) 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
Ceratomyxa protopsettae in olive flounder

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).

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**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.
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), *C. protopsettae* (Fujita, 1923), and the present study. The light micrographs of developmental stages of *C. protopsettae* are shown in Figs 15-30. The light micrographs were taken using a Leica MZ125 microscope with a Leica DMRX compound microscope. The scale bars are 25 µm.
**Table 1.** Comparison of spore characteristics between original description of Fujita (1923) and the present specimens.

<table>
<thead>
<tr>
<th></th>
<th>Fujita, 1923</th>
<th>Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spore</td>
<td>unequal valve and variation in curvature of the shell</td>
<td>11.64 ± 0.95</td>
</tr>
<tr>
<td>Length (µm)</td>
<td>10 ~ 12</td>
<td>11.64 ± 0.95</td>
</tr>
<tr>
<td>Width (µm)</td>
<td>12 ~ 13</td>
<td>-</td>
</tr>
<tr>
<td>Thickness (µm)</td>
<td>50 ~ 65</td>
<td>46.63 ± 5.8</td>
</tr>
<tr>
<td>Polar capsule</td>
<td>two large and ovate</td>
<td>two subspherical</td>
</tr>
<tr>
<td>Length (µm)</td>
<td>6</td>
<td>4.15 ± 0.34</td>
</tr>
<tr>
<td>Breadth (µm)</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Polar filament</td>
<td>-</td>
<td>5 ~ 6 coiled</td>
</tr>
<tr>
<td>Sporoplasm</td>
<td>asymmetrically distributed and binucleated</td>
<td></td>
</tr>
<tr>
<td>Host</td>
<td><strong>Paralichthys olivaceus</strong></td>
<td></td>
</tr>
<tr>
<td>Organ</td>
<td>gallbladder</td>
<td></td>
</tr>
<tr>
<td>Geographical location</td>
<td>Hokkaido, Japan</td>
<td>East Sea, South Korea</td>
</tr>
</tbody>
</table>

**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).
Figs 35–40. Electron-micrographs of presporogenic 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).
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
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 sporogenic 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. sparassaurati. 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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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...

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, Modřý 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 occurrence 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 (Divíš 1990). Habitats in locality 2 (50° 00’ N, 016° 13’ E), near the town Chocěv (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 km² oak-hornbeam forest complex (Voříšek 2000).

Population density of buzzards has differed between the study sites; the density was similar in Česká Skalice and Chocěv (20-40 breeding pairs per 100 km², Divíš 1990, Voříšek 1995) while the density in Mikulov has reached one of the highest values ever known (30-50 breeding pairs per 22 km², 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 Chocěv, 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% K₂Cr₂O₇ at 4°C. Before microscopic examination, samples were centrifuged for 10 min at 200 g, the sediment was mixed with 33% ZnSO₄, recentrifuged, and flotated for 20 min.

In Chocěv 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 Laciná 1998), the age of the oldest nestling in a nest was used for further analysis.


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 host species with the other effects were also examined. 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 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: F2,217 = 1.66, P = 0.192; year: F6,217 = 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: t1306 = -3.6, P < 0.001; 1st vs. 3rd: t1303 = -2.5, P = 0.013; 2nd vs. 3rd: t1296 = 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: t1306 = -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.

<table>
<thead>
<tr>
<th>Locality</th>
<th>Year</th>
<th>Broods (samples)</th>
<th>Prevalence (%)</th>
<th>Brood age (days)</th>
<th>Brood size</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Mean ± SD</td>
<td>Range</td>
<td>Mean ± SD</td>
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<td>15</td>
<td>60.0</td>
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<td>49</td>
<td>65.3</td>
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<td>Skalice</td>
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<td>16</td>
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<tr>
<td>Skalice</td>
<td>1993</td>
<td>10</td>
<td>60.0</td>
<td>20.5 ± 4.2</td>
<td>14 - 28</td>
</tr>
<tr>
<td>Choceň</td>
<td>1989</td>
<td>8</td>
<td>75.0</td>
<td>17.6 ± 4.0</td>
<td>12 - 25</td>
</tr>
<tr>
<td>Choceň</td>
<td>1990</td>
<td>7</td>
<td>42.9</td>
<td>18.9 ± 4.8</td>
<td>10 - 25</td>
</tr>
<tr>
<td>Choceň</td>
<td>1991</td>
<td>6</td>
<td>66.7</td>
<td>18.4 ± 5.1</td>
<td>8 - 21</td>
</tr>
<tr>
<td>Choceň</td>
<td>1992</td>
<td>15</td>
<td>73.3</td>
<td>15.9 ± 7.3</td>
<td>6 - 30</td>
</tr>
<tr>
<td>Mikulov ^a</td>
<td>1993</td>
<td>34</td>
<td>55.9</td>
<td>25.0 ± 5.7</td>
<td>3 - 36</td>
</tr>
<tr>
<td>Mikulov ^a</td>
<td>1994</td>
<td>35</td>
<td>88.6</td>
<td>29.3 ± 6.4</td>
<td>2 - 36</td>
</tr>
<tr>
<td>Mikulov ^a</td>
<td>1995</td>
<td>19</td>
<td>78.9</td>
<td>24.9 ± 7.4</td>
<td>3 - 33</td>
</tr>
<tr>
<td>Mikulov ^b</td>
<td>1993</td>
<td>120</td>
<td>28.3</td>
<td>16.2 ± 4.5</td>
<td>1 - 36</td>
</tr>
<tr>
<td>Mikulov ^b</td>
<td>1994</td>
<td>100</td>
<td>54.0</td>
<td>18.4 ± 6.4</td>
<td>2 - 36</td>
</tr>
<tr>
<td>Mikulov ^b</td>
<td>1995</td>
<td>51</td>
<td>62.7</td>
<td>18.7 ± 5.0</td>
<td>2 - 33</td>
</tr>
</tbody>
</table>

^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 x age were included as random effects.

<table>
<thead>
<tr>
<th>Fixed effect</th>
<th>Estimate ± SE</th>
<th>Type III test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DDF ^a</td>
<td>F</td>
</tr>
<tr>
<td>Model A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>0.370 ± 2.037</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.007 ± 0.087</td>
<td>218 &lt; 0.1</td>
</tr>
<tr>
<td>Brood size</td>
<td>-2.000 ± 0.999</td>
<td>221 4.0</td>
</tr>
<tr>
<td>Age × brood size</td>
<td>0.105 ± 0.046</td>
<td>221 5.3</td>
</tr>
<tr>
<td>Model B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>-5.473 ± 0.981</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.212 ± 0.039</td>
<td>242 29.4</td>
</tr>
<tr>
<td>Brood size</td>
<td>-0.413 ± 0.350</td>
<td>221 1.4</td>
</tr>
<tr>
<td>Age × brood size</td>
<td>0.070 ± 0.019</td>
<td>240 13.6</td>
</tr>
</tbody>
</table>

^a Denominator df estimated by the Satterthwaite method; numerator df = 1 in all cases.
in ecotone than in open habitat, in relatively heavier individuals and in those positive for *Frenkelia*; which effects were consistent across all host species (nonsignificant 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, t29 = 4.3, P < 0.001) but not in common shrew (0.63 ± 0.68, t17 = 0.9, P = 0.374) and bank vole (-0.03 ± 0.73, t38 = -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
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* occurred 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-

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**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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Frenkelia</th>
<th>Sarcocystis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>apofla</td>
<td>aposyl</td>
</tr>
<tr>
<td>Skalice</td>
<td>4.7 (107)</td>
<td>0.9 (115)</td>
</tr>
<tr>
<td>Chocerh</td>
<td>0.0 (15)</td>
<td>5.3 (94)</td>
</tr>
<tr>
<td>Mikulov</td>
<td>0.8 (128)</td>
<td>2.6 (77)</td>
</tr>
<tr>
<td>1st day</td>
<td>0.9 (109)</td>
<td>2.0 (149)</td>
</tr>
<tr>
<td>2nd day</td>
<td>3.6 (84)</td>
<td>3.3 (90)</td>
</tr>
<tr>
<td>3rd day</td>
<td>3.5 (57)</td>
<td>4.3 (47)</td>
</tr>
<tr>
<td>Ecotone</td>
<td>3.1 (194)</td>
<td>3.7 (218)</td>
</tr>
<tr>
<td>Open</td>
<td>0.0 (56)</td>
<td>0.0 (68)</td>
</tr>
<tr>
<td>Spring</td>
<td>7.3 (55)</td>
<td>8.7 (46)</td>
</tr>
<tr>
<td>Autumn</td>
<td>1.0 (195)</td>
<td>1.7 (240)</td>
</tr>
<tr>
<td>Male</td>
<td>0.0 (66)</td>
<td>0.0 (43)</td>
</tr>
<tr>
<td>Female</td>
<td>2.6 (77)</td>
<td>0.0 (40)</td>
</tr>
<tr>
<td>Total</td>
<td>2.4 (250)</td>
<td>2.8 (286)</td>
</tr>
</tbody>
</table>

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

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**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.

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

* 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.

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

* 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.
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% (Irovec 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 (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.

Prevalence of both _Frenkelia_ and _Sarcocystis_ is higher in heavier individuals. Body weight correlates with the age of small rodents and shrews (Śebek 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 cocididia. 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 occasionally 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% (Goszczynski 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.
Sarcosporidia in buzzards and small mammals

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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REFERENCES


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. glareoloi, 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. glareoloi is significantly higher than prevalence of F. microti in their respective intermediate

Holišová 1959, Obrtel and Holišová 1974). Insects may


Voříšek P. (2000) An extremely high population density of common buzzard (Buteo buteo) in Biosphere Reserve Palava (Czech Republic) and its possible causes. Buteo 11: 51-56


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260 M. Svobodová
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 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


Although there is considerable information on the species of Microsporidia (Lom and Matos 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 microscopy 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 brevioirostris (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 brevioirostris (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% OsO4 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 (v/v) and observed by DIC optics. The ultrathin sections were double stained with uranyl acetate and lead citrate (Reynolds 1963) and (v/v) and observed by DIC optics. The ultrathin sections were double stained with uranyl acetate and lead citrate (Reynolds 1963) and (v/v) and observed by DIC optics. The ultrathin sections were double stained with uranyl acetate and lead citrate (Reynolds 1963) and (v/v) and observed by DIC optics. The ultrathin sections were double stained with uranyl acetate and lead citrate (Reynolds 1963) and (v/v) and observed by DIC optics. The ultrathin sections were double stained with uranyl acetate and lead citrate (Reynolds 1963) and (v/v) and observed by DIC optics. The ultrathin sections were double stained with uranyl acetate and lead citrate (Reynolds 1963) and (v/v) and observed by DIC optics. The ultrathin sections were double stained with uranyl acetate and lead citrate (Reynolds 1963) and (v/v) and observed by DIC optics. The ultrathin sections were double stained with uranyl acetate and lead citrate (Reynolds 1963) and (v/v) and observed by DIC optics. The ultrathin sections were double stained with uranyl acetate and lead citrate (Reynolds 1963) and (v/v) and observed by DIC optics. The ultrathin sections were double stained with uranyl acetate and lead citrate (Reynolds 1963) and (v/v) and observed by DIC optics.

RESULTS

Irregular whitish xenomas, were macroscopically observed only in the internal muscular tissue of the abdominal cavity of the fish, Brachyhypopomus brevioirostris (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 exosporium (~ 15 nm thick) and an electronlucent endosporium (~ 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 polyribosemes 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.
~ 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).
Schematic drawings of the spore morphology (Fig. 13) were made from serial ultrathin sections.

**Taxonomy summary of Microsporidium brevirostris**<br>
**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 Ralphp et Matthews, 1986; Nosemoids 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 macrosposes, 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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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 x100 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 denticle and radial pins, the mode is given instead of the arithmetic mean. The span of the denticle 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).
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Table 1. Morphometric comparison of *Trichodinella epizootica* (Raabe, 1950) Šramek-Hušek, 1953 obtained in the present study with those of other authors.

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

**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...
Trichodinids from freshwater fishes in India

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

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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 aceanine gregarines in the Chotonagpur district of Bihar, seminal vesicles of earthworm *Amynthas robusta* 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 12-17 × 8-16 µm. Gametocysts are ovoidal, enclose two unequal gamonts and measure 71-96 × 46-83 µm. Oocysts are biconical measuring 6.5-7 × 3.5-4 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 52-66 × 33-39 µm and oocysts are navicular shaped with sharply pointed ends and measure 10-12 × 6-7 µm.

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

**INTRODUCTION**

Aceanine 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 aceanine gregarines in the oligochaete worms, seminal vesicles of earthworm *Amynthas robusta* 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 acéphaline 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 (overnight) 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 Martis, 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 granular; 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:** Monocystidae Bütschli, 1882  
**Subfamily:** Monocystinae Bhatia, 1930  
With the characters of genus *Stomatophora* Drzhevetskii, 1907, 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 granular; 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).
New Species of acephaline gregarines

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

<table>
<thead>
<tr>
<th>Parasite species/ Character</th>
<th><em>A. akaryoseminiferus</em></th>
<th><em>A. monokaryoseminiferus</em></th>
<th><em>A. chotonagpurensis</em> sp. n.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trophozoite shape</td>
<td>spherical, solitary</td>
<td>spherical, solitary</td>
<td>spheroidal or ovoid</td>
</tr>
<tr>
<td>Trophozoite size</td>
<td>54-100</td>
<td>61-115</td>
<td>42-83</td>
</tr>
<tr>
<td>Gametocyst size</td>
<td>ni</td>
<td>ni</td>
<td>ovoidal</td>
</tr>
<tr>
<td>Gametocyst shape</td>
<td>ni</td>
<td>biconical, ends slightly flattened</td>
<td>biconical, ends sharply pointed</td>
</tr>
<tr>
<td>Oocyst shape</td>
<td>biconical, ends slightly flattened</td>
<td>biconical, ends slightly flattened</td>
<td>biconical, ends sharply pointed</td>
</tr>
<tr>
<td>Oocyst size</td>
<td>33.0-39</td>
<td>10.0-12.0</td>
<td>6.5-7 × 3.5-4</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Parasite species/ Character</th>
<th><em>S. bahli</em></th>
<th><em>S. globa</em></th>
<th><em>S. pedongensis</em></th>
<th><em>S. majumdarai</em></th>
<th><em>S. janovyi</em> sp. n.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trophozoite shape</td>
<td>discoidal; flattened between poles</td>
<td>spherical, flattened between poles</td>
<td>discoidal, flattened and compressed between poles</td>
<td>spherical, flattened between poles</td>
<td>discoidal, flattened and flower like</td>
</tr>
<tr>
<td>Trophozoite size</td>
<td>43-82</td>
<td>31-97</td>
<td>50-94</td>
<td>40-63</td>
<td>80-85</td>
</tr>
<tr>
<td>Gametocyst shape</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ovoidal</td>
<td>ovoidal</td>
</tr>
<tr>
<td>Gametocyst size</td>
<td>ni</td>
<td>ni</td>
<td>90-119</td>
<td>90-130 × 90-115</td>
<td>52-66 × 33-39</td>
</tr>
<tr>
<td>Oocyst shape</td>
<td>ni</td>
<td>ni</td>
<td>navicular</td>
<td>navicular</td>
<td>navicular</td>
</tr>
<tr>
<td>Oocyst size</td>
<td>ni</td>
<td>ni</td>
<td>in</td>
<td>8 × 5</td>
<td>10-12 × 6-7</td>
</tr>
<tr>
<td>Host</td>
<td><em>Amynthas diffringens</em></td>
<td><em>Pheretima alexendri</em></td>
<td><em>Amynthas diffringens</em></td>
<td><em>Metaphire posthuma</em></td>
<td><em>Amynthas hawayanus</em></td>
</tr>
<tr>
<td>Soil type</td>
<td>podzol</td>
<td>podzol</td>
<td>podzol</td>
<td>alluvial</td>
<td>podzol</td>
</tr>
<tr>
<td>Elevation (m)</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>ni</td>
<td>3323</td>
</tr>
</tbody>
</table>

Gametocysts 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.
New Species of acephaline gregarines

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. majumdaari 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. pedogensis but larger than the nucleus of S. majumdaari and it contains some vacuolated areas. In S. janovyi the gametocyst is ovoid and smaller than in S. pedogensis and S. majumdaari. Oocyst is similar in shape in all five species but the measurements are distinctly longer than in both S. pedogensis and S. majumdaari. Based on distinct differences in comparison with species previously reported from the Indian oligochaetes, 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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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 bartoli Stépánek, 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. 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 photomicrographs 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 magnification (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
Redescription of *Difflugia tuberspinifera*

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),

---

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

<table>
<thead>
<tr>
<th>Characters¹</th>
<th>X</th>
<th>M</th>
<th>SD</th>
<th>SE</th>
<th>CV</th>
<th>Min</th>
<th>Max</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shell height (1)</td>
<td>111.4</td>
<td>112.0</td>
<td>5.71</td>
<td>0.81</td>
<td>5.13</td>
<td>94.0</td>
<td>129.0</td>
<td>50</td>
</tr>
<tr>
<td>Shell diameter (2)</td>
<td>109.4</td>
<td>110.5</td>
<td>5.33</td>
<td>0.75</td>
<td>4.88</td>
<td>94.0</td>
<td>118.0</td>
<td>50</td>
</tr>
<tr>
<td>Aperture diameter (3)</td>
<td>53.0</td>
<td>53.0</td>
<td>3.93</td>
<td>0.39</td>
<td>7.41</td>
<td>45.0</td>
<td>63.0</td>
<td>102</td>
</tr>
<tr>
<td>Spine length (4)</td>
<td>28.7</td>
<td>28.0</td>
<td>6.35</td>
<td>0.90</td>
<td>22.10</td>
<td>15.0</td>
<td>42.0</td>
<td>50</td>
</tr>
<tr>
<td>Collar height (5)</td>
<td>10.6</td>
<td>11.0</td>
<td>1.74</td>
<td>0.25</td>
<td>16.31</td>
<td>8.0</td>
<td>15.0</td>
<td>50</td>
</tr>
<tr>
<td>Rear end length (6)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Foreside length (7)</td>
<td>32.0</td>
<td>33.0</td>
<td>6.79</td>
<td>0.67</td>
<td>21.20</td>
<td>18.0</td>
<td>57.0</td>
<td>102</td>
</tr>
<tr>
<td>Number of aperture tooth-like structures (8)</td>
<td>8.4</td>
<td>8.0</td>
<td>0.78</td>
<td>0.11</td>
<td>9.32</td>
<td>7</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>Number of conical spines (9)</td>
<td>5.6</td>
<td>6.0</td>
<td>0.61</td>
<td>0.09</td>
<td>10.82</td>
<td>4</td>
<td>7</td>
<td>50</td>
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</tbody>
</table>

¹Numbers 1-9 in parenthesis designate features as shown in Figs 1 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; X - arithmetic mean.
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 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

![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.](image1)

![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).](image2)
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

<table>
<thead>
<tr>
<th>Character</th>
<th><em>Difflugia tuberspinifera</em></th>
<th><em>D. bartoši</em></th>
<th><em>D. corona cashi</em></th>
<th><em>D. corona tuberculata</em></th>
<th><em>D. corona corona</em></th>
<th><em>D. muriformis</em></th>
<th><em>Netzelia (Difflugia)</em> tuberculata</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shell height</td>
<td>94-129</td>
<td>245-385</td>
<td>126-190</td>
<td>180-230</td>
<td>120-160</td>
<td>115-150</td>
<td>91-150</td>
</tr>
<tr>
<td>Shell diameter</td>
<td>94-118</td>
<td>192-297</td>
<td>126-177</td>
<td>160-180</td>
<td>100-140</td>
<td>110-138</td>
<td>85-132</td>
</tr>
<tr>
<td>Aperture diameter</td>
<td>39-64</td>
<td>70</td>
<td>51-86</td>
<td>80-90</td>
<td>50-68</td>
<td>31-50</td>
<td>30-40</td>
</tr>
<tr>
<td>Collar diameter</td>
<td>6-18</td>
<td>Ab</td>
<td>present</td>
<td>present</td>
<td>Present</td>
<td>Ab</td>
<td>&gt;7</td>
</tr>
<tr>
<td>Neck</td>
<td>Ab</td>
<td>35-70 × 75</td>
<td>Ab</td>
<td>Ab</td>
<td>Ab</td>
<td>Ab</td>
<td>Ab</td>
</tr>
<tr>
<td>Shell shape</td>
<td>sub-spherical</td>
<td>spherical</td>
<td>spherical or ovoid</td>
<td>sub-spherical</td>
<td>sub-spherical</td>
<td>sub-spherical</td>
<td>sub-spherical</td>
</tr>
<tr>
<td>Shell color</td>
<td>yellow to brown and opaque</td>
<td>brown and opaque</td>
<td>opaque</td>
<td>opaque</td>
<td>?</td>
<td>yellow to brown and transparent</td>
<td></td>
</tr>
<tr>
<td>Shell wall</td>
<td>MS irregular SQ</td>
<td>irregular SQ</td>
<td>irregular SQ</td>
<td>MS</td>
<td>MS</td>
<td>MS</td>
<td>MS</td>
</tr>
<tr>
<td>Aperture</td>
<td>circular</td>
<td>circular</td>
<td>circular</td>
<td>circular</td>
<td>circular</td>
<td>3-5 lobes</td>
<td>hexagonal</td>
</tr>
<tr>
<td>Number of lobes</td>
<td>7-10</td>
<td>Ab</td>
<td>10-20</td>
<td>?</td>
<td>12-20</td>
<td>3-5</td>
<td>6-7</td>
</tr>
<tr>
<td>Collar or neck shape</td>
<td>TS</td>
<td>circular</td>
<td>TS</td>
<td>TS</td>
<td>TS</td>
<td>TS</td>
<td>hexagonal with sinuous lobes</td>
</tr>
<tr>
<td>Number of spines</td>
<td>4-8</td>
<td>4-8</td>
<td>2-8</td>
<td>4-6</td>
<td>variable</td>
<td>variable</td>
<td>Ab</td>
</tr>
<tr>
<td>Spine position</td>
<td>regularly at the upper</td>
<td>regularly around mid-body</td>
<td>randomly at the aboral region</td>
<td>regularly behind mid-body</td>
<td>irregularly behind mid-body</td>
<td>at the aboral region</td>
<td>Ab</td>
</tr>
<tr>
<td>Biometry</td>
<td>monomorphic</td>
<td>monomorphic</td>
<td>monomorphic</td>
<td>monomorphic</td>
<td>monomorphic</td>
<td>monomorphic</td>
<td>monomorphic</td>
</tr>
<tr>
<td>Building material</td>
<td>?</td>
<td>exogenesis</td>
<td>exogenesis</td>
<td>exogenesis</td>
<td>?</td>
<td>exogenesis</td>
<td>endogenesis</td>
</tr>
<tr>
<td>Sample location</td>
<td>China</td>
<td>Slovakia</td>
<td>Yugoslavia and G. Britain</td>
<td>Venezuela</td>
<td>G. Britain and Venezuela</td>
<td>Argentina</td>
<td>Africa</td>
</tr>
</tbody>
</table>
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
Redescription of *Difflugia tuberspinifera* 289

differs from other *Difflugia* species in its ability to endogenously synthesize the building material. *D. bartosi* 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. bartosi* is much larger than any other six taxa. *D. corona* 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.

REFERENCES


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