ACTA PROTOZOOLOGICA
International Journal on Protistology

Editor in Chief  Jerzy SIKORA
Editors Hanna FABCZAK and Anna WASIK
Managing Editor Małgorzata WORONOWICZ-RYMASZEWSKA

Editorial Board

Christian F. BARDELE, Tübingen
Linda BASSON, Bloemfontein
Helmut BERGER, Salzburg
Jean COHEN, Gif-Sur-Yvette
John O. CORLISS, Albuquerque
György CSABA, Budapest
Johan F. De JONCKHEERE, Brussels
Isabelle DESPORTES-LIVAGE, Paris
Genoveva F. ESTEBAN, Ambleside
Tom FENCHEL, Helsingør
Wilhelm FOUSSNER, Salzburg
Vassil GOLEMANSKY, Sofia
Andrzej GRĘBECKI, Warszawa, Vice-Chairman
Lucyna GRĘBECKA, Warszawa
Donat-Peter HÄDER, Erlangen

Janina KACZANOWSKA, Warszawa
Stanisław L. KAZUBSKI, Warszawa
Leszek KUŹNICKI, Warszawa, Chairman
J. I. Ronny LARSSON, Lund
John J. LEE, New York
Jiří LOM, České Budějovice
Pierangelo LUPORINI, Camerino
Kálman MOLNÁR, Budapest
Yutaka NAITOH, Tsukuba
Jytte R. NILSSON, Copenhagen
Eduardo ORIASS, Santa Barbara
Sergei O. SKARLATO, St. Petersburg
Michael SLEIGH, Southampton
Jiří VÁVRA, Praha

ACTA PROTOZOOLOGICA appears quarterly.

The price (including Air Mail postage) of subscription to Acta Protozoolgica at 2003 is: US $ 200. - by institutions and US $ 120. - by individual subscribers. Limited numbers of back volumes at reduced rate are available. Terms of payment: check, money order or payment to be made to the Nencki Institute of Experimental Biology account: 11101053-401050001074 at BPH PBK S.A. III O/Warszawa, Poland. For matters regarding Acta Protozoolgica, contact Editor, Nencki Institute of Experimental Biology, ul. Pasteura 3, 02-093 Warszawa, Poland; Fax: (4822) 822 53 42; E-mail: jurek@ameba.nencki.gov.pl For more information see Web page http://www.nencki.gov.pl/public.htm

Circulation: 300


©Nencki Institute of Experimental Biology
Polish Academy of Sciences
This publication is supported by the State Committee for Scientific Research

Desktop processing: Justyna Osmulska, Information Technology Unit of the Nencki Institute
Printed at the MARBIS, ul. Poniatowskiego 1
05-070 Sulejów, Poland
Species Separation and Identification of *Uronychia* spp. (Hypotrichia: Ciliophora) using RAPD Fingerprinting and ARDRA Riboprinting

Zigui CHEN¹, Weibo SONG¹ and Alan WARREN²

¹ Laboratory of Protozoology, College of Fisheries, Ocean University of China, Qingdao, P. R. China; ² Department of Zoology, Natural History Museum, London, UK

**Summary.** The 3 most common morphospecies of *Uronychia*, i.e. *U. setigera*, *U. transfuga* and *U. binucleata*, were examined *in vivo* and following protargol impregnation. Among these, *U. transfuga* is morphologically different to the others (large cell size, more macronuclear segments etc.). By contrast, *U. setigera* and *U. binucleata* are very similar and difficult to separate based only on their morphologies. Random amplified polymorphic DNA (RAPD) fingerprinting and amplified ribosomal DNA restriction analyses (ARDRA riboprinting) were therefore performed in order to confirm the division between them and to aid species identification. Using 4 different random primers the RAPD fingerprinting revealed 3 distinct patterns. Thus, 7 strains could be separated into 3 species with a similarity index of over 82% between different strains of the same species and only 30% to 40% between strains of different species. The unique restriction pattern of highly-conserved rDNA fragments (ARDRA) of different strains of *U. setigera* and *U. binucleata* using the enzyme Msp I was found to be species-specific and could be applicable for both species identification and species separation. According to our molecular analyses, the *Uronychia*-populations comprised at least 3 taxa (morphospecies). Moreover, the morphologically similar *U. setigera* and *U. binucleata* could be reliably separated and identified at the molecular level.

**Key words:** ARDRA riboprinting, identification, RAPD fingerprinting, species separation, *Uronychia*.

**INTRODUCTION**

The genus *Uronychia* is one of the most commonly-reported hypotrichous ciliates and is found worldwide in a range of marine and other saline biotopes (Müller 1786; Stein 1859; Quennerstedt 1867; Wallengren 1900; Buddenbrock 1920; Young 1922; Mansfeld 1923; Wang and Nie 1932; Kirby 1934; Wang 1934; Ozaki and Yagiu 1941; Fenichel 1965; Reiff 1968; Kattar 1970; Agamaliev 1971; Borror 1972a, b; Wilbert and Kahan 1981; Song 1997; Shi and Song 1999). In terms of species separation and taxonomy, however, *Uronychia* is possibly one of the most confused of all ciliate genera despite the fact that several studies concerning its taxonomy, morphology and morphogenesis have been carried out in recent years (Curds and Wu 1983, Dragesco and Dragesco-Kernéis 1986, Hill 1990, Valbonesi and Luporini 1986, Hill 1990, Valbonesi and Luporini 1990, Petz et al. 1995, Wilbert 1995, Song 1996).

Up to 1983,10 species of *Uronychia* had been described in the literature (Müller 1786, Dujardin 1841,

Following their detailed investigation of over twenty Uronychia-populations collected over a 6-year period, Song and Wilbert (1997) concluded that U. magna and U. antarctica are conspecific with U. transfuga and U. binucleata respectively. Consequently, all known putative Uronychia spp. were allocated to one of 4 revised morphospecies: U. transfuga, U. setigera, U. binucleata (all of which are common) and U. multicularis (which is rare).

Although Song and Wilbert (1997) significantly enhanced our knowledge and understanding of Uronychia-populations based on morphometric analyses, some morphological features at the species level are weak and easily overlooked. For example, the separation of U. setigera and U. binucleata is based almost exclusively on cell size and on the difference in the number of basal body pairs in the leftmost dorsal kinety, the latter character only being discernible with careful observation of high quality protargol preparations.

It has previously been reported that PCR-based methods such as RAPD (random amplified polymorphic DNA) fingerprinting and ARDRA (amplified ribosomal DNA restriction analyses, also called ‘riboprinting’) are suitable for the study of microbial diversity (Persing et al. 1993). In the field of protozoology, RAPD fingerprinting has been used to identify different parasite species (Neto et al. 1993), to separate different scuticociliate species (Song et al. 2002), to study genetic diversity within Trypanosoma spp. (Dirie et al. 1993, Mathieu-Daudé et al. 1995, Stotheard et al. 2000) and in the genus Tetrahymena (Lynch et al. 1995), and to investigate the population structure of Euploites spp. (Kusch and Heckmann 1996). Jerome and Lynn (1996) even suggested that PCR-based RFLP riboprinting (i.e. ARDRA) provides an alternative to traditional techniques for identifying and distinguishing Tetrahymena spp., while Stocke et al. (2000) successfully combined two molecular techniques (RAPD and ARDRA) to reject the hypothesis of sibling species in Paramecium caudatum. By contrast, Foissner et al. (2001) failed to separate two morphospecies of the hypotrich ciliate Gonostomum using RAPD-fingerprinting.

During the present study, RAPD and ARDRA were used in order to investigate the relatedness between 3 species of Uronychia: U. setigera (three strains), U. binucleata (three strains) and U. transfuga (one strain). The main objective was to determine whether the separation of these 3 morphospecies is supported at the molecular level.

MATERIALS AND METHODS

Origin of isolates

Seven strains of Uronychia were isolated from diverse marine biotopes between 1998 and 2002. Three strains of U. setigera were from: (i) a crab-farming pond at Xunshan (strain 99062701); (ii) off the coast of Qingdao (strain 99102305); (iii) a shrimp-farming pond at Laizhou (strain 01041607). Three strains of U. binucleata were from: (i) a shrimp-farming pond at Huangdao (strain 010424001); (ii) a shrimp-farming pond at Huangdao (strain 010424106); (iii) a shrimp-farming pond at Weihai (strain 01051801). One strain of U. transfuga (strain 00042803) was from the shellfish-farming pond at Hongdao. Clonal cultures of each strain were established and maintained in sterile seawater at room temperature with rice grains to enrich natural bacteria as food for the ciliates. The protargol method according to Wilbert (1995) was used to reveal the infraciliature. The infraciliature and other morphological features of each strain were compared to those reported in previous papers (Song 1996, Song and Wilbert 1997).

DNA extraction and RAPD reaction

The nucleotide extraction protocols used in this study have been described in detail elsewhere (Chen and Song 2001) and involved the following steps: cells were rinsed 3 times with sterile artificial marine water after being starved overnight and were then pelleted by centrifugation (about 1000 g), 0.5 ml lysis buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl2, 0.6 % Tween 20, 0.6 % Nonidet P40, 60 µg/ml Proteinase K) was added and the mixture was incubated at 56°C for 1-2 h. After incubation, the DNA was extracted using an equal volume of a mixture of phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with 70% ethanol. DNA samples were stored at -20°C.

Amplifications by PCR were carried out in a total volume of 25 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.1% Triton X-100, 3 mM MgCl2, 0.2 mM dNTP, 0.5 µM of one oligonucleotide primer, 15 ng of genomic DNA and 1.5 units of Taq DNA polymerase (TaKaRa, Japan). For amplification the reaction mixtures were denatured at 94°C for 5 min, followed by the first 5 cycles consisting of denaturation for 30 s at 94°C, primer annealing for 30 s at 35°C and extension for 1 min at 72°C. The subsequent 35 cycles comprised denaturation for 30 s at 94°C, primer annealing for 30 s at 40°C and extension for 1 min at 72°C. Cycling was followed by a final extension step for 5 min at 72°C. Two or 3 repetitions of the PCR reaction were performed in order to assess the reproducibility of the data.
Species separation among *Uronychia* spp. 85

(Chen et al. 2000). Four random primers were used, the sequences of which were as follows: S040 - 5' GTT GCG A TC C - 3'; S103 - 5' AGA CGT CCA C - 3'; S104 - 5' GGA AGT CGC C - 3'; S113 - 5' GAC GCC ACA C - 3' (Sangon Bio Co., Canada).

Ribosomal DNA amplification and digestion (ARDRA)

PCR amplifications were performed in a PCR thermal cycler. Genome DNA (50 ng) was mixed with 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.1% Triton X-100, 3 mM MgCl₂, 0.2 mM dNTP, and 0.5 mM of each oligonucleotide primer (16S-like F: 5' - AAC CTG GTT GA T CCT GCC AGT - 3'; 23S-like R: 5' - TTG GTC CGT GTT TCA AGA CG - 3') and 5 units of Taq Ex polymerase (TaKaRa, Japan). Distilled water was added to the mixture to a total volume of 100 µl. The reaction mixtures were denatured at 94°C for 5 min before the polymerase was added, followed by the first 5 cycles consisting of denaturation for 1 min at 94°C, primer annealing for 2 min at 56°C and extension for 2 min at 72°C. In the subsequent 35 cycles, the annealing temperature was raised to 62°C. The circulation was followed by a final extension step for 5 min at 72°C (Elwood et al. 1985, Medlin et al. 1988, Jerome and Lynn 1996).

The amplified ribosomal DNA products were purified using the UNIQ-5 DNA Gel Extraction Kit (Sangon Bio. Co., Canada) and dissolved in 50 µl Tris-EDTA solution (pH 8.0). Restriction of amplified ribosomal DNA was carried out by separate incubations at 37°C for 1.5 h with each of 10 restriction enzymes: *Kpn* I, *Taq* I (65°C), *Xba* I, *Bam* HI (30°C), *Eco* R I, *Hind* III, *Eco* R V, *Hae* III, *Msp* I, *Pst* I (TaKaRa Bio. Co., Japan). The total volume of restriction mixtures was 10 µl containing 0.5 µl of each restriction enzyme solution, 1 µl 10x buffer for each enzyme, 1 µl BSA (0.1% w/v) and 7.5 µl PCR product (Shang et al. 2002).

Data analysis

The band-sharing index of RAPD fingerprinting for two individuals is based on that used by Wetton et al. (1987) for comparing DNA fingerprints and is given by the formula: \( S = 2 N_{AB} / (N_A + N_B) \), where \( N_A \) and \( N_B \) are the number of bands scored in ciliates A and B respectively and \( N_{AB} \) is the number shared by both.

RESULTS AND DISCUSSION

Morphological comparison

The strain of *U. transfuga* could be clearly separated from the 6 strains belonging to the other two species by the following characters: more elongated, rectangular body shape; body size in *vivo* 110-250 x 80-180 µm; macronucleus moniliform comprising several to many (6-13) segments which form a horseshoe shape; base of buccal cirrus conspicuously long and narrow (Fig. 2, Table 1). The number of macronuclear segments is considered a particularly reliable character for *U. transfuga* thereby confirming its identity unequivocally (Song and Wilbert 1997).

*Uronychia setigera* and *U. binucleata* are morphologically similar and are characterized as follows: two macronuclear segments often connected by short funiculus, but sometimes only one segment in globular forms of *U. setigera* (Song and Wilbert 1997); invariably with one small micronucleus between the macronuclear segments; rapidly crawling or very fast swimming movement; 4 frontal and 2 ventral cirri; 4 large and one small transverse cirri; 3 strong left marginal cirri; constantly 6 dorsal kineties. *Uronychia setigera* differs from *U. binucleata* by having fewer basal body pairs in the leftmost dorsal kinety (Fig. 1, arrowed), smaller body size, a conspicuous (vs inconspicuous) spur on the left cell margin and its characteristic buccal apparatus (Figs 1, 2; Table 1).

Thus, our findings confirm those of Song and Wilbert (1997) who also reported that *U. transfuga* is morphologically distinct from *U. binucleata* and *U. setigera*, and that the latter two are so morphologically similar that they can only be separated following meticulous observations of cells in *vivo* and of the infraciliature following protargol impregnation. It has been suggested that in cases such as this, molecular methods, which have the potential to provide informative data independent of the morphological organisation, might be usefully employed (Clark 1997).

RAPD fingerprinting

The RAPD fingerprinting patterns obtained with the 4 random primers revealed different polymorphic band patterns (Fig. 3). The control with no DNA template amplified no bands. Lane 7 (*U. transfuga*) was distinctly different from the other 6 samples for each of the
4 primers. Lanes 4 to 6 (U. binucleata) shared similar band patterns though the intensity of some amplified bands varied due to the different concentration of template DNA. The same is true for lanes 1 to 3 (U. setigera).

Calculating the mean similarity index of the DNA banding patterns for each pair of strains, we found a genetical relatedness of 82-100% between different strains of the same species (Table 2, in bold) and only 30-40% between strains of different species (Table 2, in italics). Specifically, there was an 82-89% similarity among the 3 strains of U. setigera, and over 92% among the 3 strains of U. binucleata. The 11-18% dissimilarity among U. setigera strains and <8% dissimilarity among U. binucleata strains in the RAPD patterns can be explained by genotypical variance and genetic diversity within these species (Mathieu-Daudé et al. 1995, Kusch and Heckmann 1996, Kusch 1998, Stoeck and Schmidt 1998, Stoeck et al. 2000). On other hand, there was a mean similarity of only 35% between U. setigera and U. transfuga, 39% between U. binucleata and U. transfuga and 34% between U. setigera and U. binucleata (Table 2).

Kusch and Heckmann (1996) revealed that the similarity index was 0.38-0.46 (i.e. a 38-46% similarity) when different species of Euplotes were compared, and 0.60-0.78 (60-78% similarity) among strains of the same species. Our findings are consistent with these results. Thus, based on the polymorphic RAPD patterns we can confidently separate the 3 Uronychia species. Notably, our results confirm the separation of uronychias with two macronuclear segments into two distinct species, U. setigera and U. binucleata. This is in contrast to the findings of Foissner et al. (2001) who were unable to
Table 1. Morphological comparison of three *Uronychia* species. Data based on Song and Wilbert (1997).

<table>
<thead>
<tr>
<th>Species</th>
<th>Cell length <em>in vivo</em> (µm)</th>
<th>Number and appearance of macronuclear segments</th>
<th>Base of buccal cirrus</th>
<th>Body shape</th>
<th>Number of basal body pairs in leftmost dorsal kinety</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Uronychia setigera</em></td>
<td>40-70 (mostly 45-55)</td>
<td>1 or 2 (usually 2) sausage-like (occasionally as a single spherical mass)</td>
<td>short, <em>ca</em> 2-3 µm long</td>
<td>oval, lateral spur conspicuous</td>
<td><em>ca</em> 15 (12-19)</td>
</tr>
<tr>
<td><em>Uronychia binucleata</em></td>
<td>70-120 (mostly 90-110)</td>
<td>constantly 2, sausage-like sometimes in two-doublets form</td>
<td>short, <em>ca</em> 3 µm long</td>
<td>long oval, lateral spur inconspicuous</td>
<td><em>ca</em> 30 (23-40)</td>
</tr>
<tr>
<td><em>Uronychia transfuga</em></td>
<td>110-250 (mostly 150-210)</td>
<td>6-13, moniliform, forming a C-shape</td>
<td>conspicuously long, 10-14 µm in length</td>
<td>elongated rectangular, lateral spur inconspicuous</td>
<td><em>ca</em> 50 (46-56)</td>
</tr>
</tbody>
</table>

Table 2. Similarity indices (S) of pairwise comparisons of RAPD patterns of 7 *Uronychia* strains. S was calculated according to Nei and Li (1979) from RAPD fingerprinting with 4 different primers with a total 56 polymorphic bands (*U. setigera* - strains 99062701, 99102305, 01041607; *U. binucleata* - strains 01042001, 01042106, 01051801; *U. transfuga* - strain 00042803).

<table>
<thead>
<tr>
<th></th>
<th>99062701</th>
<th>99102305</th>
<th>01041607</th>
<th>01042001</th>
<th>01042106</th>
<th>01051801</th>
<th>00042803</th>
</tr>
</thead>
<tbody>
<tr>
<td>99062701</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>99102305</td>
<td>0.82</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>01041607</td>
<td>0.89</td>
<td>0.86</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>01042001</td>
<td>0.30</td>
<td>0.31</td>
<td>0.35</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>01042106</td>
<td>0.36</td>
<td>0.37</td>
<td>0.42</td>
<td>0.92</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>01051801</td>
<td>0.30</td>
<td>0.31</td>
<td>0.35</td>
<td>1.00</td>
<td>0.92</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>00042803</td>
<td>0.36</td>
<td>0.37</td>
<td>0.33</td>
<td>0.39</td>
<td>0.40</td>
<td>0.39</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Z. Chen et al.

separate two morphospecies of the hypotrich ciliate *Gonostomum* using RAPD. Nothing is known about the DNA sequences amplified in RAPD fingerprinting, however, and specific bands cannot be associated with particular loci in the genome (Lynch 1991). Consequently RAPD data on their own are not sufficiently reliable to unambiguously identify *Uronychia setigera* or *U. binucleata*.

**ARDRA**

Two primers were used in order to amplify the complete small subunit (16S), and part of the large subunit (23S), ribosomal RNA gene (including internal transcribed spacers). The lengths of these amplified products were similar (ca 3000 bp) for each of the 7 strains. Ten restriction enzymes were used to select informative bands between two species (strain 99102305 vs strain 01042106) beforehand (Fig. 4). All enzymes with the exception of *Kpn I* could digest the DNA product while only *Msp I* revealed different restriction patterns between the two strains. Subsequently the amplified products were restricted with *Msp I* enzyme which revealed two kinds of patterns among the 7 strains; the 3 strains of *U. setigera* and the *U. transfuga* strain had one pattern, whereas the 3 strains of *U. binucleata* had another (Figs 5a,b). By using a second restriction enzyme (*Alu I*) it was possible to separate *U. transfuga* from *U. setigera* and *U. binucleata* since the former had a species-specific restriction band of about 400 bp which was absent in the other two (Figs 5c,d). None of the other restriction enzymes tested produced riboprints with identifiable patterns.

ARDRA riboprinting, has previously been successfully applied for the separation and identification of various protozoan taxa including species of *Entamoeba* (Clark and Diamond 1997), trypanosomes (Clark *et al.* 1995) and sibling species belonging to the *Tetrahymena pyriformis* species complex (Jerome and Lynn 1996). The results of the present study suggest that the morphologically similar *U. setigera* and *U. binucleata* can be identified among *Uronychia*-populations by their species-specific ARDRA patterns using restriction enzyme *Msp I*. Interestingly, the riboprint pattern for *U. transfuga* is similar to that of *U. setigera* (compare lanes 1-3 and lane 7, Figs 5a,b), although the two are morphologically distinct.

In conclusion, although we have shown that different strains of *Uronychia* can be characterized by the unique digestion patterns of their rDNA fragments, it cannot be stated unequivocally that these riboprints are absolutely species-specific since currently no published riboprint data are available for *Uronychia multicirrus*. Furthermore, although we were unable to separate the 3 *Uronychia* spp. using a single enzyme, *U. transfuga* could be separated from *U. setigera* and *U. binucleata* using *Alu I*, while the restriction patterns produced with *Msp I* enzyme provide a reliable basis for the identification of these latter two.

**Acknowledgements.** This work was supported by the “Key Program of the Education Ministry of China”, the “Nature Science Foundation of China” (Project Number 30170114) and a Royal Society/NNSFC Joint Project Grant (Project Number Q822). We are grateful to Miss Huimin Shang, a graduate student of the Laboratory of Protozoology, OUC, for her assistance with the PCR procedure.

![Fig. 5. ARDRA riboprint patterns and schematic representation of 7 *Uronychia* strains by *Msp I* enzymes (a, b) and by *Alu I* enzyme (c, d). Lane M - 100 bp molecular markers. Lanes 1-3 - three strains of *U. setigera* (strains 99062701, 99102305, 01041607). Lanes 4-6 - three strains of *U. binucleata* (strains 01042001, 01042106, 01051801). Lane 7 - *U. transfuga* (strain 00042803).](image-url)
Species separation among *Uronychia* spp.

Kirby H. (1934) Some ciliates from salt marshes in California. *Arch. Protistenkd.* **82**: 114-133


Mansfeld K. (1923) 16 neue oder wenig bekannte marine Infusorien. *Z. Parasitenkd.* **46**: 97-140


Quennerstedt A. (1867) Vidrag till Sveriges Infusorie-fauna. II. *Arch. Protistenkd.* **413-444


Taylor C. V. (1928) Protoplasmic reorganization of *Uronychia uncinata* sp. nov. during binary fission and regeneration. *Physiol. Zool.* **1**: 1-25


Received on 24th September, 2002; revised version on 31st January, 2003; accepted on 2nd March, 2003
Comparison of Lectin Induced Chemotactic Selection and Chemical Imprinting in *Tetrahymena pyriformis*

László KŐHIDAI, Csaba BÁNKY and György CSABA

Department of Genetics, Cell and Immunobiology, Semmelweis University Budapest, Hungary

**Summary.** In case of hormonal (chemical) imprinting, the first encounter of a mixed cell population with a bioactive molecule provokes changes in the receptor-signal transduction system. In case of chemotactic selection, from the mixed cell culture the chemotactically most affine cells are selected and maintained. In the present experiments chemotactic effects elicited by three lectins (Concanavalin-A = Con A, lens, Helix) were studied in *Tetrahymena pyriformis* GL unicellular model, for comparing the effect of imprinting and selection. *Tetrahymena* (without selection or imprinting) showed a molecule-dependent (positive) chemotactic reaction to lectins. Subpopulations gained by chemotactic selection were inducible in a ligand-specific scale with preference of [α-Man-α-Glu] or N-Ac-Gal specificity lectins, producing a significant affinity to Helix and non-significant affinity to Con A, and negative chemotaxis to lens. Similar results were shown in case of selection and imprinting with Con-A (except the significant affinity of mixed population), nevertheless in case of Helix and lens lectins (having similar, however not identical sugar specificity) differences had been observed between selection and imprinting. These accordances and deviations of results in imprinting and selection studies suggest that chemotaxis receptors share functional moieties with lectin receptors of unicellular organisms. The experiments call attention to the possible role of selection in the development of imprinting however, this is dependent on the nature of the imprinter molecule.

**Key words:** chemotactic selection, chemotaxis, hormonal imprinting, lectin, *Tetrahymena pyriformis* GL.

**INTRODUCTION**

Lectins are multifunctional monovalent or complex peptide-type substrates possessing carbohydrate specificity. Wide ranges of biological entities (plants, animals, bacteria or viruses) have the ability to synthesize these molecules. Their roles are very diverse as some of these ligands are essential intracellular regulatory components of the protein traffic and sorting in the rER (L-type lectins, calnexin) or in the Golgi/post-Golgi system (P type lectins), while others have role in the ER associated degradation of glycoproteins (M-type lectins). Some lectins have the ability to influence the turnover of glycoproteins; others are effector molecules of enzyme targeting (R-type lectins). Other distinct group of lectins have cell surface or extracellular matrix associated functions: several types of lectins contribute to the cell adhesion (e.g. C- and I-type lectins); others are members of the innate immunity (cell lectins) or promote
cross-linking between glycan components (galectins) of the extracellular matrix (Drickamer and Taylor 1998).

Extracellular activities of lectins with their specific recognition of saccharide components provides a primordial, “immune system”-like significance of these molecules in organisms possessing no genuine humoral and cellular immune network (e.g. sponges, tunicates). Other basic cell-physiological functions like phagocytotic behaviour, the target reaction of chemotaxis, and growing of cells are also influenced by lectins at higher and lower levels of phylogeny (Agrell 1966). Chemoattractant or chemorepellent moieties of lectins are essential even on these levels; distinct subpopulations of cells are mobilized or localized by them. This activity of lectins is also detectable at unicellular level; animal and plant type lectins - Helix, Con A etc., can induce or suppress chemotactic responses of the eukaryotic ciliate *Tetrahymena pyriformis* (Köhidal and Csaba 1996). Ciliary membrane of these cells possess numerous binding sites for lectins (Pagliaro and Wolfe 1987, Csaba and Kovács 1991, Kovács et al. 1995, Driscoll and Hufnagel 1999), in one of the experiments sixteen Con-A binding polypeptides were demonstrated (Dentler 1992), in an other experiment the Con-A binding protein seemed to be a 66 kDa glycoprotein (Leick et al. 2001). Lectins are also applied to follow changes of carbohydrate composition or membrane fluidity in the ciliate surface membrane as a result of treatments with bioactive substances (Köhidal et al. 1986).

*Tetrahymena* is a frequently used model cell in the study of cellular signaling (Csaba 1985, 1994). Its homologies to the higher vertebrates is present at membrane receptor level (i.e. insulin receptor) (Kovács and Csaba 1990a, b; Christopher and Sundermann 1995; Leick et al. 2001; Christensen et al. 2001) in intracellular second messenger systems as cAMP (Csaba and Lantos 1976), cGMP (Köhidal et al. 1992), Ca-calmodulin system (Schultz et al. 1983; Kovács and Csaba 1987a, b), inositol lipids (Kovács and Csaba 1990a, b) and in homologies of hormonally influenced metabolic responsiveness (Köhidal and Csaba 1985). On the basis of the mentioned characteristics was observed the hormonal imprinting, which develops at the cell’s first encounter with a biologically active molecule and persists also after hundreds of generations. As a result of imprinting the binding capacity of receptors is influenced permanently as well, as signal transduction and responsiveness (Csaba 1994, 2000).

Chemotactic activity of these unicellular ciliates is one of the most essential cell-physiological properties. They can distinguish slight diversities of small and relatively big ligands i.e. amino acids (Levandowsky et al. 1984) and insulins (Csaba et al. 1994). Chemotactic selection, a recently developed technique provides the possibility to select these cells upon their chemotactic responsiveness towards bioactive molecules (e.g. hormones or chemokines) (Köhidal and Csaba 1998, Köhidal et al. 2000). Selected subpopulations are dedicated groups of cells for studying backgrounds of chemotaxis, short- and long-term signalling in eukaryotic models (Köhidal 1999).

On the basis of the above described phylogenetical and cell-physiological characteristics of lectins in the present study our objectives were: (i) to study ability of lectins to select subpopulations *via* their chemotactic potency; (ii) to study the effect of pretreatments (imprinting) with lectins on the chemotactic responsiveness of subpopulations; (iii) to compare the results of imprinting and chemotactic selection.

### MATERIALS AND METHODS

#### Cells and culturing

Cells of *Tetrahymena pyriformis* GL strain were maintained in axenic cultures containing 1% tryptone (Difco, Michigan, USA) and 0.1% yeast extract, without addition of antibiotics, at 28 °C. According to the certificate analysis, the two basic components of media were free of lectins. However, the potential of interference of the used lectins with the media composing peptides is given, the non-synthetic type of media was used as our earlier experiments (Köhidal and Csaba 1998, Köhidal 1999) and reference works of the chemotaxis-literature of *Tetrahymena* were done in such systems (Almagor et al. 1981, Francis and Hennessey 1995, Kuruvilla and Hennessey 2001). Cultures were in logarithmic phase of growth; density of samples was 10⁶ cells/ml.

#### Chemicals and buffers

The studied lectins were purified from plants *Canavalia ensiformis* (Con A), *Lens culinaris* (lens) and snail *Helix pomatia* (Helix). All the three lectins were obtained from Sigma Chemical Co., St. Louis, MO, USA. In the experiments (PBS), 0.05 M phosphate buffer containing 0.9% NaCl at pH 7.2 was also used.

#### Chemotaxis assay

The chemotactic ability of cells was determined in a two-chamber capillary assay system modified by us (Köhidal et al. 1995). According to this setup tips of a multi-8-channel automatic pipette served as an inner chamber to minimize the standard error of sampling, while microtitration plates were used as outer chambers. The outer chamber was filled with the cells to be tested; the inner one contained the test substance of the lectins. In control experiments culture medium was...
used as attractant (absolute control). For validation of the assay two chemotactartants 10^{-9} M f-Met-Leu-Phe (f-MLF; Sigma, St. Louis, USA) and 10^{-11} M interleukin-8 (IL-8; Promega, Madison, USA) were also tested as positive controls. After 15 min incubation the samples of inner chambers, containing the chemotactically positive responder cells, were fixed in 4% formaldehyde containing PBS. The samples were evaluated in a Neubauer hemocytometer.

**Chemotactic selection**

This technique deals with the chemotactic capacity of different signal molecules to form sub-populations from mixed cultures of cells. First we applied the chemotaxis assay (see above). The inner chamber of the system contained the test substance of the lectins (10^{-9} M in case of Con-A and 10^{-6} M in case of lens and Helix). In control experiments culture medium was used as attractant (absolute control). At the end of incubation, the positive responder cells were transferred to fresh culture medium for cultivation. Both cultures selected with a lectin (in general L) and controls (C) were consecutively transferred in every 48 h. The chemotactic response of cultures was studied again after one week in the following combinations: L/L - cells selected with the lectin in the first run and assayed to the signal substance in the second run; L/C - cells selected with the lectin in the first run and assayed to the control substance in the second run; C/L - cells selected with the control substance in the first run and assayed to the signal substance in the second run; C/C - cells selected with the control substance in the first run and assayed to the control substance in the second run.

**Pretreatment (imprinting) with lectins**

*Tetrahymena* cells were pretreated with 10^{4} M lectins (Con A, lens and Helix) for 1 h at room temperature. Controls were treated with fresh culture medium. After treatments the samples were washed trice with PBS and then aliquots of cell-suspensions were transferred into fresh culture medium. Pretreated and control cultures were transferred consecutively in every 48 h. After one week chemotactic responsiveness of cultures was tested by the chemotaxis assay described above. Similar combinations were applied as in the chemotactic selection study: C/C, C/L, L/C, L/L (abbreviations are given above).

**Statistical evaluation**

Each lectin was tested in ten replica assays, the figures demonstrate the averages of these results. The statistical analysis was done by ANOVA of Origin 4.0.

**RESULTS AND DISCUSSION**

In an earlier experiment (Köhidal and Csaba 1996) all the three investigated lectins proved to have chemotactartant ability, however the concentration optimum was characteristic to each chemotactartant ligand (Table 1). The effective chemotactartant concentration was wide in the case of both plant lectins. In the case of the Con A - possessing α-mannose and α-glucose specificity - a two-peak (10^{-9} and 10^{-6} M) dose-dependency was detected with the peak at 10^{-9} M. In contrast, the α-mannose specific lens lectin had a wide range chemotactartant character, its maximal chemotactartant effect was elicited only in the higher concentration range at 10^{-6} M. Profile of concentration dependency of chemotaxis elicited by the N-acetyl-galactose-amine specific Helix lectin was similar to lens, its maximal chemotactartant effect was observed also at the highest tested concentration (10^{-4} M). In this study Helix proved to be the only chemorepellent lectin, this negative responsiveness of the model cells was elicited at 10^{-11} M. The most positively effective concentration was chosen for the chemotactic selection assay in each case.

Chemotactic selection provides the possibility to distinguish signaling mechanisms used by different ligands. In classical way chemotaxis is a result of a specific interaction of ligands and their receptors in the plasma membrane. As a consequence of the downstream signaling in the cytoplasm reorganisation of cytoskeletal network is triggered and migratory behaviour of cells is changed. Dynamic presence of chemotaxis-receptor components can determine consistence of chemotactic responsiveness in progeny generations, unstable structure or fast turn-over of some binding sites make them responsible for “short-term” chemotactic responsiveness, while other fixed, constant and genetically well preserved units of the plasma membrane might work as receptors of “long-term” acting chemotactartant ligands. During chemotactic selection the most responsive cells are selected and maintained, forming a special group of the original cell population.

In the present study the three lectins could select subpopulations chemotactically in different ways (Fig. 1). Chemotactic responses were detected in sub-

---

**Table 1.** Concentration course study on chemotactic activity of three lectins in *Tetrahymena pyriformis* GL cells. (x- p<0.05; y- p<0.01; z- p<0.001).

<table>
<thead>
<tr>
<th>Conc. log molar [M]</th>
<th>Con A [%]</th>
<th>Lens [%]</th>
<th>Helix [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>-12</td>
<td>120</td>
<td>127</td>
<td>106</td>
</tr>
<tr>
<td>-11</td>
<td>109</td>
<td>109</td>
<td>79</td>
</tr>
<tr>
<td>-10</td>
<td>128</td>
<td>154</td>
<td>98</td>
</tr>
<tr>
<td>-9</td>
<td>159</td>
<td>172</td>
<td>131</td>
</tr>
<tr>
<td>-8</td>
<td>144</td>
<td>209</td>
<td>137</td>
</tr>
<tr>
<td>-7</td>
<td>131</td>
<td>190</td>
<td>167</td>
</tr>
<tr>
<td>-6</td>
<td>150</td>
<td>227</td>
<td>181</td>
</tr>
</tbody>
</table>

---
populations selected with the plain culture medium. We considered these results as relative controls of the successful chemotaxis assay with the identical lectin. However, it was found that the chemotactic potency and activity of the subpopulations selected with chemotaxis was characteristically different. In control-medium-selected cells, the control medium itself (composed of tryptone and yeast extract) was observed to have a potent selector effect on *Tetrahymena*. Comparing the results of our selection study determining different chemotactic responses of *Tetrahymena* with varying concentration courses of the same lectins shows that the response of cells selected with this control substance was ligand-dependent. In the present experiment the responsiveness was decreased with Con A (159% vs. 122.9%; not significant) and lens (227 vs. 49.8; p<0.01) and increased with Helix (136.9%; vs. 181.2% p<0.05).

Fig. 1. Chemotactic selection of *Tetrahymena* cells with lectins (Con A, lens, Helix). Chemotactic responsiveness of cells selected with the identical lectin (L) or the control substance (C) was assayed in four combinations: the first letter refers to the type of ligand applied at selection; the second letter refers to the ligand used at the second chemotaxis assay 1 week after selection. (x- p<0.05; y- p<0.01; z- p<0.001).

Fig. 2. Effect of imprinting with 10^{-6} M lectins (Con A, lens, Helix) on *Tetrahymena*. Chemotactic responsiveness of pretreated cells with the identical lectin (L) or the control substance (C) was assayed in four combinations: the first letter refers to the type of ligand applied at pretreatments; the second letter refers to the ligand used at the second chemotaxis assay 1 week after pretreatments. (x- p<0.05; y- p<0.01; z- p<0.001).

Subpopulations selected with lectins expressed also ligand-dependent responsiveness. Chemotactic responsiveness of subpopulations selected with Con A or lens sharing in ligand-specificity to α-mannose was significantly decreased (Con A/C and lens/C), while a moderate, increased chemotactic ability (124.7%) was detected in subpopulations selected with the oligosaccharide specific Helix lectin (Hel/C).

Results of the repeated encounter with the selector lectin showed that the diverse membrane level changes responsible for different forms of chemotactic responsiveness in selection studies are detectable even for lectin-type ligands. In Con A selected group (Con A/Con A) the identical lectin could elicit a statistically significant and increased chemotactic response, while lens lectin (lens/lens) could not induce positive response, in contrast, the selected subpopulation showed negative chemotactic behaviour to the lens lectin. Subpopulations selected with Helix lectin expressed positive chemotaxis towards the selector ligand, however this activity of cells was not enhanced compared to their relative controls.
suggest that in our ciliated model a concurrent
the membrane play also important role. Data obtained
ment of chemotactic signaling carbohydrate moieties of
59x682] vs lens/lens = 66.2%) suggests that in the fine adjust-
ment of chemotactic signaling carbohydrate moieties of
the membrane play also important role. Data obtained
suggest that in our ciliated model a concurrent
59x669] α-mannose specific binding fails to do so. On the other
hand, relative positive effectiveness of Helix lectin raise
the possibility that plasma membrane is furnished with
constant, however non-inducible constituents e.g.
N-acetyl-galactose-amine residues.

While in case of chemotactic selection a rapid incu-
bation is used to assort cells from a mixed population
with high responsiveness, in case of imprinting (pretreat-
ment) with biologically active substances are still applied
to a mixed population to tune/adjust cells in a time- and
concentration-dependent way, however the investigated
cultures are still mixed. Series of former experiments
proved that Tetrahymena is a sensitive model for im-
printing; long-lasting or transient modifications of the
plasma membrane were characterized in binding studies
with hormones (Kovács et al.1984) or lectins (Csaba
and Kovács 2000). In the present experiment our goal
was to evaluate whether the lectin induced chemotactic
selection and imprinting have homologies or deviations in
signaling.

In this setup chemotactic responsiveness of lectin-
pretreated cells was tested to the identical lectin used for
imprinting or to the fresh culture medium as control. To
validate the experiments an absolute control (C/C) and
a second control (C/L) were applied, in which the
chemoattractant ability of the used lectin was tested.
Proper sensitivity of our system was confirmed by the
significant positive chemotactic indices of all the three
second controls (C/Con A, C/lens and C/Hel) (Fig. 2).

In case of Con A, imprinting had dual effect: the
pretreated cells expressed an increased chemotactic
responsiveness to Con A at the second encounter
(Con A/Con A=198.4%), while the plain culture medium
was recognized as chemorepellent (Con A/C = 84.6%).

Lens lectin could not induce long-lasting positive
effects like Con A. Pretreatment with lens abdolished
the positive chemotactic responsiveness of Tetrahymena
cells to the lectin (lens/lens = 92.3%), nevertheless a
decrease in the native chemotactic ability of the lens
pretreated cells (lens/lens = 92.3% or lens/C = 101.2%
vs. C/lens = 227.2%) was also detected.

Pretreatments with Helix lectin presented also a
unique and characteristic outcome. In contrast Con A
and lens lectins the native chemotactic activity of the
Helix pretreated cultures (Hel/C = 160.7%) was en-
hanced, whereas we failed to apply the identical lectin as
a chemoattractant ligand, it worked as a very strong
chemorepellent substance (Hel/Hel = 42%), what means
that a negative imprinting developed.

As it was presented above, interaction of the three
lectins with the target cells has a long lasting effect
considering their chemotactic ability. Results of repeated
encounters with the ligand applied at the pretreatment
suggest that lectins possessing slight diversities in carbo-
hydrate specificity (see variance of α-glucose specific-
ity of Con A to lens) can result an increase or decrease
of chemotaxis receptors however, the diversity in the
protein configurations of lectins also have to be consid-
ered, as this can also influence the affinity or repellence.
This means that only the comparison of the results
gained by imprinting or selection leads to clear and exact
conclusions, the results of inter-lectin comparison sug-
gest only possible explanations. One of this explanations
is that while selection of sensitive cells might have a role
in the development of imprinting (e.g. in case of Con-A),
this is not regular, as other factors also could play a role,
which turned to negative imprinting of the positive
selection (e.g. in case of Helix lectin).

Cultures of the present study were maintained for one
week after pretreatments, the reexposed cells, due to the
short cell cycle of Tetrahymena, were ~70th generation
of the culture, this made possible to study long-term
persisting receptors and the associated activities. Poten-
tial significance of lectin receptors in chemotaxis- work-
ing as a probable subclass of chemotaxis receptors - is
underlined in two reasons (i) the investigated group of
lectins could characteristically induce the expression of
saccharide components of the plasma membrane ca-
pable as components of the responsible binding sites
(e.g. coexistence of α-mannose and α-glucose is re-
quired for the positive outcome) and (ii) can elicit
chemotactic responses in a high carbohydrate-specific
manner (e.g. the amino-sugar N-acetyl-galactose-amine
linked signaling could “freeze” the required chemotactic
pathways).

Comparison of results gained by chemotactic selec-
tion and pretreatment show that ligands e.g. lectins,
possessing deep phylogenetical backgrounds have mole-
cule-specific potency to act as chemotactic agents.
Chemotactic responsiveness induced by imprinting has
similarities and diversities to chemotactic selection: con-
gruence of positive Con A/Con A results of the two different experiments raises the possibility that imprinting might have a selector capacity, too; however other ligand (Helix lectin) could work also as a good selector but a reversed, negative effect was registered after imprinting. It is not unusual that different lectins having similar sugar affinity disparately influence cell functions: this was observed in case of cell aggregation (Csaba and Kovács 1991), and conjugation (Ofer et al. 1976).

In conclusion, present data point out that three lectins, that are phylogenetically ancient signal molecules, can elicit molecule-specific chemotactarant responses in unicellular model. Investigations of chemotactic selection and imprinting with the lectins call attention thatchemotaxis is based upon fine receptor-lectin interactions and that carbohydrate moieties are potent functional components of chemotaxis receptors. In earlier experiments similar phenomenon was demonstrated in case of insulin receptors (Kovács et al. 1987, 1996). The experiments demonstrate that in contrast to the similarities in the provocation of reaction (treatment with a bioactive molecule and study of later response), the selected population can show similar or disparate reaction in chemotactic assay, depending on the sugar affinity (and maybe on the protein structure) of the provoking molecule.

Molecular-genetic backgrounds of the described phenomena is still obscure. As both selection and pretreatments have the potency to interfere with the genetic mechanisms of this protozoon, causing changes in long-term cell-physiological responsiveness, we intend to broaden our analysis into genetic aspects.

Acknowledgements. This work was supported by grant from the Hungarian Research Fund (OTKA, T032533 and T037303).

REFERENCES

Csaba G., Lantos T. (1976) Effect of cAMP and theophylline on phagocytotic activity of Tetrahymena pyriformis. Experimental 32:
Csaba G., Kovács P., Kőhidai L. (1994) Tetrahymena cells distinguish insulins according to their amorphous and crystalline condition or their bovine and porcine origin. Study of imprinting in aspects of hormone binding and chemotaxis. Microbios 80: 215-221
Kőhidai L. Csaba G. (1998) Chemotaxis and chemotactic selection induced with cytokines (IL-8, RANTES and TNFα) in the unicellular Tetrahymena pyriformis. Cytokine 10: 481-486


Received on 4th October, 2002; revised version on 24th January, 2003; accepted 4th February, 2003
Population Dynamics and Food Preferences of the Testate Amoeba *Nebela tincta major-bohemia-collaris* Complex (Protozoa) in a *Sphagnum* Peatland

Daniel Gilbert¹, Edward A. D. Mitchell², Christian Amblard³, Gilles Bourdier³ and André-Jean Francez⁴

¹Laboratoire de Biologie et Écophysiologie, USC INRA, Université de Franche-Comté, Besançon, France; ²Department of Biological Sciences, University of Alaska Anchorage, Anchorage, U.S.A.; ³Laboratoire de Biologie Comparée des Protistes, UMR CNRS, Université de Clermont-Ferrand, Aubière Cedex, France; ⁴Équipe Interactions Biologiques et Transferts de Matière, UMR CNRS Ecobio, Université de Rennes I, Rennes Cedex, France

**Summary.** Population dynamics and food preferences of the testate amoeba species complex *Nebela tincta major-bohemia-collaris* ("*Nebela collaris sensu lato*") were described from a *Sphagnum* peatland over one growing season. The average abundance of *Nebela collaris sensu lato* was 29582 ind. l⁻¹ active, and 2263 ind. l⁻¹ encysted forms. On average, 17.4% of *Nebela collaris sensu lato* specimens were observed associated with prey, 71% of which could not be identified because of their poor preservation state. Among the identified prey, those most frequently ingested were micro-algae (45% of the total predator-prey associations, especially diatoms: 33%), and spores and mycelia of fungi (36%). Large ciliates, rotifers and small testate amoebae were also ingested, but mainly in summer. The seasonal variations in the proportions of prey categories in the ecosystem and the percentage of identifiable prey lead us to hypothesise that (1) *Nebela collaris sensu lato* ingest mainly immobile, senescent or dead organisms, and (2) that the more mobile micro-organisms such as ciliates and micro-Metazoa become more accessible, in relatively dry conditions, when the water film is thin.

**Key words:** microbial food web, Nebelidae, population dynamics, Rhizopoda, soil micro-organisms, Wetland.

**INTRODUCTION**

In aquatic environments, knowledge on the trophic interactions within microbial food webs has benefited from the use of artificial or fluorescent-labelled prey. This approach is used to study the bacterivorous activity of micro-organisms in marine and lacustrine pelagic ecosystems (Borsheim 1984, Pace and Bailiff 1987, Sanders *et al.* 1989, Simek *et al.* 1990, Carrias *et al.* 1996). More recently the same principle has been applied using labelled eukaryotes (Premke and Arndt 2000). By contrast in environments where an interface between the solid and liquid exists (including the benthic zone of fresh- and salt-water bodies in aquatic ecosystems, the upper horizons of soils in terrestrial ecosystems, bryophytes, and lichens) these techniques are difficult to use mainly because many predators feed on
fixed and/or mobile prey and that the potential range of these prey is impossible to reconstruct artificially.

As a result, although the functional importance of the microbial loop has been established in several interface environments such as soils (Clarholm 1994, Coleman 1994, Bonkowski and Brandt 2002), and peatlands (Gilbert et al. 1998a, b), little data is available on the feeding habits of protozoa in these environments. Information on the feeding habit of testate amoebae especially is very scarce (Coûteaux and Pussard 1983, Coûteaux 1984, Chardez 1985, Yeates and Foissner 1995, Gilbert et al. 2000), although this group represents an important part of the protozoan biomass in these interfaces (Gilbert et al. 1998a, b; Mitchell et al. 2003). This study therefore aimed at establishing the feeding habits of one of the most abundant testate amoeba group in Sphagnum peatland, the Nebela tincta major-bohemica-collaris complex (hereafter “Nebela collaris sensu lato”) (Deflandre 1936, Heal 1964, Warner 1987), in natural conditions, in relation to the seasonal dynamics of their potential prey.

MATERIALS AND METHODS

The study was carried out in the Pradeaux peatland (Puy de Dôme, France, 3°55 E, 45°32 N, altitude 1350 m. a. s. l.), a drained Carex rostrata/Sphagnum fallax fen covering a surface of 10 ha (Francez 1992).

Three PVC tubes 25 cm in diameter and 30 cm in length were permanently inserted vertically in the peat in macroscopically identical locations in order to get triplicate measurements. Once a month, from April to November 1995, 50 ml water samples were collected in each tube by pressing the moss surface with a sieve (mesh size 1.5 mm) and sucking the water up with a syringe. During each site visit pH and conductivity were measured in situ with a Chekmate M90 multiparametric probe (pH: ± 0.01 unit; conductivity: ± 0.01 µScm⁻²). Water levels were measured with 30 cm piezometers inserted in the peat. Because of the high water holding capacity of Sphagnum and the relatively high water table levels it was possible to sample water every month except in August. This method, which was already used by Grolière (1977) for ciliates and by Francez (1988) for rotifers, allows the recovery of most micro-organisms. However, this method probably underestimates the densities of at least some microbial groups because they may be attached to the Sphagnum mosses, or because they may be living inside the large, empty cells of Sphagnum called hyalocysts. Conversely, an advantage of this method is that as the Sphagnum carpet is very little affected by the sampling, repeated sampling at the exact same place is possible. This is important because fine-scale heterogeneity in testate amoeba distributions may occur even in a macroscopically homogeneous Sphagnum moss carpet (Mitchell et al. 2000). The water samples were fixed with glutaraldehyde (2% final concentration) and stored at 4°C in the dark. Cyanobacteria, fungi, micro-algae, protozoa, rotifers, and nematodes were identified using a sedimentation (plankton) chamber and an inverted microscope (Utermöhl 1958).

Bioluminescence of morphotypes within each community were estimated by assuming geometric shapes and converted to biomass using conversion factor according to Gilbert et al. (1998a). For each sample, we observed a minimum of 20 specimens of Nebela collaris sensu lato (total for this study: 684 active or encysted specimens). Among the active specimens, we distinguished those with an attached prey. We defined as “prey” any organic matter particle, even if precise identification was not possible because of poor preservation (e.g. decomposed organic matter or partly decomposed micro-organisms). The glutaraldehyde solution fixes efficiently all micro-organisms and we were able to verify that this method does not separate the prey from the predator, even after homogenizing the sample through the vortex.

RESULTS AND DISCUSSION

Physico-chemical environment

The water table level fluctuates during the year according to the relative importance of precipitations and evapotranspiration and the drainage of the site. These fluctuations are especially important in the case of drained peatlands such as the site we studied. At our site, the water table level remained high in spring (11 cm below the surface of the moss carpet). It then became gradually lower, until the beginning of summer when it reached 19.3 cm. During August, the water table fell below 30 cm, and it was impossible to sample surface water. The autumn rainfall did not fully compensate for this water deficit and the water level had only risen to 14 cm by November (Table 1). The water temperature at the surface of the peatland fluctuated between 0.7 and 24.4°C during the course of the study. The mean values for water pH and conductivity were 4.7 and 43.1 µS cm⁻¹ respectively. The concentration of dissolved oxygen fluctuated between 2.0 mg l⁻¹ in June and 9.8 mg l⁻¹ in November (Table 1). The dissolved oxygen concentration was logically negatively correlated to water temperature (n = 12, r = -0.99, P < 0.001). The conductivity was also negatively correlated to water temperature (n = 18, r = -0.457, P = 0.05), probably because the activity of Sphagnum, and therefore the nutrient uptake, increases with the temperature.

Population dynamics

Nebela collaris sensu lato was regularly present in Sphagnum mosses (averages: 29582 ± 9650 ind l⁻¹, for the active forms, and 2263 ± 1620 ind l⁻¹, for the encysted forms) (Fig. 1). The highest abundance of active
Nebela food preferences

Table 1. Temporal variations in physico-chemical variables (mean ± SE, ND not determined).

<table>
<thead>
<tr>
<th>Months</th>
<th>Water table depth (cm)</th>
<th>Temperature (°C)</th>
<th>pH</th>
<th>Dissolved oxygen (mg l⁻¹)</th>
<th>Conductivity (µS cm⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>April</td>
<td>11.0 ± 2.6</td>
<td>5.4 ± 0.6</td>
<td>4.5 ± 0.2</td>
<td>ND</td>
<td>56.4 ± 4.7</td>
</tr>
<tr>
<td>May</td>
<td>14.0 ± 2.0</td>
<td>13.2 ± 0.5</td>
<td>4.7 ± 0.1</td>
<td>5.8 ± 0.6</td>
<td>33.7 ± 8.6</td>
</tr>
<tr>
<td>June</td>
<td>16.3 ± 4.7</td>
<td>20.7 ± 0.1</td>
<td>4.5 ± 0.0</td>
<td>2.1 ± 0.2</td>
<td>26.7 ± 3.9</td>
</tr>
<tr>
<td>July</td>
<td>19.3 ± 2.1</td>
<td>19.8 ± 0.6</td>
<td>4.4 ± 0.2</td>
<td>ND</td>
<td>48.5 ± 7.0</td>
</tr>
<tr>
<td>August</td>
<td>&gt; 30.0 ± 0.0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>September</td>
<td>22.3 ± 2.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>October</td>
<td>22.3 ± 3.5</td>
<td>11.9 ± 0.4</td>
<td>4.3 ± 0.1</td>
<td>6.3 ± 0.2</td>
<td>47.2 ± 11.5</td>
</tr>
<tr>
<td>November</td>
<td>14.3 ± 2.1</td>
<td>6.7 ± 0.6</td>
<td>5.4 ± 0.2</td>
<td>9.5 ± 0.1</td>
<td>43.6 ± 10.7</td>
</tr>
</tbody>
</table>

forms was observed at the end of June (up to 37947 ± 9843 ind. l⁻¹) and the lowest in July (13172 ± 3137 ind. l⁻¹) (Fig. 1). This pattern seems to be characteristic for drained peatland, with winters being too cold and summers being too dry for microbial development. Similar temporal patterns were described before in Sphagnum for microalgae (Schoenberg and Oliver 1988) and ciliates (Grolière 1977). In the same way, the encysted forms were most abundant at the beginning of spring (4634 ± 2347 ind. l⁻¹ in April) when the water temperatures were still low, and after the dry period (mean for September, October and November: 2934 ± 2845 ind. l⁻¹) (Fig. 1). Cyst abundance was negatively correlated with temperature (r = -0.86, P = 0.03) and positively correlated with conductivity (r = 0.86, P = 0.03).

Seasonal feeding activity and general feeding habits

The frequencies of Nebela collaris sensu lato specimens observed associated with a prey (M=17.4 ± 6.0%) were low in spring (8.0% in April), rising until July (24.1%), and then decreasing in autumn (Fig. 2). However, these variations were not significant, and the number of specimens observed in association with a prey was positively correlated with the number of active individuals (n = 21, r = 0.53, P = 0.01). Thus our data suggest that the fraction of Nebela collaris sensu lato actively feeding is relatively constant throughout the year.

Among the identified prey, those most frequently ingested were micro-algae (45% of the total identified predator-prey associations, especially diatoms 33%), and spores and mycelia of fungi (36%). Predation on large ciliates (12%), rotifers (3%) and small testate amoebae (4%, among which Trinema spp. and Euglypha spp.) appeared to be more marginal (Figs 3, 4). However, on average, 71 ± 27% of the prey could not be identified because of their poor preservation state. These unidentified prey were most likely Sphagnum leaf fragments, other plant cells, or prey without rigid skeleton, such as green algae, ciliates, and most bdelloid.
rotifers. Fungi spores and mycelia as well as diatoms and testate amoebae were easily identifiable, even dead, because of their internal or external skeleton, or rigid cell walls. Thus the ingestion frequencies obtained for fungi, diatoms, and testate amoebae are probably quite accurate, whereas those obtained for rotifers and ciliates are most likely underestimated. However, glutaraldehyde being a very efficient fixative, we do not believe that the poor preservation stage of the majority of the prey is due to their degradation between the time of sampling and the time of analysis. We rather infer that the unrecognisable prey might have been dead before the amoeba started to feed on them. If true this would suggest that *Nebela collaris sensu lato* is mainly detritivorous. Nevertheless, in vivo observations of carnivorous Nebelidae populations (*Nebela* spp., *Hyalosphenia papilio*) showed that they are also capable of attacking living or senescent ciliates (Gilbert et al. 2000).

**Seasonal feeding habits**

The dominant kind of ingested prey varied with time. Because of the relatively low numbers of predators-prey associations observed in each sample, we grouped the sampling periods into three categories: spring (April and May), summer (June and July), and autumn (September, October and November). In spring and autumn, identified prey were dominated by fungi and autotrophic microorganisms, while during the summer period the main prey were autotrophic microorganisms, protozoa, and micro-metazoa (Fig. 5a). The biomass of different categories of selected prey was estimated in order to compare the nature and variations of their feeding habits to the seasonal dynamics of their prey. Figure 5b shows the biomass variations of the same microbial groups: fungi (mycelia and spores), micro-algae, protozoa and micro-metazoa. A significant relationship was found between the biomass of fungi in the environment and the
frequency of their ingestion by *Nebela collaris sensu lato* \((r = 0.99, P = 0.04)\), but no such relationship was found for the other groups. Because the size of our data set is limited, it is difficult to draw strong conclusions regarding the ability of *Nebela collaris sensu lato* to select their prey. Direct observations of microcosms with a range of prey types could clarify this question. Furthermore we described the predator-prey relationships with a low taxonomic resolution for the prey, but *Nebela collaris sensu lato* may select their prey at the species level. Thus, it remains possible that *Nebela collaris sensu lato* selects specific species of ciliates and rotifers that are more abundant or more accessible in summer when the lower water content of mosses makes these prey types more accessible. Indeed, the water content of *Sphagnum* mosses may play an important role in determining the ability of predators to catch their prey. *In vivo* observations showed that micro-organisms were located primarily between the leaves and on the stems of *Sphagnum*. In dry periods the water film in which micro-organisms live becomes thinner and their concentration therefore increases. In such conditions slow moving organisms like testate amoebae may be able to catch mobile predatory micro-organisms, such as ciliates or rotifers, otherwise too fast for them, whereas immobile, senescent or dead organisms, are accessible all year round.

The methodology we used neither allowed us to quantify the consumption of bacteria, because they are invisible, nor of naked amoebae, because they are difficult to observe, even alive. Furthermore, although glutaraldehyde is a fixing agent widely used in predation experiments of micro-organisms (*Borsheim 1984, Pace and Bailiff 1987*), the interpretations of results is difficult because many prey were not identifiable when they were fixed. Nevertheless, our study establishes that (1) *Nebela collaris sensu lato* feed on a wide range of living, senescent, or dead micro-organisms (fungi, microalgae, ciliates, other testate amoebae, rotifers) and organic remains, and (2) the proportion of predators among the identified prey is higher during the summer. Some prey seems to be selected, but further studies are needed to determine the importance of this phenomenon.

**Acknowledgements.** This study was conducted as part of a European Community environmental programme (3rd Framework, Contract No. EV5V-CT92-0099) and was financed by the CEREMCA Association. We also thank two anonymous reviewers for valuable comments on an earlier version of this paper.

**REFERENCES**


**Fig. 4.** Relative proportions (%) for the different identified prey categories (ND - not determined).

**Fig 5 a, b.** Temporal relative proportion of variations (a) of identified ingested prey and (b) of the relative proportion of the biomass of the same categories of prey in *Sphagnum* (ND - not determined).


Received on 18th November 2002; revised version on 11th February, 2003; accepted on 25th February 2003
Morphology, Biometry and Ecology of *Arcella excavata* Cunningham, 1919 (Rhizopoda: Arcellinida)

Milcho Todorov and Vassil Golemansky
Institute of Zoology, Bulgarian Academy of Sciences, Sofia, Bulgaria

**Summary.** Testate amoeba *Arcella excavata* Cunningham, 1919, isolated from the aeration tanks of the Sofia’s Wastewater Treatment Plant and its morphology, biometry and ecology have been investigated. Based on the rich live material (120 specimens), a detailed morphological description of *A. excavata* and new data about the cytoplasm and the nuclei of the species are supplied. It was found out that *A. excavata* is a multinuclear species and the nuclei are usually 3 or 4, rarely 5-6 (1-2% only). These new data, as well as the characteristic shape of the shell and its bigger depth, show clearly that *A. excavata* is a separate, well distinguishing species. The size frequency distribution analysis indicates that *A. excavata* is a size-monomorphic species, characterized by a main-size class and a small-size range (all measured individuals have a shell length of 60-70 µm and 98% have a shell breadth of 55-66 µm). The present study shows that *A. excavata* inhabits polluted waters also and this fact does not confirm the conclusion of Snegovaya that it can be used as a bioindicator of the waters with the saprobity β-α. Probably, it is also a freshwater eurybiont as well as the majority of the known species of family *Arcellidae*.

**Key words:** *Arcella excavata*, biometry, ecology, morphology, Rhizopoda, Testacea.

**INTRODUCTION**

The genus *Arcella* is one of the most numerous testacean genera. More than 130 taxa of the genus have been described till now. The majorities of them are cosmopolite and inhabit mainly the freshwater pools, moist mosses and rarely soil litter (Deflandre 1928, Chardez 1989).

Besides, some rare species, with a restricted distribution, have been described. The scanty data about their morphology, biometry, distribution and ecological preferences for many of them exist in the literature. Furthermore, the cytoplasm, the types of pseudopodia and nuclei for the majority of these species have not been observed and this causes difficulties in their systematical identification. *Arcella excavata* Cunningham, 1919, is one of these rare and poorly studied species of the genus *Arcella*.

*Arcella excavata* was observed and described in a small swamp near Durham, N. Carolina (USA) (Cunningham, 1919). The empty shells were observed only. According to the original description the shape of the shell is “somewhat like a quarter-section of cantaloupe, the mouth being situated in the cup. The color is brown to almost black. The sizes of the shell are: length
55 µm, width 50 µm, total depth 45 µm, depth of depression 25 µm, mouth 15 x 20 µm”.

According to Cunningham A. excavata is close to A. curvata Wailes, 1913, but differs from it by the considerably smaller sizes and by the height of the shell. Later Deflandre (1928) described A. curvata as a variety of A. polypora, Penard and reported that the sizes of this species range from 125 to 130 µm in diameter.

Deflandre (1928) included A. excavata in his monograph of the genus Arcella after the description and the figures of Cunningham (1919). Deflandre had a doubt about his independence and noted that: “il serait intéressant de voir si elle est réellement fixée dans la station d’où elle provient et si des exemplaires cultivés ne rétrograderaient pas vers une Arcella discoides type ou plutôt var. scutelliformis, dont les dimensions sont approchantes”.

More than forty years after the original description A. excavata was not observed in USA and Europe. Štěpánek (1953, 1954) reported the finding of empty shells of A. excavata in Moravice River and in aquatic mosses near the Janské Lazni, Krkonoše (Czech Republic). According to Štěpánek the sizes of the observed specimens range as follows: diameter 63-67 µm, depth 35-42 µm, diameter of aperture 16-24 µm, depth of the aperture collar 3 µm. Living specimens have not been observed.

In his monograph Bartoš (1954) also reported A. excavata in the Czech Republic (on the data of Štěpánek). He added that it inhabited the sapropel among the aquatic plants. The sizes pointed by Bartoš were borrowed from Štěpánek (1954). Later Geltzer and Korganova (1976), and Geltzer et al. (1985) reported the presence of A. excavata in Russia also.

Recently Alekperov and Snegovaya (1999, 2000) observed A. excavata in four of the studied 5 freshwater pools from Apsheron Peninsula (Azerbaijan). Snegovaya (2001) described A. excavata also in a dam of Djeiran-Batan (Azerbaijan). According to Snegovaya (2001) A. excavata occurs in comparatively pure water and can be used as a bioindicator of water with saprobity β-α. However, in the above papers no data about the cytoplasm and detailed morphometrical characterization was provided.

During our investigation on the testate amoebae from the aeration tanks of Sofia’s Wastewater Treatment Plant we have observed an abundant material of living specimens of A. excavata with a high population density. This allows us to make more detailed studies on their morphology, on the variation of his sizes and on the cytoplasm. The results of our studies are the subject of the present paper.

**MATERIALS AND METHODS**

The material for the present study was collected in July and August 2001 from the aeration tank No 1 of the Sofia’s Wastewater Treatment Plant. The water in this aeration tank during the period of the study is characterized by the following chemical parameters: ammonium nitrogen (NH₄-N) - between 8.7 and 13.1 mg N/l; nitrite nitrogen (NO₂-N) - between 0.07 and 0.09 mg N/l; nitrate nitrogen (NO₃-N) - between 0.5 and 0.6 mg N/l; undissolved substances - between 7.0 and 15.1 mg/l.

The fresh material was cultivated in a laboratory at room temperature. Thus we were able to observe living specimens, young forms and variability of the shells of A. excavata. Some of the observations, measurements and photos were made by optical microscope. For scanning electron microscopy the shells were isolated, cleaned by several transfers through distilled water, mounted directly on stubs and air-dried. The shells were coated evenly with gold in a vacuum coating unit. The microphotographs were obtained by using a Phillips SEM 515, operating at 25 kV.

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

**RESULTS AND DISCUSSION**

**Morphology of the shell**

The shell is colourless or yellowish in young specimens and brown in older specimens, oval or circular in apertural view and croissant-like in lateral view (Figs 1-4, 7-10). The large axis of the shell (length) in almost all observed specimens is in perpendicular direction to the shell’s protuberances, when the shape of the shell in apertural view is not circular. Rarely (about 5% of the observed individuals) the large axis of the shell is parallel to the shell’s protuberances. The apertural surface is deeply invaginated forming two pronounced protuberances with circular or elliptical aperture at the bottom of invagination. The aperture is bordered by a small lip, of about 3-4 µm (Fig. 5). Štěpánek (1953) notes that small
pores border the aperture, however in our study we do not observe such pores. The aboral hemispherical region usually has a series of regular depressions (Fig. 7). The shell wall is composed of numerous alveoli in diameter of 1-1.5 µm, made of a proteinaceous material, and arranged in one layer. The shell surface is smooth or irregular and has numerous small pores (Figs 6, 9).

**Cytoplasm**

The cytoplasm does not quite fill the shell cavity and numerous thin cytoplasmic strands (epipodes) attach it to the inner shell wall (Fig. 7).

The nuclei are 3 or 4, rarely 5-6 (1-2% only) and are large, spherical, between 9.5 and 10.5 µm in diameter.
Figs 5-6. *Arcella excavata*. 5 - apertural view to illustrate the small lip, bordering the aperture; 6 - surface of shell showing proteinaceous alveoli and small pores. Scale bars 10 µm (5); 1 µm (6).

**Table 1.** Morphometric characterization of *Arcella excavata* (measurements in µm).

<table>
<thead>
<tr>
<th>Character</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>length</td>
<td>64.8</td>
<td>65</td>
<td>2.6</td>
<td>0.2</td>
<td>4.0</td>
<td>60</td>
<td>70</td>
<td>120</td>
</tr>
<tr>
<td>breadth</td>
<td>61.1</td>
<td>61</td>
<td>2.8</td>
<td>0.3</td>
<td>4.6</td>
<td>53</td>
<td>70</td>
<td>120</td>
</tr>
<tr>
<td>diameter of aperture</td>
<td>19.5</td>
<td>20</td>
<td>0.9</td>
<td>0.1</td>
<td>4.5</td>
<td>18</td>
<td>22</td>
<td>120</td>
</tr>
<tr>
<td>depth</td>
<td>40.1</td>
<td>40</td>
<td>1.8</td>
<td>0.2</td>
<td>4.5</td>
<td>36</td>
<td>45</td>
<td>120</td>
</tr>
<tr>
<td>apertural invagination</td>
<td>18.9</td>
<td>19</td>
<td>1.7</td>
<td>0.2</td>
<td>9.0</td>
<td>14</td>
<td>25</td>
<td>120</td>
</tr>
</tbody>
</table>

**Table 2.** Measurements (in µm) of *Arcella excavata* according to different authors.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Length of shell</th>
<th>Breadth of shell</th>
<th>Depth of shell</th>
<th>Diameter of aperture</th>
<th>Depth of depression</th>
<th>Number of measured specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cunningham 1919</td>
<td>55</td>
<td>50</td>
<td>45</td>
<td>15x20</td>
<td>25</td>
<td>no data</td>
</tr>
<tr>
<td>Deflandre 1928</td>
<td>55</td>
<td>50</td>
<td>45</td>
<td>15x20</td>
<td>25</td>
<td>no data</td>
</tr>
<tr>
<td>Štěpánek 1953</td>
<td>67</td>
<td>67</td>
<td>35</td>
<td>16</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Štěpánek 1954</td>
<td>63</td>
<td>63</td>
<td>42</td>
<td>24</td>
<td>-</td>
<td>no data</td>
</tr>
<tr>
<td>Bartoš 1954</td>
<td>63</td>
<td>63</td>
<td>42</td>
<td>24</td>
<td>-</td>
<td>no data</td>
</tr>
<tr>
<td>Present work</td>
<td>60-70</td>
<td>53-70</td>
<td>36-45</td>
<td>18-22</td>
<td>14-25</td>
<td>120</td>
</tr>
</tbody>
</table>
They have one central nucleolus (about 5 µm in diameter), easily visible in each of the nuclei (Figs 8, 9). The number of the nuclei, as well as the characteristic shape of the shell and its bigger depth, show clearly that *A. excavata* is a separate, well distinguished species and it has nothing common with *A. discoides* and *A. discoides* var. *scutelliformis* (as Deflandre, 1928 doubt) or with *A. polypora* var. *curvata* (with that it was compared by Cunningham 1919).

Normally one big contractile vacuole, between 12 and 14 µm in diameter, occurs in the cytoplasm.

The lobopodia are usually 3 or 4, but sometimes they may fuse to form a single fan-like pseudopodium with a ruffled edge.
Table 1 shows the morphometric characterization of *Arcella excavata* according to our studies. The ideal individual of this species is constructed from median values of all characters in Fig. 11. Shell measurements with the exception of character (5) are fairly constant and have low variability (CV between 4.0 and 4.5). Our values agree well with those of Štěpánek (1953, 1954) and Bartoš (1954), and are bigger than those of Cunningham (1919) and Deflandre (1928) (Table 2).

Size frequency distribution analysis indicates that *A. excavata* is a size-monomorphic species characterized by a main-size class and a small size range (Figs 12, 13). Figure 12 shows that all measured individuals have a shell length 60-70 µm and more than half of them (55%) are within the limits of 63-66 µm. The frequency analysis of the shell breadth shows almost the same results (Fig. 13). Ninety-eight percent of all measured individuals have a shell breadth between 55 and 66 µm and about three-fourths of them (75%) are within the limits of 60-65 µm. Only 0.8% has a shell

Biometry

Table 1 shows the morphometric characterization of *Arcella excavata* according to our studies. The ideal individual of this species is constructed from median values of all characters in Fig. 11. Shell measurements with the exception of character (5) are fairly constant and have low variability (CV between 4.0 and 4.5). Our values agree well with those of Štěpánek (1953, 1954) and Bartoš (1954), and are bigger than those of Cunningham (1919) and Deflandre (1928) (Table 2).
breadth less than 55 µm and 0.8% has a shell breadth above 66 µm.

**Ecology**

Since the original descriptions of *A. excavata* there have been scanty data about the ecology of this species. Cunningham (1919) and Deflandre (1928) pointed out that this species occurred in small swamps. According to Štěpánek (1953, 1954) and Bartoš (1954) *A. excavata* inhabits the aquatic mosses and the sapropele among the aquatic plants. Štěpánek (1953) noted that this species is betamesosaprobic. Snegovaya (2001) reported that *A. excavata* occurred in comparatively pure water and could be used as a bioindicator of water with the saprobity β-α.

Our investigation shows that *A. excavata* inhabits also polluted waters of the aeration tanks of Sofia’s Wastewater Treatment Plant. This fact does not confirm the conclusion of Snegovaya (2001) that *A. excavata* can be used as a bioindicator of water with the saprobity β-α. Probably *A. excavata* is also a freshwater eurybiont, as well as the majority of the known species of the family *Arcellidae*.

Geographical distribution: Azerbaijan, Bulgaria, Czech Republik, Russia and United States of America.

**Acknowledgements.** We thank to the student from the University of Sofia Ms I. Christova for the collection of living material from the aeration tanks of the Sofia’s Wastewater Treatment Plant.

**REFERENCES**


Received on 28th October, 2002; revised version on 11 December 2002; accepted on 23rd December, 2002
The Myriokaryonidae fam. n., a New Family of Spathidiid Ciliates (Ciliophora: Gymnostomatea)

Wilhelm FOISSNER
Universität Salzburg, Institut für Zoologie, Salzburg, Austria

Summary. The new family Myriokaryonidae is based on a thorough literature review and the (re)investigation of Myriokaryon lieberkuehnii (Bütschli, 1889) Jankowski, 1973 and Cephalospatula brasiliensis, a new genus and species discovered in soils of South America. The key features of the new family, which belongs to the order Spathidiida Foissner and Foissner, 1988, are the truncated anterior body end and the spoon-shaped oral bulge, respectively, circumoral kinety. The anterior truncation causes a highly characteristic bend of the oral bulge and circumoral kinety in the transition zone of spoon-shovel and spoon-handle, that is, subapically where the oral bulge and the circumoral kinety enter the ventral side of the cell. Based on literature data, Holophrya emmae Bergh, 1896 and Pseudoprorodon armatus Kahl, 1930a are classified as representatives of two further new myriokaryonid genera, viz., Berghophrya gen. n. and Kahlophrya gen. n. Genera within the Myriokaryonidae are distinguished by details of the dorsal brush and the arrangement of the somatic ciliary rows and extrusomes. Pseudoprorodon emmae (Bergh, 1896), as redescribed by Song and Wilbert (1989), is recognized as a new genus and species, Songophrya armata gen. n., sp. n., likely belonging to the gymnostomatous family Pseudoholophryidae.

Key words: biodiversity, Haptorida, infraciliature, Litostomatea, soil protozoa, South America.

INTRODUCTION

The haptorid gymnostomes (“Litostomatea”) are a highly diverse ciliate assemblage, ranging from the simple Enchelys to the complex Homalozoon and from common freshwater and marine to highly specialized enterozoic species (Corliss 1979, Grain 1994). However, many gymnostomes are rather inconspicuous and thus attracted few specialists, most notably Kahl (1926, 1930a, b), Lipscomb and Riordan (1990), and myself (Foissner 1984, 1996; Foissner and Foissner 1988). Accordingly, the group contains a huge amount of undescribed taxa, as shown by a recent study on soil ciliates from Namibia, describing 10 new genera and many new species (Foissner et al. 2002). Detailed observations and much experience are necessary to unravel this hidden diversity. This is evident also from the present study, where a new family and four new genera will be established.

Large ciliates, such as Myriokaryon lieberkuehnii and Cephalospatula brasiliensis, are difficult to present by ordinary figures. Thus, I fragmented them into many details, which, however, strongly increased the number of pages. But this is the sole way to document such species, which tend to be misidentified, to an extent that detailed biogeographic comparisons of species and populations will be possible in future.
MATERIALS AND METHODS

Myriokaryon lieberkuehni was found in the microaerobic mud of a boggy drainage ditch at the margin of a small bog near the village of Franking (13°E 48°2’N), Upper Austria, in April 1990. Specimens were numerous and survived in the collecting jar for weeks, but did not reproduce.

Cephalospatula brasiliensis was discovered in soil samples from three sites of South America. The type population occurred in a sample, kindly collected by Dr. L. Felipe Machado Velho (Maringá State University) in May 2001, from the high Paraná River floodplain near the town of Maringá (53°15’W 22°40’S, altitude about 500 m), State of Mato Grosso do Sul, Brazil. The sample was taken from the Aurelio Lagoon, that is, a marginal lagoon associated with the Baia River, a tributary of the Paraná River. The dark, humic soil was mixed with much partially decomposed plant litter, had pH 5.1 (in water), was air-dried in the Salzburg laboratory for about one month, and stored in a plastic bag. In November 2001, the about 300g soil were put in a Petri dish and saturated, but not flooded with distilled water to obtain a “non-flooded Petri dish culture”, as described in Foissner et al. (2002). About two weeks after rewetting, a small population of C. brasiliensis developed. The second population of C. brasiliensis occurred in the surroundings of Rio de Janeiro (43°W 23°S), that is, in the Restinga area about 100 m inshore the Atlantic Ocean, where the ground is partially covered by grass and an up to 1 cm high litter layer. The sample, which was taken on 16.11.1996 and treated as described above, contained sandy soil, surface litter, and plant residues sieved off the very sandy soil up to a depth of 10 cm. A weak population of C. brasiliensis developed 8 days after rewetting the sample, which had pH 5.2 (in water), in May 1997. The third population of C. brasiliensis was found near the airport of Puerto Ayacucho (68°W 5°N), Venezuela, on 28.5.1997. Here are large, granitic rocks (Lajas) with many pools. The sample comprised litter, mud and soil accumulated in a pool between the cushion-like root layer of a species of the endemic Velloziaceae family. The material, which was treated as described above and had pH 5.3 (in water), was rewetted in June 1997, when a small population of C. brasiliensis developed after 5 days.

Specimens were studied in vivo using a high-power, oil immersion objective and differential interference contrast optics. The ciliary pattern and various cytological structures were revealed by protargol impregnation, as described in Foissner (1991). Counts and measurements on prepared specimens were performed at a magnification of x1000. In vivo measurements were conducted at magnifications of x100-1000. Although these provide only rough estimates, it is worth giving such data as specimens may change in preparations. Illustrations of live specimens were based on free-hand sketches, while those of prepared cells were made with a camera lucida. Terminology is mainly according to Corliss (1979).

RESULTS

Genus Myriokaryon Jankowski, 1973

Improved diagnosis: vermiform Myriokaryonidae with long mouth on steeply slanted anterior body region and many (>3) isomorphic dorsal brush rows converging to an acute pattern anteriorly. Extrusomes scattered in oral bulge. At left side of circumoral kinety many transverse kinetofragments partially connected with left side ciliary rows (basically Supraspathidiurn pattern).

Type species (by monotypy): Prorodon Lieberkühni Bütschli, 1889.

Redescription of Myriokaryon lieberkühni (Bütschli, 1889) Jankowski, 1973 (Figs 1-53; Table 1)

1859 Enchelys gigas Stein, Organismus der Infusionsthierie, p. 80 (a nomen nudum because too briefly described without figure).

1889 Prorodon Lieberkühni Bütschli, Protozoa, explanation to figure 6 of plate LVII (single figure, here reproduced as figure 91; without description).

1914 Spathidium gigas (Stein 1859) - Cunha. Mem. Inst. Osw. Cruz, 6: 173 (synonymy doubtful; possibly a “true”, large Spathidium/Arcuospathidiurn; Fig. 96).

1930 Pseudoprorodon (Prorodon) lieberkühni Bütschli, 1889 - Kahl, Tierwelt Dtl., 18: 71 (first reviser and important new observations; Figs 97, 99).


1973 Myriokaryon lieberkühni (Bütschli, 1889) Jankowski comb. n. - Jankowski, Zool. Zhl., 52: 424 (solid redescription and transfer to the new genus Myriokaryon; in Russian with English summary; Fig. 101).

1986 Myriokaryon lieberkühni (Bütschli, 1889) Jankowski, 1973 - Dragesco and Dragesco-Kernéis, Fauna tropicale, 26: 169 (brief review and description of a Benin population; Fig. 94).

Material and types: Jankowski (1973) stored 29 neotype slides with 70 conventionally (various hema-toxylin methods, Feulgen reaction, bromphenol) prepared specimens of M. lieberkühni at the marine biological laboratory of the Russian Academy of Sciences in St. Peterburg. Although these slides do not show all the details necessary for a reliable identification, I suggest to accept them as a neotype because the known data agree with my material. The specimens of Dragesco (1972) and Dragesco and Dragesco-Kernéis (1986) are likely still in the private collection of Prof. Jean Dragesco. Furthermore, they are poorly prepared, as obvious from the mistakes in the description (see discussion). My material is excellently prepared and fully described below. Five slides with protargol-impregnated
A new spathidiid ciliate family

Wilbert’s method specimens have been deposited in the Biologiezentrum of the Oberösterreichische Landesmuseum in Linz (LI), Austria. Relevant specimens are marked with black ink on the cover glass. Description of Austrian population: true size difficult to ascertain because contractile by about one third of body length and contraction, respectively, extension occur so slowly that is is impossible to recognize whether or not the cell is fully extended, as also mentioned by Engelmann in Bütschli (1889). Thus, size variability is high, viz., 900-2000 x 70-120 µm in vivo, usually near 1500 x 90 µm, with conspicuous length:width variation of 9-24:1 in protargol preparations, where cell width is rather distinctly shrunken (Table 1). Jankowski (1973), however, mentions that it does not contract when touched, but he might have overlooked the slow contraction described above. He measured 34 specimens and noted also a pronounced variability: length 625-1250 µm, usually 825-950 µm; width at anterior end 37-52 µm; width at proximal oral bulge end 47-62 µm; width in mid-body 100-175 µm, usually 100-125 µm.

Based on protargol-impregnated (Wilbert’s method; see Foissner 1991), mounted, morphostatic field specimens. 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 arithmetic mean, x - arithmetic mean.

Table 1. Morphometric data on Myriokaryon lieberkuehnnii.

<table>
<thead>
<tr>
<th>Characteristics</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>Body, length</td>
<td>1321.0</td>
<td>1320.0</td>
<td>280.1</td>
<td>61.1</td>
<td>21.2</td>
<td>860.0</td>
<td>1800.0</td>
<td>21</td>
</tr>
<tr>
<td>Body, width about 100 µm posterior of anterior end</td>
<td>52.8</td>
<td>53.0</td>
<td>5.1</td>
<td>1.1</td>
<td>9.7</td>
<td>45.0</td>
<td>63.0</td>
<td>21</td>
</tr>
<tr>
<td>Body, maximum width</td>
<td>77.1</td>
<td>75.0</td>
<td>9.3</td>
<td>2.0</td>
<td>12.0</td>
<td>68.0</td>
<td>110.0</td>
<td>21</td>
</tr>
<tr>
<td>Body length:maximum width, ratio</td>
<td>17.3</td>
<td>16.9</td>
<td>3.9</td>
<td>0.8</td>
<td>22.5</td>
<td>9.1</td>
<td>24.3</td>
<td>21</td>
</tr>
<tr>
<td>Mouth, length (anterior end to proximal end of circumoral kinety, distance)</td>
<td>328.3</td>
<td>330.0</td>
<td>57.1</td>
<td>12.5</td>
<td>17.4</td>
<td>240.0</td>
<td>450.0</td>
<td>21</td>
</tr>
<tr>
<td>Body length:mouth length, ratio</td>
<td>4.1</td>
<td>3.9</td>
<td>0.9</td>
<td>0.2</td>
<td>21.4</td>
<td>3.0</td>
<td>6.3</td>
<td>21</td>
</tr>
<tr>
<td>Mouth, anterior width (distance between circumoral kinety in head area)</td>
<td>10.5</td>
<td>11.0</td>
<td>2.0</td>
<td>0.6</td>
<td>19.0</td>
<td>7.0</td>
<td>14.0</td>
<td>13</td>
</tr>
<tr>
<td>Mouth, mid-region width (distance between circumoral kinety)</td>
<td>6.9</td>
<td>7.0</td>
<td>1.3</td>
<td>0.3</td>
<td>18.6</td>
<td>5.0</td>
<td>10.0</td>
<td>18</td>
</tr>
<tr>
<td>Mouth, posterior width (distance between circumoral kinety)</td>
<td>11.2</td>
<td>10.0</td>
<td>2.2</td>
<td>0.5</td>
<td>19.5</td>
<td>8.0</td>
<td>15.0</td>
<td>17</td>
</tr>
<tr>
<td>Macronuclei, length</td>
<td>8.9</td>
<td>8.0</td>
<td>4.1</td>
<td>0.9</td>
<td>46.6</td>
<td>4.0</td>
<td>18.0</td>
<td>21</td>
</tr>
<tr>
<td>Macronuclei, width</td>
<td>3.6</td>
<td>4.0</td>
<td>0.6</td>
<td>0.1</td>
<td>16.7</td>
<td>3.0</td>
<td>5.0</td>
<td>21</td>
</tr>
<tr>
<td>Micronuclei, length</td>
<td>3.0</td>
<td>3.0</td>
<td>0.5</td>
<td>0.1</td>
<td>16.0</td>
<td>2.2</td>
<td>4.0</td>
<td>21</td>
</tr>
<tr>
<td>Micronuclei, width</td>
<td>3.0</td>
<td>3.0</td>
<td>0.5</td>
<td>0.1</td>
<td>16.0</td>
<td>2.2</td>
<td>4.0</td>
<td>21</td>
</tr>
<tr>
<td>Excretory pores, number per vacuole</td>
<td>2.3</td>
<td>2.0</td>
<td>0.6</td>
<td>0.1</td>
<td>28.2</td>
<td>1.0</td>
<td>3.0</td>
<td>21</td>
</tr>
<tr>
<td>Somatic kineties, number in mid-oral area</td>
<td>82.8</td>
<td>82.0</td>
<td>-</td>
<td>-</td>
<td>73.0</td>
<td>100.0</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Somatic kineties, number in mid-body</td>
<td>94.3</td>
<td>95.0</td>
<td>-</td>
<td>-</td>
<td>83.0</td>
<td>110.0</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Kinetids, number in 10 µm in oral area</td>
<td>6.1</td>
<td>6.0</td>
<td>1.0</td>
<td>0.2</td>
<td>15.7</td>
<td>5.0</td>
<td>9.0</td>
<td>21</td>
</tr>
<tr>
<td>Kinetids, number in 10 µm in mid-body</td>
<td>5.0</td>
<td>5.0</td>
<td>0.8</td>
<td>0.2</td>
<td>15.5</td>
<td>4.0</td>
<td>7.0</td>
<td>21</td>
</tr>
<tr>
<td>Kinetids, number in 10 µm near posterior end</td>
<td>3.1</td>
<td>3.0</td>
<td>0.2</td>
<td>0.1</td>
<td>7.2</td>
<td>3.0</td>
<td>4.0</td>
<td>21</td>
</tr>
<tr>
<td>Dorsal brush kineties, number near anterior body end</td>
<td>7.5</td>
<td>7.5</td>
<td>0.6</td>
<td>0.1</td>
<td>8.1</td>
<td>6.0</td>
<td>8.0</td>
<td>20</td>
</tr>
<tr>
<td>Anterior body end to end of dorsal brush, distance</td>
<td>508.3</td>
<td>500.0</td>
<td>-</td>
<td>-</td>
<td>300.0</td>
<td>680.0</td>
<td>21</td>
<td></td>
</tr>
</tbody>
</table>

* Data based on protargol-impregnated (Wilbert’s method; see Foissner 1991), mounted, morphostatic field specimens. 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 arithmetic mean, x - arithmetic mean. 

Basicall elongate knife-shaped with blade (=oral area) occupying 25% of body length on average, handle thus much longer than blade; anterior and posterior quarter flattened laterally up to 2:1, anterior body end acute due to steeply slanted mouth area and indistinct oral bulge gradually merging into body proper proximally (Figs 1-4, 12, 20); usually widest in or near mid-body, rarely in blade-like oral area making specimens somewhat club-shaped (Fig. 11); contracted cells widened in mid-body (Figs 2, 3). Largest specimens with about 5000 (!) macronuclear nodules scattered throughout body in an about 15 µm thick, subcortical layer; individual nodules globular to elongate ellipsoidal with several minute and small nucleoli of various shape (Figs 1, 10, 14, 18, 33, 35, 40, 45, 46; Table 1). Micronuclei less numerous than macronuclear nodules, likely between 100-200, minute, that is 3-4 µm across. A large contractile vacuole with several excretory pores in rear end and about 60 small contractile vacuoles each with one to three intrakinetal excretory pores scattered throughout cortex (Figs 1, 10, 16, 19, 22-24, 33). Extrusomes (likely toxicysts) accumu-
lated in both sides of oral bulge and scattered throughout cytoplasm in conspicuous bundles; individual extrusomes rod-shaped and very flexible, straight to slightly curved, about 80 x 0.5 µm in size; those attached to oral bulge never impregnate with the protargol method used, while cytoplasmic bundles often stain deeply with individual rods frequently somewhat disordered and irregularly curved (Figs 1, 5-7, 10, 29, 30, 33-37, 42, 43). A second type of minute (about 1-1.5 x 0.4 µm), rod-shaped structures, likely mucocysts (Fauré-Fremiet and André 1968), forms oblique rows in the oral bulge and is scattered throughout the cortex and cytoplasm, where rods are up to 2 µm long and occasionally impregnated with protargol (Figs 7, 8, 10, 33, 36, 39, 46). Cortex conspicuous because 3-4 µm thick and sharply separated from granular cytoplasm by a distinct tela corticalis described by Fauré-Fremiet and André (1968); in most specimens rather densely covered with 2-4 µm long bacterial rods (Figs 1, 8, 10, 29, 31, 33, 39). Cytoplasm colourless, packed with lipid droplets 1-10 µm across and surprisingly small, viz., 10 µm-sized food vacuoles containing colourless, granular remnants and golden lipid droplets, indicating heterotrophic and autotrophic protists as main food source (Figs 1, 10, 30), unfortunately, definite food inclusions were neither found in vivo nor prepared cells, indicating that prey is rapidly lysed either outside the cell or in the food vacuoles. Swims and crawls worm-like in and on the sediment and microscope slide, showing great flexibility, viz., may curl-up and/or spiralize along main body axis (Fig. 4). Cilia about 12 µm long in vivo and narrowly spaced, especially right of oral bulge, arranged in an average of 95 rows in mid-body and of only 82 rows in mid-oral area due to gradual shortening along circumoral kinety (Figs 15, 17, 25, 37, 40, 41, 44; Table 1), while some rows are added postorally and along left margin of dorsal brush (Figs 16, 48-53). Ciliary rows equidistantly and narrowly spaced (about 2 µm), extend meridionally; right side rows loosely ciliated anteriorly and shortened successively abutting on circumoral kinety in steep angles, while densely ciliated and strongly curved anterior end of left side ciliary rows abuts on circumoral kinety at almost right angles; furthermore, many additional, small kinetofragments of unknown origin occur along left side of circumoral kinety, a special feature also found in Cephalospatula brasiliensis (Figs 15, 17, 19, 25-28, 37, 38, 40, 41, 44). These fragments look like dileptid “adesmokineties”, a term coined by Jankowski earlier than the American “paratenes”. Kinetids of posterior body region associated with long fibres forming conspicuous, subcortical bundles extending anteriorly (Fig. 22). Dorsal brush at anterior portion of six to eight slightly left laterally located, ordinary somatic ciliary rows, longest brush kineties occupy 38% of body length on average (Table 1); composed of narrowly spaced dikinetids with posterior bristles up to 5 µm long and inflated distal end curved anteriorly (Figs 1, 9, 29, 39); kineties gradually shortened from left to right, except of the three rightmost rows, bristles mixed with ordinary cilia posteriorly, that is, before brush kineties extend as ciliary rows to rear body end; middle brush rows, additionally, slightly shortened anteriorly, where the unshortened marginal rows curve right and converge subapically near the circumoral kinety, providing the brush with an acute end, another unique feature of the somatic ciliary pattern of Myriokaryon (Figs 16, 20, 21, 23, 24, 26, 27, 29, 38, 39, 48-53; Table 1). Mouth occupies steeply slanted and slightly convex anterior 25% of body, in vivo distinct due to the numerous and long extrusomes contained, basically, however, indistinct because oral bulge hardly set off from body proper, that is, only about 5 µm high and 12 µm wide. Oral bulge elongate dumbbell-shaped in frontal view, curved anterior portion slightly thicker (higher) and wider than straight proximal part; oral slit stands out as a whitish (unimpregnated) cleft from brownish impregnated bulge wall, containing a fibrous reticulum and innumerable extrusomes, as described above. Circumoral kinety composed of very narrowly spaced, oblique dikinetids having ciliated only one basal body, likely consists of many kinetofragments of varying length, as indicated by minute breaks making kinety somewhat irregular; of same shape as oral bulge, that is, elongate dumbbell-shaped with highly characteristic, sharp bend in the transition zone of transverse-truncate anterior body end and curved ventral portion. Oral basket rods originate from circumoral dikinetids, very fine and short (about 30 µm) as compared to size of cell, basket thus inconspicuous in protargol preparations and invisible in vivo, where it is easily confused with the more distinct and longer, rod-shaped extrusomes (Figs 1-5, 7, 11-13, 15, 17, 25-29, 32, 37, 40, 41, 43-45, 47; Table 1).

**Distribution and ecology:** all reliable records of *M. lieberkuehnii* are from the Holarctic and African region (Bütschli 1889, Kahl 1930a, Dragesco 1972, Jankowski 1973, Dragesco and Dragesco-Kernéis 1986, present study). Cunha (1914) reported it from freshwater in the surroundings of Manguinhos, Brazil, where it occurred only once, but in great numbers (Fig. 96). However, I agree with Kahl (1930a) that conspecificity
A new spathidiid ciliate family

Figs 1-11. *Myriokaryon lieberkuehnii* from life (1-10) and after protargol impregnation (11). 1 - right side view of a representative specimen, length 1400 µm. For details, see figure 10; 2, 3 - a specimen extended and contracted; 4 - a slightly spiralized specimen; 5 - ventral view of oral area; 6 - oral bulge extrusomes, length 80 µm; 7 - ventral view of oral bulge showing cortical granules (upper half) and extrusomes underneath (lower half); 8 - surface view showing cortical granules and epicortical bacteria; 9 - part of a dorsal brush row with posterior bristles 5 µm long and inflated distally; 10 - optical section showing the 3-4 µm thick cortex and main cytoplasmic inclusions. Note that the extrusome bundle (80 µm) is not shown in full length; 11 - a specimen widest in oral area. B - dorsal brush; BA - epicortical bacteria; C - cortex; CK - circumoral kinety; CV - contractile vacuoles; E - extrusomes; FV - food vacuole; G - cortical granules; L - lipid droplets; MA - macronuclear nodule; N - nematodesmata; OB - oral bulge; OS - oral slit; P - pellicle; T - tela corticalis. Scale bars 10 µm (10); 400 µm (1, 11).
Figs 12-19. *Myriokaryon lieberkuhnii*, main voucher specimen after protargol impregnation. 12, 13 - ventrolateral overview (length 1800 µm) and oral area at higher magnification; 14 - upper layer of macronuclear nodules in mid-body; 15, 16 - oral and somatic ciliary pattern of ventral and dorsal anterior body portion; 17 - ciliary pattern of mid-oral area at high magnification. Arrowhead marks kinetofragments, some obviously connected with the ciliary rows, at left margin of circumoral kinety. Asterisks denote oral slit; 18 - variability of macronuclear nodules, drawn to scale (6 µm); 19 - ciliary pattern in mid-body. B - dorsal brush; CK - circumoral kinety; EP - excretory pore; OB - oral bulge. Scale bars 6 µm (17, 18); 70 µm (13, 14-16, 19); 400 µm (12).
A new spathidiid ciliate family

Figs 20-24. *Myriokaryon lieberkuehni*, infraciliature after protargol impregnation. 20, 21, 23, 24 - dorsolateral overview (20) and details of dorsal brush in anterior (21), middle (23), and posterior (24) region. The brush rows gradually decrease in length from left to right (arrowheads) and continue posteriorly as ordinary somatic ciliary rows (24). Anteriorly, the middle brush rows are slightly shortened, while the marginal ones converge, forming a highly characteristic, pointed anterior brush end (21); 22 - posterior body region with subcortical fibre bundles originating from basal bodies of cilia. B - dorsal brush; C - cortex; CI - cilia; CK - circumoral kinety; CV - contractile vacuole; EP - excretory pores; F - fibre bundles; OB - oral bulge. Scale bars 30 µm (21-24); 400 µm (20).
Figs 25-28. *Myriokaryon lieberkuehni*, details of ciliary pattern after protargol impregnation. At right, the ciliary rows abut on the circumoral kinety in very steep angles, whereas at left many small kinetofragments, some obviously connected with the ciliary rows, occur and abut on the circumoral kinety at right angles (arrowheads). 25, 28 - ventral and frontal view showing the slightly widened posterior and anterior mouth end; 26, 27 - lateral views showing the main family features, viz., the strongly curved anterior portion of the oral bulge and circumoral kinety. Note the pointed anterior brush end. B - dorsal brush; CK - circumoral kinety; OB - oral bulge. Scale bars 30 µm.
Figs 29-32. *Myriokaryon lieberkuehnii* from life (interference contrast). **29** - anterior body portion of a slightly squashed specimen showing the 80 µm long extrusomes attached to the oral bulge. The opposed arrowheads mark the unusually thick cortex; **30** - the cytoplasm is packed with extrusome bundles and about 10 µm-sized food vacuoles containing golden (and thus dark in the micrograph) lipid droplets, indicating that this specimen fed on autotrophic protists; **31** - surface view showing the cortex covered with bacterial rods; **32** - ventral view of posterior mouth end. The oral bulge, marked by arrowheads, is finely striated by rows of cortical granules, likely mucocysts; underneath are the extrusomes, whose anterior ends appear as minute granules (cp. figure 7). Asterisk denotes the mouth slit. B - dorsal brush; E - extrusomes; FV - food vacuoles; L - lipid droplets; OB - oral bulge.
Figs 33-36. *Myriokaryon lieberkuehnhii* from life (33) and after protargol impregnation (34-36). 33 - optical section in mid-body showing the 3-4 μm thick cortex, marked by opposed arrowheads, and many about 80 μm long extrusome bundles; 34, 35 - overviews in mid-body, showing the cell packed with macronuclear nodules and extrusome bundles; 36 - long (toxicysts, arrows) and short (likely mucocysts, arrowheads) extrusomes occur in the cytoplasm. CV - contractile vacuoles; E - extrusome bundles; FV - food vacuoles; G - cortical granules, likely mucocysts; L - lipid droplets; MA - macronuclear nodules.
Figs 37-39. Myriokaryon lieberkuehni after protargol impregnation (37, 38) and from life (39). 37, 38 - ventral and dorsal overview of oral body portion showing the slightly dumbbell-shaped mouth and the dorsal brush rows shortening from left to right (arrowheads); 39 - part of dorsal brush showing the curved and distally inflated bristle pairs. Opposed arrowheads mark the 3-4 μm thick cortex. B - dorsal brush; CK - circumoral kinety; E - extrusomes; MA - macronuclear nodules; OS - oral slit.
Figs 40–43. Myriokaryon lieberkuehnii after protargol impregnation (40–42) and from life (43). 40, 41 - anterior and posterior portion of mouth at high magnification (for an overview, see figure 37). At right, the somatic ciliary rows abut to the dikinetid circumoral kinety in steep angles; while at left many transversely oriented, monokinetidal kinetofragments occur (some marked by arrowheads), some of which are obviously connected with the ciliary rows. Opposed arrows mark the mouth slit sensu stricto; 42 - extrusome bundles in the cytoplasm; 43 - surface view of posterior portion of oral bulge packed with extrusomes. CK - circumoral kinety; E - extrusomes; MA - macronuclear nodules.
Figs 44-47. *Myriokaryon lieberkuehnii* after protargol impregnation. 44, 45, 47 - oral infraciliature at high magnification and two focal planes, viz., at level of basal bodies (44) and tightly underneath (45, 47) to show the peripherically arranged macronuclear nodules and the very fine oral basket rods (nematodesmata) associated with the circumoral dikinetids (kinety). The circumoral kinety consists of dikinetidal kinetics, of which one is marked by arrowheads (44). Opposed arrows in figure 44 denote the faintly impregnated oral slit sensu stricto. At right, the somatic ciliary rows abut on the circumoral kinety at steep angles, while at left many transversely oriented, monokinetidal kinetics occur, some of which are obviously connected with the somatic ciliary rows (asterisks). This pattern reminds on *Spathidium* and *Supraspathidium*; 46 - macronuclear nodules and 2 µm long rods (arrows), likely developing mucocysts, in subcortical layer. CK - circumoral kinety; MA - macronuclear nodules; N - nematodesmata.
Figs 48-50. *Myriokaryon lieberkuehni*, dorsal brush after protargol impregnation. The brush consists of narrowly spaced dikinetids in the anterior third of six to eight dorsal ciliary rows (Table 1). Kineties (asterisks) are successively inserted left of the brush. Thus and due to successive addition of kineties along the right mouth margin, kinety number is higher in mid-body than in the oral area. 48 - anteriorly, the middle brush rows are shortened, while the unshortened marginal rows converge (arrowheads) and produce a unique, pointed brush end; 49, 50 - subapical brush region of two other specimens, showing that the brush rows are successively shortened from left to right (arrowheads) and continue as ordinary somatic kineties posteriorly (see also following figures). Arrow in figure 50 marks a fine fibre (?) between the brush rows. B - dorsal brush; CK - circumoral kinety.
A new spathidiid ciliate family must be confirmed by more detailed investigations. Indeed, *M. lieberkuehnii* has been confused with other large, likely not yet described species, for instance, by Kahl (1930a; the small form, Fig. 99) and Al-Rasheid (2000), whose identification did not withstand a thorough reinvestigation (slide kindly provided by Dr. K. Al-Rasheid). The specimen illustrated (Fig. 100) and some others contained in the slide are insufficiently impregnated, but observation with interference contrast optics reveals a three-rowed polykinetid along the long and narrow mouth, indicating that it might be a poorly preserved geleiid. These data suggest that misidentifications are common and several not yet described or recognized *Myriokaryon* or *Myriokaryon*-like ciliate species exist, such as *Cranotheridium elongatum* Penard, 1922 and the new genus described below.

*Myriokaryon lieberkuehnii* is likely restricted to freshwater, occurring in ponds and pond-like habitats (Kahl 1930a; present study) or the lentic zones of running waters (Buck 1961, Dragesco 1972). Only Jankowski (1973) found it in plankton of ponds and in a lake near Leningrad. He observed up to 10 specimens in 1l lake water, where they hanged vertically in the water.

**Figs 51-53.** *Myriokaryon lieberkuehnii*, dorsal brush after protargol impregnation. The figures are from the same specimen as shown in figure 49, but demonstrate the middle (51) and posterior (52) region, where the brush, due to successive shortening of the rows from left to right (arrowheads), consist of only five, respectively, three rows. Figure 53 shows the post-brush area, where kinetids are slightly narrower spaced and some dikinetids (arrows) are interspersed in the basically monokinetidal rows extending to rear body end as ordinary somatic kineties. Kineties are successively inserted along the left brush margin (asterisks). Thus and due to the successive addition of kineties along the right mouth margin (Figs 37, 40, 44), kinety number is higher in mid-body than in the oral area. B - dorsal brush.
column, indicating negative geotaxis. The records available show that M. lieberkuehnii has a wide ecological range from clean lake water to microaerobic bog environment (present record); it is thus surprising that it is so rare.

Detailed data on food and environmental requirements are unknown, although Buck (1961) classified M. lieberkuehnii as a beta-mesosaprobic indicator species in rivers of Germany. Like me, Jankowski (1973) observed bacteria, organic debris, small diatoms, and chrysomonads in the food vacuoles. Colpidium, when added in mass, was not ingested; thus, pure cultures failed. Jankowski (1973) observed globular inclusions 3-6 µm across, possibly endosymbionts.

**Cephalospatula gen. n.**

**Diagnosis:** vermiform Myriokaryonidae with long mouth on golfclub-shaped anterior (oral) body portion and 3 isomorphic dorsal brush rows. Extrusomes bundled in inflated anterior portion of oral bulge. At left side of circumoral kinety few oblique kinetofragments more or less distinctly connected with left side ciliary rows (basically Spathidium pattern).

**Type species:** Cephalospatula brasiliensis sp. n.

**Etymology:** composite of the Greek noun cephalo (head) and the Latin generic name Spathidium (spatulate organism), referring to both, the inflated anterior end and the similarity with members of the family Spathidiidae. Feminine gender.

**Description of Cephalospatula brasiliensis sp. n.** (Figs 54-90; Table 2)

**Diagnosis:** size about 350 x 35 µm in vivo. Rod-shaped with flat, inconspicuous oral bulge extending about one third of body length. Macronucleus filiform. On average 4 contractile vacuoles in line with dorsal brush. Two types of extrusomes: type I acicular and about 10 x 1 µm in vivo; type II rod-shaped and 3 µm long. On average 37 ciliary rows and about 50 dikinetids in each brush row.

**Type location:** floodplain soil of Paraná River in Brazil, near the town of Maringá, 53°15’W 22°40’S.

**Type material:** named after the native country.

**Type material:** 1 holotype slide and 4 paratype slides with protargol-impregnated specimens from type location and 6 voucher slides with protargol-impregnated specimens from the other populations have been deposited in the Biologiezentrum of the Oberösterreichische Landesmuseum in Linz (LI). Relevant specimens are marked by a black ink circle on the cover glass.

**Description:** I studied 3 populations of this species. However, only few specimens were found, altogether 16 cells. Thus, morphometry of the individual populations is incomplete (Table 2). A closer analysis of the data shows that the two Brazilian populations tend to be more similar to each other than to the Venezuelan specimens, indicating some biogeographic specialization. However, variability of most characters is high, as usual in long and slender ciliates, suggesting that morphometric differences should not be over-interpreted. Nonetheless, I base the diagnosis and description only on the two Brazilian populations. The Venezuelan specimens are considerably longer and thinner than the Brazilian ones (439 x 27 µm vs. 375 x 31 µm and 287 x 32 µm) and have also fewer ciliary rows (32 vs. 36-38). All other main features, viz., the nuclear apparatus and extrusome size, shape, and location are very similar.

Size 200-500 x 20-45 µm in vivo, depending on population, Brazilian specimens frequently near 250 x 35 µm and distinctly stouter than Venezuelan cells (Table 2). Usually rod-shaped and slightly curved, rarely vermiciform; unflattened and acontractile. Anterior body end obliquely truncate and slightly inflated, especially in protargol preparations, inflation (“head”) and oblique truncation difficult to recognize in vivo because specimens are very flexible and restless; posterior end narrowly rounded, widest near or in mid-body (Figs 54, 68, 78, 83, 86, 90). Nuclear apparatus usually in posterior two thirds of cell. Macronucleus filiform, tortuous, often somewhat spiralized and nodulated, ribbon-like flattened (2:1) in most specimens from type location and in some cells from the other populations, a conspicuous feature found also in several slender spathidiids (Foissner et al. 2002); nucleoli scattered, small, inconspicuous. On average 18 globular to broadly ellipsoidal micronuclei attached to or near macronuclear strand, some scattered (Figs 54, 67, 69, 80, 83, 86). Three to eight, usually three to five contractile vacuoles in line with dorsal brush, first vacuole, occasionally also second within brush rows; each vacuole with several closely spaced, interkinetal excretory pores in line, except of scattered pores of last vacuole in posterior body end (Figs 54, 57, 66, 68, 71). Cortex rather thick, contains about three rows of pale, 0.8-1 x 0.4 µm-sized granules between each two ciliary rows; granules form short, transverse rows (type location) or arrow-like pattern (other populations) in cortex of oral bulge (Figs 55, 56, 59, 60, 64). Two shape and size types of extrusomes scattered in cytoplasm and assembled to highly conspicuous bundle in inflated anterior portion of oral bulge, definitely lacking in ventral bulge.
A new spathidiid ciliate family

Table 2. Morphometric data on three populations of *Cephalospatula brasiliensis*.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Pop(^a)</th>
<th>x M SD SE CV Min Max n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body, length</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRP</td>
<td>375.0</td>
<td>390.0</td>
</tr>
<tr>
<td>BRR</td>
<td>287.5</td>
<td>310.0</td>
</tr>
<tr>
<td>VEN</td>
<td>439.0</td>
<td>435.0</td>
</tr>
<tr>
<td>TOT</td>
<td>373.1</td>
<td>380.0</td>
</tr>
<tr>
<td><strong>Body, maximum width</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRP</td>
<td>31.0</td>
<td>31.0</td>
</tr>
<tr>
<td>BRR</td>
<td>32.0</td>
<td>30.0</td>
</tr>
<tr>
<td>VEN</td>
<td>26.8</td>
<td>28.0</td>
</tr>
<tr>
<td>TOT</td>
<td>29.9</td>
<td>30.0</td>
</tr>
<tr>
<td><strong>Body length:width, ratio</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRP</td>
<td>11.9</td>
<td>12.3</td>
</tr>
<tr>
<td>BRR</td>
<td>9.2</td>
<td>9.3</td>
</tr>
<tr>
<td>VEN</td>
<td>16.5</td>
<td>15.0</td>
</tr>
<tr>
<td>TOT</td>
<td>12.7</td>
<td>12.6</td>
</tr>
<tr>
<td><strong>Mouth, length (anterior body end to proximal end of circumoral kinety, distance)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRP</td>
<td>108.0</td>
<td>115.0</td>
</tr>
<tr>
<td>BRR</td>
<td>98.5</td>
<td>107.5</td>
</tr>
<tr>
<td>VEN</td>
<td>128.2</td>
<td>125.0</td>
</tr>
<tr>
<td>TOT</td>
<td>111.9</td>
<td>111.0</td>
</tr>
<tr>
<td><strong>Body length:mouth length, ratio</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRP</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>BRR</td>
<td>2.9</td>
<td>3.0</td>
</tr>
<tr>
<td>VEN</td>
<td>3.6</td>
<td>3.5</td>
</tr>
<tr>
<td>TOT</td>
<td>3.4</td>
<td>3.5</td>
</tr>
<tr>
<td><strong>Mouth, anterior width (distance between circumoral kinety in head area)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRP</td>
<td>12.0</td>
<td>-</td>
</tr>
<tr>
<td>BRR</td>
<td>14.5</td>
<td>-</td>
</tr>
<tr>
<td>VEN</td>
<td>12.5</td>
<td>-</td>
</tr>
<tr>
<td>TOT</td>
<td>13.0</td>
<td>12.5</td>
</tr>
<tr>
<td><strong>Mouth, mid-region width (distance between circumoral kinety)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRP</td>
<td>4.7</td>
<td>5.0</td>
</tr>
<tr>
<td>BRR</td>
<td>5.3</td>
<td>4.5</td>
</tr>
<tr>
<td>VEN</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>TOT</td>
<td>4.7</td>
<td>1.1</td>
</tr>
<tr>
<td><strong>Anterior body end to macronucleus, distance</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRP</td>
<td>117.9</td>
<td>125.0</td>
</tr>
<tr>
<td>BRR</td>
<td>110.5</td>
<td>122.5</td>
</tr>
<tr>
<td>VEN</td>
<td>147.6</td>
<td>138.0</td>
</tr>
<tr>
<td>TOT</td>
<td>125.3</td>
<td>125.0</td>
</tr>
<tr>
<td><strong>Macronuclear figure, length</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRP</td>
<td>209.2</td>
<td>200.0</td>
</tr>
<tr>
<td>BRR</td>
<td>143.0</td>
<td>147.5</td>
</tr>
<tr>
<td>VEN</td>
<td>232.0</td>
<td>240.0</td>
</tr>
<tr>
<td>TOT</td>
<td>199.1</td>
<td>190.0</td>
</tr>
<tr>
<td><strong>Macronucleus spread, length (uncoiled but not despiralized, very approximate values)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRP</td>
<td>270.0</td>
<td>270.0</td>
</tr>
<tr>
<td>BRR</td>
<td>176.3</td>
<td>180.0</td>
</tr>
<tr>
<td>VEN</td>
<td>266.0</td>
<td>295.0</td>
</tr>
<tr>
<td>TOT</td>
<td>245.3</td>
<td>255.0</td>
</tr>
<tr>
<td><strong>Macronucleus, width</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRP</td>
<td>8.7</td>
<td>9.0</td>
</tr>
<tr>
<td>BRR</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>VEN</td>
<td>7.6</td>
<td>8.0</td>
</tr>
<tr>
<td>TOT</td>
<td>7.7</td>
<td>8.0</td>
</tr>
<tr>
<td><strong>Macronucleus, number (in 2 out of 16 specimens broken into two long pieces)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRP</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>BRR</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>VEN</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>TOT</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Micronuclei, diameter</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRP</td>
<td>3.6</td>
<td>3.5</td>
</tr>
<tr>
<td>BRR</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>VEN</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>TOT</td>
<td>3.6</td>
<td>3.5</td>
</tr>
<tr>
<td><strong>Micronuclei, number</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRP</td>
<td>19.3</td>
<td>20.0</td>
</tr>
<tr>
<td>BRR</td>
<td>17.8</td>
<td>17.5</td>
</tr>
<tr>
<td>VEN</td>
<td>19.0</td>
<td>17.0</td>
</tr>
<tr>
<td>TOT</td>
<td>18.8</td>
<td>19.0</td>
</tr>
</tbody>
</table>
portion of all populations and specimens investigated (Figs 54, 57, 58, 61-63, 79, 81, 82, 90): type I extrusomes conspicuous because acicular, slightly curved and 12 ± 1 µm in size (9-11 ± 1 µm in second Brazilian population, 12-14 ± 1 µm in Venezuelan specimens), rarely faintly impregnate with the protargol method used; type II extrusomes rod-shaped, inconspicuous because fine and only 3-4 µm long. Exploded type I extrusomes 25-35 ± 1 µm in size, of typical toxicyst structure (Figs 62, 79, 82). Cytoplasm colourless, usually crammed with lipid droplets 1-2 µm across and food vacuoles containing remnants of rotifers and oral baskets of small ciliates, likely Drepanomonas (Figs 54, 55, 68). Swims rather rapidly by rotation about main body axis, showing great flexibility.

Cilia about 8 µm long in vivo, loosely spaced in oral area, arranged in an average of 37 ± 5 in Venezuelan specimens) ordinarily and equidistantly spaced rows abutting on circumoral kinety in Spathidium pattern, that is, in steep angles on right side and at almost right angles
A new spathidiid ciliate family

Figs 54-67. *Cephalospatula brasiliensis* from life (54-64) and after protargol impregnation (65-67). 54 - right side view of a representative specimen from type location. Note the large food vacuole with a decomposing rotifer in mid-body. Arrowheads mark contractile vacuoles; 55, 56 - optical section and surface view of cortex. Note food vacuole with a decomposing oral basket of a microthoracid ciliate; 57, 58 - lateral and frontal view of oral area, showing the conspicuous, spoon-shaped oral bulge containing extrusomes only in the anterior inflation. Arrow marks posterior brush region, where bristles and ordinary cilia alternate; 59, 60 - cortical granulation of oral bulge in a Venezuelan (59) and Brazilian (60) specimen; 61-63 - resting (61, 63) and exploded (62) type I (61, 62) and type II (63) oral bulge extrusomes, drawn to scale, length 9-12 µm, 25 µm, 3-4 µm; 64 - cortical granule, about 0.8-1 x 0.4 µm; 65, 66 - ventral and dorsal view of ciliary pattern in oral body portion of a Venezuelan specimen; 67 - ventrolateral view of a Venezuelan specimen with almost straight macronucleus. Arrowhead marks end of oral bulge. B - dorsal brush; CK - circumoral kinety; CV - contractile vacuole; E - extrusomes; EP - excretory pores; FV - food vacuole; G - cortical granules; MA - macronucleus; MI - micronucleus; N - nematodesmata; OB - oral bulge. Scale bars 100 µm.
Figs 68-73. *Cephalospatula brasiliensis*, ciliary pattern after protargol impregnation. 68, 69 - dorsal and ventral view of holotype specimen. Arrowheads mark excretory pores of the contractile vacuoles; 70, 71 - ventral and dorsal view of oral body portion of holotype specimen; 72, 73 - left side view of anterior body portion of two other specimens from type location. Note the sharp angle in the oral bulge (arrows) and circumoral kinety caused by the oblique truncation of the body end. Arrowheads mark indistinct left side kinetofragments. B - dorsal brush; CK - circumoral kinety; EP - excretory pores; F - oral bulge fibres; M - mastax of a rotifer; MA - macronucleus; MI - micronuclei; OB - oral bulge. Scale bars 100 µm (68, 69); 40 µm (70, 71); 20 µm (72, 73).
A new spathidiid ciliate family

on left; left side pattern, however, distinct only in apical region, where cilia form kinetofragment-like condensations (Figs 54, 65, 68-73, 76-78, 83-85, 87, 88; Table 2). Three dorsolateral ciliary rows anteriorly differentiated to dorsal brush occupying 35% of body length on average; brush, however, inconspicuous in vivo because bristles merely up to 4 µm long; dikinetidal and monokinetidal bristles irregularly alternate in posterior region of brush, and some monokinetids are interspersed in whole length of brush (Figs 54, 57, 66, 68, 71, 76, 77, 89; Table 2). Brush rows 1 and 2 of very similar structure, that is, composed of about 2 µm long, paired, inflated bristles, row 2 slightly longer than rows 1 and 3; row 3 composed of 3-4 µm long, fusiform bristles and a monokinetidal tail extending to second body third with about 3 µm long bristles; rarely, brush rows have a short, monokinetidal tail anteriorly (Figs 54, 57, 74, 75).

Mouth extends on anterior third of body, indistinct, except of inflated anterior portion containing the conspicuous extrusome bundle, due to the flat, hardly projecting oral bulge. Oral bulge distinctly spoon-shaped, conspicuous when viewed ventrally, both in vivo and protargol preparations, due to the anterior inflation; contains fine, short fibres originating from circumoral dikinetids and extending obliquely anteriorly. Circumoral kinety composed of comparatively widely spaced, oblique dikinetids having ciliated only one basal body; of same shape as oral bulge, shows highly characteristic,
Figs 79-85. *Cephalospatula brasiliensis* from life (79, 81, 82) and after protargol impregnation (80, 83-85). 79 - anterior body (oral bulge) end of a squashed specimen with released type I extrusomes, which have typical toxicyst structure and are about 25 µm long; 80 - middle portion of nuclear apparatus. The macronucleus is spiralized and ribbon-like flattened showing either the broad (arrowhead) or narrow (arrow) side; 81, 82 - resting (arrows) and extruded (asterisks) type I extrusomes and resting type II extrusomes (arrowheads), which are 3–4 µm long; 83 - overview showing some main features, such as the obliquely truncated anterior end and the spiralized macronucleus; 84, 85 - ventral views of anterior body portion showing the sharp bend (arrowheads) in the oral bulge and circumoral kinety, a main feature of the family Myriokaryonidae. Arrow denotes proximal end of mouth (circumoral kinety). CK - circumoral kinety; MA - macronucleus; MI - micronuclei; N - nematodesmata; OB - oral bulge.
A new spathidiid ciliate family

Figs 86-90. *Cephalospatula brasiliensis* after protargol impregnation. 86, 87 - overview and oral detail of a slender specimen. Arrowheads mark the mouth, respectively, oral bulge, which is hardly set off from body proper proximally, and thus difficult to recognize *in vivo*. Note the conspicuous widening of oral bulge and circumoral kinety in the truncated anterior body end, a main feature of the family; extrusomes are contained only in the widened area, a main feature of the genus (Figs 57, 90); 88-90 - right (88) and left (89) side view of ciliary pattern and optical section (90) of a laterally oriented specimen showing details of oral area, viz., the anteriorly distinctly curved dikinetidal circumoral kinety associated with conspicuous nematodesmata; the three-rowed dorsal brush; and the extrusomes in the anterior widening of the oral bulge. B1-3 - dorsal brush rows; CK - circumoral kinety; E - extrusomes; MA - macronucleus; MI - micronucleus; N - nematodesmata (oral basket rods); OB - oral bulge.
sharp bend subapically, that is, in the transition zone of oblique anterior and straight ventral portion. Oral basket conspicuous because of long nematodesma bundles originating from circumoral dikinetids (Figs 54, 57, 58, 65-73, 76-78, 83-90; Table 1).

Occurrence and ecology: as yet found only at three sites in South America, as described in the material and methods section, although I investigated similar biotopes worldwide (Foissner 1998, Foissner et al. 2002, and unpubl. data), suggesting that this conspicuous ciliate might be restricted to South America and/or Gondwana. Two of the three sites, and to a certain extent also the Restingha area, are definitely semiterrestrial (floodplain soil, mud and soil from a rock-pool), indicating that C. brasiliensis might occur also in ordinary freshwaters, although the slender shape suggests a preference for soil (Foissner 1987).

DISCUSSION

Myriokaryonidae fam. n.

Diagnosis: Spathidiina with spoon-shaped oral bulge (circumoral kinety) and oblique to transverse-truncate anterior body end, causing a more or less sharp bend in oral bulge and circumoral kinety.


Nomenclature: Jankowski (1975) already erected a family Myriokaryonidae, but without any characterization. Thus, it is a nomen nudum.

Suprafamiliar classification: Myriokaryon and related genera belong to the gymnostomatous holotrichs, as defined by Corliss (1979), Grain (1994), and Lynn and Small (2002), because they have a holotrichious ciliature composed of monokinetids, a rhabdos-type oral ciliature, a dorsal brush, and toxicysts. Today, this assemblage is usually ranked as a class, viz., Litotomatea or Gymnostomatea (Foissner et al. 2002). Many distinct groups are recognizable within this large clade, which contains about 1000 described and many undescribed species (Foissner et al. 2002). Unfortunately, within-class classification differs considerably, likely because few detailed data are available on ultrastructure, character states (pleiomesomorphies, apomorphies), and gene sequences (Corliss 1979, Foissner and Foissner 1988, Grain 1994, Lynn and Small 2002). My own cladistic attempts (unpubl., Hennig’s method) failed because I could not unequivocally determine the state of most characters. Similarly, the cladistic analysis by Lipscomb and Riordan (1990) obviously confused character states because spathidiids and pleurostomatids form a clade, which is, in my opinion, unlikely. Gene sequence data are available only from few species and thus cannot be used in the present context. Thus, my classification of the Myriokaryonidae follows the system of Foissner and Foissner (1988), which is based on light microscopical and ultrastructural data. However, I emphasize that any classification must be considered as preliminary at the present state of knowledge.

In spite of these problems, most recent authors agree that Myriokaryon belongs to the group which contains Spathidium and related genera. This is in accordance with the present results, which suggest that the Myriokaryonidae should be classified as a family of the order Spathidiida Foissner and Foissner (1988): “Cytostome apical, round or slit-like, in suborder Didiniina on top of cone-like proboscis; rhabdos made of three microtubular components: transverse ribbons originating from the nonciliated kinetosomes of the oral dikinetids, nematodesmal bundles originating exclusively from the same source, and bulge microtubules; somatic ciliation uniform or limited to dense bands which, however, rest within longitudinally running kinetics composed of nonciliated kinetids; dorsal brush composed of two to many kinetics; toxicysts localized, typically in or near oral area; free-living”. Foissner and Foissner (1988) distinguish three suborders within the Spathidiida, viz., the Belonophryina (mainly Actinobolina and related genera), the Didiniina (Didinium and related genera), and the Spathidiida, which they define as follows: “Cytostome apical, round, oval or slit-like, in some genera covering the ventral body margin; somatic ciliation usually uniform”. Obviously, the Myriokaryonidae match this definition.

Comparison with related families: the Myriokaryonidae are established to include Myriokaryon Jankowski, 1973 and three new genera, viz., Cephalospatula, Berghophrya, and Kahlophrya. The key features uniting these genera, viz., the transverse-truncate anterior body end and the spoon-like anterior widening of the circumoral kinety and oral bulge are, unfortunately, difficult to recognize because the truncated part, viz., the spoon-shovel, is small and the oral bulge flat (Figs 1, 5, 13, 20, 40, 48, 54, 57, 70, 76-78, 83-85). The truncation of the body end causes a highly characteristic bend of the oral bulge and circumoral kinety in the transition zone of spoon-shovel and spoon-handle, that is, the region where the oral bulge and the circumoral kinety enter the ventral side of the cell (Figs 15, 26-28, 57, 72, 73, 76, 84,
85, 87, 88); again, the bend is not easily recognized neither in vivo or silver preparations, especially in the elliptical species, viz., Bergophrya and Kahlphrya (Figs 102, 103, 107, 109-111).

As discussed above, the Myriokaryonidae match the diagnosis of the suborder Spathidiina, which contains the families Spathidiidae, Lacrymariidae, Homalozoonidae, and Trachelophyllidae; the latter family was raised to subordinal rank recently (Foissner et al. 2002). Within this assemblage, the Myriokaryonidae are obviously most closely related to the Spathidiidae, as shown by the general body plan and ciliary pattern, which basically match those of “classical” spathidiids, such as Spathidium and Arcuospathidium, as described by Foissner (1984) and Foissner et al. (2002). Interestingly, most generic patterns found in the Spathidiidae occur also in the Myriokaryonidae, indicating highly convergent evolution.

Admittedly, the features separating the Myriokaryonidae from the Spathidiidae (preliminary characterization: Spathidiina with roundish to elongate elliptical oral bulge and circumoral kinety) are rather inconspicuous. However, this must be considered under a more general view, viz., that haptorid gymnostomes have, compared with hypotrichs for instance, fewer distinct features, which, additionally, are more difficult to recognize and reveal.

Kahl (1930a) classified Myriokaryon lieberkuehnii in Pseudoprorodon (now Prorodon for nomenclatural reasons, see Aescht 2001 and Foissner et al. 1994), a still poorly known genus of unclear affinities. However, if the redescription of the type species, P. niveus, by Groliere (1977) is accepted, then Prorodon is highly different from Myriokaryon and, also, likely from the spathidiids. This is emphasized by Pseudoprorodon (now Prorodon) arenicola, which, at first glance, looks like a myriokaryonid (Dragesco 1960), but has a prorodontid oral basket (Kattar 1972).

Based on a reinvestigation, Jankowski (1973) classified Prorodon lieberkuehnii into a new genus, Myriokaryon, which he assigned to the Trachelidae because of the supposed dileptid oral ciliary pattern. This was not accepted by Lynn and Small (2002), who classified Myriokaryon in the Spathidiidae, a relationship also discussed by Jankowski (1973). My investigations show that the short, transverse kineties at the left side of the circumoral kinety are not dileptid preoral ciliary rows, as supposed by Jankowski (1973), but the polymerized anterior end of the left side somatic kineties. Such polymerization is frequent in spathidiids (Foissner 1984, Foissner et al. 2002).

Cranotheridium foliosum (Foissner, 1983), as redescribed by Wirnsberger et al. (1984), has very similar generic features as Myriokaryon, viz., a slightly spoon-shaped oral bulge and many isomorphic dorsal brush rows. However, I hesitate to transfer it to Myriokaryon because it is a small (length near 80 µm), Spathidium-shaped species and thus quite dissimilar to M. lieberkuehnii. Possibly, C. foliosum evolved convergently and belongs to the Spathidiidae, as proposed by Wirnsberger et al. (1984). Unfortunately, details of the oral and somatic ciliary pattern of Cranotheridium taeniatum, type of the genus, are unknown. However, if the observations of Schewiakoff (1893) are correct, Cranotheridium likely neither belongs to the Myriokaryonidae nor Spathidiidae because it has a distinct, prostomatid or nassulid pharyngeal basket. Indeed, very recent observations on a Cranotheridium foliosum-like ciliate from soil suggest that such species represent a new spathidiid genus different from Cranotheridium Schewiakoff, 1893.

Myriokaryon lieberkuehnii

My population of M. lieberkuehnii basically matches the original figure by Bütschli (1889) and several redcriptions (Kahl 1930a, Dragesco 1972, Jankowski 1973, Dragesco and Dragesco-Kernéis 1986), especially in having a large, slender body with a minute, transverse truncation anteriorly; more than 2000 macronuclear nodules; many contractile vacuoles; long, fine extrusomes; and about 100 ciliary rows. Thus, identification is beyond reasonable doubts.

However, more or less pronounced differences are found in details, all likely caused by incomplete and/or incorrect observations of the above mentioned authors. I shall not discuss all these mistakes, but some must be mentioned for the sake of identification and taxonomic classification; a few doubtful identifications and synonyms are mentioned in Kahl (1930a) and the distribution and ecology section.

Bütschli (1889) and Kahl (1930a) illustrated a sort of conical oral basket at the anterior end of the organisms (Figs 91, 97, 99). Such basket is definitely absent in my specimens. I do not have any explanation for this difference, except of that these are different species, which is, however, unlikely considering that many other features match well. Jankowski (1973) suggests that previous authors misinterpreted the apical extrusome bundles (“trichites”) as a nematodesmal apparatus. Dragesco (1972) and Dragesco and Dragesco-Kernéis (1986), whose in vivo observations match my data, did
not see the dorsal brush and the special ciliary pattern at the left side of the circumoral kinety, although they used protargol impregnation (Figs 92-95). Likely, their preparations were too weak. The data of Jankowski (1973) are much better than those of Dragesco, although he misinterpreted the whole mouth organization, possibly because he related *Myriokaryon* to the dileptids (Fig. 101). Specifically, he did not observe the circumoral kinety and the very fine nematodesmata. Thus, he interpreted the narrow, central mouth slit as “mouth free of any nematodesmal armature”. Further, he misinterpreted the densely ciliated anterior end of the left side ciliary rows as tracheliid preoral kineties, and the long toxicysts as “trichites”. On the other hand, Jankowski (1973) recognized, inter alia, the widened anterior end of the oral bulge, the polymerized anterior end of the left side kineties, and the high number (about 10) of dorsal brush rows (thigmotactic stripe).

*Cephalospatula brasiliensis* as a new genus and species

*Cephalospatula* is obviously related to *Myriokaryon*, as shown by distinct similarities in shape of body and oral bulge and the kinetofragments along the left margin of the circumoral kinety, although these are less conspicuous. On the other hand, there are also considerable differences, mainly in the number of dorsal brush rows (three vs. many) and the arrangement of the oral

---

**Figs 91-100.** *Myriokaryon lieberkuehni*, figures from literature. 91 - from Bütschli (1889), length 1250 µm. Arrow marks tubular “mouth”; 92, 93, 95, 98 - from Dragesco (1972), shape variability from life (92), ciliary pattern in anterior body region after protargol impregnation (93), general *in vivo* view, length 1100 µm (95), and nuclear pattern after Feulgen reaction (98); 94 - from Dragesco and Dragesco-Kernéis (1986), ciliary pattern of ventral side after protargol impregnation; 96 - from Cunha (1914), general *in vivo* view, length 600-800 µm; 97 - from Kahl (1930a), general *in vivo* view of an ordinary specimen, length 1100 µm; 99 - from Kahl (1930a), *in vivo* view of a small specimen, length 600 µm (likely a misidentification); 100 - from Al-Rasheid (2000), left lateral view after protargol impregnation, length 600-800 µm (misidentification, see discussion). CK - circumoral kinety; MA - macronuclear nodules; MI - micronucleus; OB - oral bulge.
Fig. 101. Myriokaryon lieberkuhni, Russian neotype population investigated by Jankowski (1973). Figures without scale bars and explained in text. Thus, the reader is referred to the original publication (English translation available from Foissner).
extrusomes (single apical bundle vs. scattered in whole bulge). Three brush rows are the usual brush pattern in haptoral gymnostomes and are thus likely the plesiomorphic state of this important specialization (Foissner and Foissner 1988, Foissner et al. 2002). Thus, any deviation from this pattern, as in Myriokaryon, should be rated rather high, viz., at genus level. Likewise, the extrusome pattern is widely used for separation of genera and even families, for instance, in the spathidiid genera Legendrea and Cranotheridium (see Kahl 1930a for literature), and the definition of the family Pleuroplitidae Foissner, 1996.

There is only one species in the literature, viz., Spathidium vermiforme Penard, 1922, bearing some resemblance to C. brasiliensis. However, S. vermiforme is leaf-like flattened and has the extrusomes scattered in the short, oblique oral bulge. Thus, C. brasiliensis is a very distinct species easy to recognize by the large, slender body; the extrusome bundle in the widened apical end; the filiform macronucleus; and the dorsal row of contractile vacuoles.

**Berghophrya gen. n. (Figs 102-109)**

**Diagnosis:** ellipsoidal Myriokaryonidae with short mouth on obliquely truncated anterior body end and 3 dorsal brush rows accompanied by 3 rows of papillae. Extrusomes scattered in oral bulge and postoral bundles. Infraciliature likely in Arcuospathidium pattern.

**Type species:** Holophrya Emmae Bergh, 1896.

**Dedication:** I dedicate this genus to Dr. R. S. Bergh, the Danish protozoologist who discovered and described the type species so well that the data are useful even today. Berghophrya is a composite of Bergh and the Greek noun ophrya (eyebrow ~ cilia ~ ciliate). Feminine gender.

**Comparison with related genera:** the excellent observations of Bergh (1896) were confirmed and supplemented by Kahl (1930a). They show that Holophrya emmae has the same main generic feature as Myriokaryon lieberkuehni and Cephalospatula brasiliensis, viz., a spoon-like oral bulge (Figs 102-109). Kahl (1930a) even recognized the similarity with M. lieberkuehni and classified both in Pseudoprorodon. Accordingly, the redescription of H. emmae by Song and Wilbert (1989), which shows a ciliate with a circular oral bulge, is based on a misidentification (see the new genus Songophrya below). Holophrya emmae is sufficiently different in details of the dorsal brush and extrusome pattern to be transferred to a new genus: Berghophrya emmae (Bergh, 1896) comb. n.

Details of the infraciliature from silver-impregnated B. emmae are not available. However, Kahl (1930a) shows that it likely has an Arcuospathidium pattern, like Kahlophrya armata, that is, the ciliary rows abut on the circumoral kinety at steep angles at both sides of the oral bulge (Fig 107).

**Kahlophrya gen. n. (Figs 110-112)**

**Diagnosis:** elongate Myriokaryonidae with short mouth on obliquely truncated anterior body end and many (more than 3) dorsal brush rows, most of which are heteromorphic. Extrusomes scattered in oral bulge and equidistant body stripes. Infraciliature in Arcuospathidium pattern.

**Type species:** Pseudoprorodon armatus Kahl, 1930a.

**Dedication:** I dedicate this genus to Alfred Kahl (1877-1946), the famous German ciliate taxonomist, whose detailed observations greatly facilitated the present study. Kahlophrya is a composite of Kahl and the Greek noun ophrya (eyebrow ~ cilia ~ ciliate). Feminine gender.

**Comparison with related genera:** Kahlophrya is based on the redescription of Pseudoprorodon armatus Kahl, 1930a by Foissner (1997). This study shows the features mentioned in the genus diagnosis and the spoon-shaped oral bulge characteristic for the family (Figs 110-112). Thus, Pseudoprorodon armatus Kahl, 1930a and Prorodon armatides Foissner, 1997 are combined to Kahlophrya armata (Kahl, 1930a) comb. n. The main generic feature of Kahlophrya is the dorsal brush, which consists of about 20 rows. This resembles Myriokaryon, whose rows are, however, isomorphic, that is, consist of dikinetids with short cilia (bristles). Kahlophrya has only one isomorphic brush row, all others are heteromorphic, that is, composed of irregularly alternating monokinetids with ordinary cilia and dikinetids with short bristles. A tendency for heteromorphy is recognizable also in the posterior region of the brush rows of Myriokaryon and Cephalospatula (Figs 51-53, 57). Another important feature of Kahlophrya are the about 20 stripes of body extrusomes not found in any other genus of the family, although the genus Berghophrya shows such a tendency (see above); further, somatic extrusome stripes occur in several genera of related families, viz., in Apospathidium of the family Spathidiidae and in Apobryophyllum of the family Bryophyllidae (Foissner et al. 2002). The ciliary pattern of Kahlophrya is as in the spathidiid genus...
A new spathidiid ciliate family

Figs 102-109. *Berghophrya emmae* from life, according to Bergh 1896 (102-104) and Kahl 1930a (105-109). 102 - general left side view, length up to 200 µm; 103, 104 - ventral and lateral view of squashed specimens showing the spoon-shaped oral bulge, the dorsal papillae (arrowheads), and postoral extrusome bundles; 105-107 - small variety (length 160 µm), lateral, dorsal, and frontal view of oral area with dorsal papillae marked by arrowhead; 108 - medium-sized variety, length 300 µm; 109 - vermiform variety, length 320 µm.

Figs 110-112. *Kahlophrya armata* from life (110, 111) and after protargol impregnation (112), according to Foissner (1997). 110-111 - general left side view (length 210 µm) and frontal view of oral area; 112 - ciliary pattern of anterior ventral side. Note the complicated dorsal brush composed of many rows with bristles and ordinary cilia.

Figs 113-116. *Songophrya armata* from life (114) and after protargol impregnation (113, 115, 116), according to Song and Wilbert (1989). This species has a circular oral bulge (113) and is thus highly different from *Berghophrya emmae*, which has a spoon-shaped oral bulge (103, 107). *Songophrya armata* has a size of 90-200 x 60-120 µm and the same brush type as *Kahlophrya armata*, that is, many rows composed of short, paired bristles (arrows) alternating with ordinary cilia (112, 114-116). B - dorsal brush; CK - circumoral kinety; E - extrusomes; OB - oral bulge.
Arcuospathidium and the myriokaryonid genus Berghophrya (see above), that is, the somatic ciliary rows abut on the circumoral kinety in steep angles at both sides of the oral bulge.

**Songophrya gen. n. (Figs 113-116)**

**Diagnosis:** Pseudoholophryidae with circular oral opening and a row of extrusome bundles extending from anterior to posterior body end.

**Type species:** *Songophrya armata* sp. n.

**Dedication:** I dedicate this genus to Prof. Dr. Weibo Song (Ocean University of China), an eminent Chinese protozoologist, who significantly contributes to ciliate alpha-taxonomy since 15 years and discovered the type species. *Songophrya* is a composite of *Song* and the Greek noun *ophrya* (eyebrow ~ cilia ~ ciliate). Feminine gender.

**Comparison with related genera:** Foissner and Gschwind (1998) and Foissner et al. (2002) suggested that *Pseudoprorodon emmae* Bergh, 1896, as re-described by Song and Wilbert (1989), is likely another species and the representative of a new genus belonging to the family Pseudoholophryidae, as redefined by Foissner et al. (2002). With the new knowledge available from Myriokaryon and Cephalospatula, it is evident that the species described by Song and Wilbert (1989) is not *Holophrya emmae* Bergh, 1896 because it has a simple, circular oral bulge, while that of *H. emmae* is conspicuously spoon-shaped (Figs 103, 113). Unfortunately, Song and Wilbert (1989) did not discuss this important difference, and thus their identification remains obscure; possibly, they did not know Bergh’s original description. Within the family Pseudoholophryidae, which contains the genera *Pseudoholophrya*, *Paraenchelys*, and *Ovalorhabdos*, *Songophrya* is unique in having a row of extrusome bundles. Further, the ciliary rows extend almost meridionally, while distinctly spirally in the other genera. As concerns the oral bulge, it is circular in *Songophrya*, *Pseudoholophrya* and *Paraenchelys*, while distinctly elliptical in *Ovalorhabdos* (Foissner 1984, Foissner and Gschwind 1998, Foissner et al. 2002). The extrusomes, which distinguish the genera *Pseudoholophrya* and *Paraenchelys* (basically rod-shaped vs. basically drumstick-shaped), were not studied *in vivo* by Song and Wilbert (1989). Likely, they are filiform, but it cannot be excluded that a second fusiform type is present, which would be a further important genus character.

**Songophrya armata sp. n.**

**Diagnosis:** size 90-200 x 60-120 μm *in vivo*; ellipsoidal. Macronucleus filiform. Extrusome row composed of 5-7 (x 5.3) bundles. On average 102 ciliary rows, more than 20 differentiated to dorsal brush in anterior half.

**Type location:** pond (Poppelsdorfer Weiher) in Bonn, Germany (7°E 51°N).

**Etymology:** the Latin adjective *armata* (armed) refers to the conspicuous extrusomes.

**Description:** see Song and Wilbert (1989) and figures 113-116.

**Acknowledgements.** The technical assistance of Dr. B. Moser and Dr. E. Herzog is gratefully acknowledged. Financial support was provided by the Austrian Science Foundation (FWF-Project P-15017).

**REFERENCES**


Foissner W. (1997) Faunistische and taxonomic studies on ciliates (Protozoa, Ciliophora) from clean rivers in Bavaria (Germany), with descriptions of new species and ecological notes. Limnologica 27: 179-238


Foissner W., Agatha S., Berger H. (2002) Soil ciliates (Protozoa, Ciliophora) from Namibia (Southwest Africa), with emphasis on two contrasting environments, the Etosha region and the Namib Desert. Denisia, Linz 5: 1-1459


Received on 2nd September, 2002: revised version on 11th November, 2002; accepted on 19th February, 2003
Two Remarkable Soil Spathidiids (Ciliophora: Haptorida), *Arcuospathidium pachyoplites* sp. n. and *Spathidium faurefremieti* nom. n.

Wilhelm FOISSNER

Universität Salzburg, Institut für Zoologie, Salzburg, Austria

Summary. This paper continues a series of studies on spathidiids, a group of free-living, rapacious ciliates with a high biodiversity. *Arcuospathidium pachyoplites* sp. n. was discovered in saline coastal soil from the Henry Pittier National Park in Venezuela, South America. *Spathidium faurefremieti*, originally described by Tucolesco (1962) from Rumanian cave water, was rediscovered in savannah soil from the Shimba Hills National Reserve in Kenya (Africa) and in floodplain soils of Brazil (South America) and Australia. The morphology of these species was investigated using live observation and protargol impregnation. The South American *A. pachyoplites* differs from the African *A. vlassaki*, possibly the nearest relative, mainly by the extrusomes and dorsal brush. *Spathidium faurei* Tucolesco, 1962 is an objective homonym of *Spathidium faurei* Kahl, 1930 and thus re-named: *Spathidium faurefremieti* nom. n. This species is remarkable in having a second contractile vacuole in anterior body half. However, conspecificity of the European and Kenyan populations is questionable; likewise, the Kenyan and Brazilian populations differ considerably, suggesting that further research might prove that all are different subspecies or even species. The present study shows that (i) an increased number of contractile vacuoles likely evolved independently three times, viz., in *Spathidium*, *Arcuospathidium*, and *Supraspathidium*, and (ii) the bivacuolate species should be separated from the polyvacuolate species, which can be referred to the genus *Supraspathidium*.

Key words: Australia, biodiversity, Brazil, Kenya, *Supraspathidium*, terrestrial Protozoa, Venezuela.

INTRODUCTION

This paper continues a series of studies on spathidiid and *Spathidium*-like ciliates, whose full diversity is still not known (Buitkamp 1977; Dragesco and Dragesco-Kernéis 1979; Foissner 1984, 2000, 2003a; Foissner et al. 2002). The two species described here are remarkable in several ways. *Arcuospathidium pachyoplites* from a saline site in Venezuela is rather similar to *A. vlassaki* Foissner, 2000 and *A. etoschense* Foissner et al. 2002 from saline inland habitats of Africa. These species might be examples for post-Gondwanan speciation. The second species, *Spathidium faurefremieti* is outstanding in having two contractile vacuoles, a feature which, however, obviously evolved independently at least twice because it is found in spathidiids with either an *Arcuospathidium* or *Spathidium* ciliary pattern, viz., in *A. bulli* Foissner, 2000 and *S. faurefremieti* re-described here.

Both species are very slender showing that many soil ciliates have the same main morphological adaptation as many metazoan soil inhabitants, viz., a worm-like body.
Further, they are able to produce dormant stages (resting cysts) to survive periods of dryness, a main physiological adaptation of soil organisms in general (Foissner 1987).

MATERIALS AND METHODS

See type locations and distribution sections for collectors and detailed site descriptions. The samples were air-dried in the Salzburg laboratory and stored in plastic bags until investigation. The ciliates were reactivated from the resting cysts by the non-flooded Petri dish method, as described in Foissner (1987) and Foissner et al. (2002). Briefly, this simple method involves placing soil in a Petri dish (10-20 cm wide, 2-3 cm high) and saturating, but not flooding it, with distilled water. These cultures were analyzed for ciliates by inspecting about 2 ml of the run-off (soil percolate) on days 2, 7, 14, 21, and 28. The descriptions of the species are based on material obtained from such cultures, i.e. no clones were set up.

Morphological methods followed those used in our previous studies (e.g. Foissner 1984, 1991; Foissner et al. 2002), and thus need not to be detailed here. Briefly, live specimens were studied in bright field and interference contrast, and permanent preparations were made with protargol (Protocol A in Foissner 1991).

RESULTS

Description of *Arcuospathidium pachyoplites* sp. n.
(Figs 1-16, 23-25, 27-34, 37-43; Table 1)

Diagnosis: size about 170 x 20 µm *in vivo*. Knife-shaped with steep, slightly cuneate oral bulge occupying about 22% of body length. Macronucleus tortuous, figure formed about 80 µm long. Extrusomes conspicuous because lanceolate and 7 x 1.4 µm in size, scattered in both sides of oral bulge and attached to bulge cortex with narrowed anterior end. On average 10 ciliary rows; dorsal brush inconspicuous, occupies 17% of body length on average.

Type location: saline coastal soil in the surroundings of the village of Choroni (67°45'W 10°15'N), Henry Pittier National Park, north coast of Venezuela, South America.

Etymology: appositive noun composed of the Greek words pachy (thick) and (h) oplites (soldier ~ extrusome), referring to the conspicuous extrusomes.

### Table 1. Morphometric data on *Arcuospathidium pachyoplites*.

<table>
<thead>
<tr>
<th>Characteristics</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>Body, length</td>
<td>158.9</td>
<td>160.0</td>
<td>25.5</td>
<td>5.6</td>
<td>16.1</td>
<td>115.0</td>
<td>210.0</td>
<td>21</td>
</tr>
<tr>
<td>Body, width</td>
<td>19.3</td>
<td>18.0</td>
<td>6.1</td>
<td>1.3</td>
<td>31.6</td>
<td>12.0</td>
<td>38.0</td>
<td>21</td>
</tr>
<tr>
<td>Body length:width, ratio</td>
<td>9.0</td>
<td>8.2</td>
<td>3.3</td>
<td>0.7</td>
<td>36.7</td>
<td>4.8</td>
<td>17.5</td>
<td>21</td>
</tr>
<tr>
<td>Oral bulge, length</td>
<td>34.4</td>
<td>35.0</td>
<td>6.2</td>
<td>1.4</td>
<td>18.0</td>
<td>21.0</td>
<td>44.0</td>
<td>21</td>
</tr>
<tr>
<td>Body length:oral bulge length, ratio</td>
<td>4.7</td>
<td>4.6</td>
<td>0.7</td>
<td>0.2</td>
<td>15.4</td>
<td>3.2</td>
<td>6.2</td>
<td>21</td>
</tr>
<tr>
<td>Oral bulge, width</td>
<td>4.6</td>
<td>4.5</td>
<td>0.7</td>
<td>0.1</td>
<td>14.6</td>
<td>3.0</td>
<td>5.5</td>
<td>21</td>
</tr>
<tr>
<td>Oral bulge, height at anterior end</td>
<td>2.0</td>
<td>2.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.5</td>
<td>3.0</td>
<td>21</td>
</tr>
<tr>
<td>Circumoral kinety to end of brush row 1, distance</td>
<td>17.2</td>
<td>18.0</td>
<td>3.2</td>
<td>0.7</td>
<td>18.5</td>
<td>11.0</td>
<td>23.0</td>
<td>21</td>
</tr>
<tr>
<td>Circumoral kinety to end of brush row 2, distance</td>
<td>27.2</td>
<td>27.0</td>
<td>4.8</td>
<td>1.0</td>
<td>17.6</td>
<td>18.0</td>
<td>38.0</td>
<td>21</td>
</tr>
<tr>
<td>Circumoral kinety to end of brush row 3, distance</td>
<td>17.0</td>
<td>18.0</td>
<td>3.8</td>
<td>0.8</td>
<td>21.2</td>
<td>12.0</td>
<td>27.0</td>
<td>21</td>
</tr>
<tr>
<td>Anterior body end to macronucleus, distance</td>
<td>57.1</td>
<td>56.0</td>
<td>10.6</td>
<td>2.3</td>
<td>18.5</td>
<td>43.0</td>
<td>84.0</td>
<td>21</td>
</tr>
<tr>
<td>Macronucleus figure, length</td>
<td>72.4</td>
<td>72.0</td>
<td>14.0</td>
<td>3.0</td>
<td>19.3</td>
<td>52.0</td>
<td>98.0</td>
<td>21</td>
</tr>
<tr>
<td>Macronucleus, length (spread)</td>
<td>111.3</td>
<td>110.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>80.0</td>
<td>150.0</td>
<td>21</td>
</tr>
<tr>
<td>Macronucleus, width in middle</td>
<td>4.2</td>
<td>4.0</td>
<td>0.8</td>
<td>0.2</td>
<td>18.3</td>
<td>3.0</td>
<td>5.0</td>
<td>21</td>
</tr>
<tr>
<td>Somatic kineties, number (including brush)</td>
<td>10.0</td>
<td>10.0</td>
<td>0.9</td>
<td>0.2</td>
<td>8.7</td>
<td>9.0</td>
<td>12.0</td>
<td>21</td>
</tr>
<tr>
<td>Ciliated kineties in a lateral kinety, number</td>
<td>56.2</td>
<td>56.0</td>
<td>10.8</td>
<td>2.4</td>
<td>19.2</td>
<td>36.0</td>
<td>78.0</td>
<td>21</td>
</tr>
<tr>
<td>Dorsal brush rows, number</td>
<td>3.0</td>
<td>3.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>3.0</td>
<td>3.0</td>
<td>21</td>
</tr>
<tr>
<td>Dikinetids in brush row 1, number</td>
<td>11.4</td>
<td>11.0</td>
<td>2.2</td>
<td>0.5</td>
<td>18.9</td>
<td>7.0</td>
<td>15.0</td>
<td>21</td>
</tr>
<tr>
<td>Dikinetids in brush row 2, number</td>
<td>28.0</td>
<td>28.0</td>
<td>5.7</td>
<td>1.2</td>
<td>20.4</td>
<td>18.0</td>
<td>38.0</td>
<td>21</td>
</tr>
<tr>
<td>Dikinetids in brush row 3, number</td>
<td>13.3</td>
<td>13.0</td>
<td>2.0</td>
<td>0.4</td>
<td>14.7</td>
<td>11.9</td>
<td>19.0</td>
<td>21</td>
</tr>
</tbody>
</table>

aData based on mounted, protargol-impregnated, selected (see description of species) specimens from a non-flooded Petri dish culture.

bVery approximate values. cOnly full rows counted. 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 arithmetic mean, x - arithmetic mean.
Figs 1-12. *Arcuospathidium pachyoplites* from life (1-5, 11) and after protargol impregnation (6-10, 12). 1 - left side view of a representative specimen, i.e., “constructed” from live observations and morphometric data shown in table 1. The oral bulge is very conspicuous due to the massive extrusomes contained; 2 - posterior half of brush row 2, longest bristles 4 µm; 3 - exploded toxicyst, length 20 µm; 4 - frontal view of the indistinctly cuneate oral bulge studded with extrusomes; 5 - oral bulge extrusomes, length 6-8 µm; 6, 7 - a broad and a slender specimen; note variability of macronucleus; 8-10 - ciliary pattern of ventral and dorsal side and nuclear apparatus of holotype specimen. Note the oblong circumoral kinety and the dorsal brush dikinetids, which are much more closely spaced in middle row 2 than in rows 1 and 3; 11 - surface view showing cortical granulation; 12 - left side view of another specimen with rather distorted dorsal brush and typical macronucleus with coiled and inflated ends. B - dorsal brush, B1-3 - dorsal brush rows, CK - circumoral kinety, CV - contractile vacuole, E - extrusomes, MA - macronucleus, MI - micronucleus, OB - oral bulge. Scale bars - 50 µm.
Figs 13-16. *Arcuospathidium pachyoplites*, right and left side view of oral body portion of two specimens after protargol impregnation. Dotted lines connect individual basal bodies of ciliary rows. These figures show: the rather long and very steep oral bulge; the dorsal brush dikinetids much more closely spaced in middle row 2 than in rows 1 and 3; that brush row 2 (arrows) is of about same length as the oral bulge; and, finally, the indistinct *Spathidium* pattern produced by the left side kineties, whose first basal body is very near to the circumoral kinety. B - dorsal brush, B2, 3 - dorsal brush rows, CK - circumoral kinety, MA - macronucleus, N - nematodesmata, OB - oral bulge. Scale bars 30 µm.

Figs 17-22. *Arcuospathidium vlassaki* from life (17-19; from Foissner 2000) and after protargol impregnation (20-22; new drawings from type population). 17 - left side view of a representative specimen (length 190 µm), showing that the oral bulge is much less conspicuous than in *A. pachyoplites*, where it is longer and studded with thick extrusomes (Fig. 1); 18 - frontal view showing extrusomes restricted to the left half of the oral bulge, while they are scattered throughout the bulge in *A. pachyoplites* (Fig. 4); 19 - extrusomes are 5 x 1 µm in size and attached by the broad end to the oral bulge; in *A. pachyoplites*, they are attached by the narrow end and considerably larger (6-8x1-1.7 µm), making the oral bulge very conspicuous (Figs 1, 5, 23, 25); 20-22 - the *Arcuospathidium* ciliary pattern is more distinct in *A. vlassaki* than in *A. pachyoplites* (Figs 14, 16) because the anterior end of the left side kineties is more distinctly directed dorsally (basal bodies of individual ciliary rows connected by dotted lines). Arrows mark end of dorsal brush row 2, which is distinctly longer than the oral bulge, a main difference to *A. pachyoplites*, where it is shorter or of same length as the oral bulge (Figs 14, 16). B - dorsal brush, B1-3 - dorsal brush rows, CK - circumoral kinety, E - extrusomes, MA - macronucleus, N - nematodesmata (oral basket rods), OB - oral bulge. Scale bar 30 µm (20-22).
Soil spathidiids

Figs 23-36. *Arcuospathidium pachyoplites* (23-25, 27-34) and *A. vlassaki* (26, 35, 36; from Foissner 2000) from life. 23-25 - overview and oral details of a slightly squashed and thus broadened specimen. *Arcuospathidium pachyoplites* is conspicuous due to the thick and numerous extrusomes contained in the oral bulge. Figure 24 is a surface view showing the closely spaced rows of cortical granules and the right margin of the oral bulge (arrows). Arrowheads in figure 23 mark the long, tortuous macronucleus. 26 - *Arcuospathidium vlassaki* is usually more slender than *A. pachyoplites* and the oral bulge, marked by arrowheads, is less conspicuous because it contains fewer and smaller extrusomes (cp. figures 32-36); 27 - right side view showing dorsal brush rows 2 and 3 with bristles up to 4 µm long; 28-31 - oral bulge extrusomes are 6-8 x 1-1.7 µm in size and rather variable in shape, usually, however, they are lanceolate; 32-36 - same scale comparison of extrusomes of *A. pachyoplites* (32-34) and *A. vlassaki* (35, 36). The extrusomes of *A. pachyoplites* are considerably larger (7 x 1.4 µm) than those of *A. vlassaki* (5 x 1 µm). B1, 2 - dorsal brush rows, CV - contractile vacuole, E - extrusomes, MA - macronucleus, OB - oral bulge.
Type material: 1 holotype slide and 2 paratype slides with protargol-impregnated specimens (Foissner’s method) have been deposited in the Oberösterreichische Landesmuseum in Linz (LI). All specimens illustrated and some other well-impregnated cells are individually marked by a black ink circle on the cover glass. For comparison, we add a slide of protargol-impregnated Arcuospathidium vlassaki from type population, showing that dorsal brush dikinetids are much more closely spaced in row 2 than in rows 1 and 3. Note that dorsal brush row (2) is shorter than the oral bulge, a main difference to A. vlassaki, where it is longer (Figs 20-22); 42, 43 - frontal views of circumoral kinety and oral bulge. The white dots within the circumoral kinety (oral bulge) are optical transverse sections of the large, unstained extrusomes. B1-3 - dorsal brush rows, CK - circumoral kinety, CV - contractile vacuole, MA - macronucleus, N - nematodesmata (oral basket rods), OB - oral bulge, R - ciliary rows.

Description: this species does not fix well, as is often the case with saline material. Some specimens look rather distorted and/or inflated by large food inclusions and/or insufficient preservation. Furthermore, the slides contain some very small specimens (postdividers?) and cells with a distinctly shorter oral bulge, possibly belonging to another species. All these poorly preserved and unusual specimens, roughly 10% of the population, are excluded from the description and morphometry.

Size 120-230 x 15-40 µm in vivo, usually near 170 x 20 µm, as calculated from some in vivo measurements and the morphometric data (Table 1). Knife-shaped with an average length:width ratio of 9:1, but “handle” much longer than “blade”, that is 4.7:1, flattened only in oral region; oral bulge bluntly pointed anteriorly and rather distinctly set off from narrowed neck, strongly oblique, that is, almost parallel with main body axis; posterior end
narrowly rounded, in preparations occasionally almost globular when the contractile vacuole is filled (Figs 1, 6, 7, 12, 37, 38). Macronucleus extending in posterior two thirds of body, basically a long, slightly tortuous rod with ends frequently coiled, spiralized and/or inflated; occasion ally in two long pieces. Nucleoli small, globular and numerous, rarely reticulate (Figs 1, 6-8, 12, 23, 37, 38). Probably, 5-10 micronuclei not unequivocally distinguishable from extrusomes in vivo and protargol preparations; usually fusiform and about 3 x 1-1.5 µm in impregnated specimens (Figs 7, 8). Contractile vacuole in rear body end, several excretory pores in pole area; definitely no second contractile vacuole in anterior body half. Extrusomes accumulated in both sides of oral bulge and scattered in cytoplasm, attached to bulge cortex with pointed anterior end; basically lanceolate, but with several modifications within and between specimens, as shown in figure 5; 6-8 x 1-1.7 µm in size and compact, that is, rather long, thick, and highly refractive, making them very conspicuous at even low magnification (x100; Figs 23, 25) and in silver preparations, where they appear as strongly refractive inclusions (Figs 42, 43); mature (bulge) extrusomes never impregnate with the protargol method used, while a certain cytoplasmic developmental stage impregnates brownish, like the micronuclei (see above). Exploded extrusomes about 20 µm long and of typical toxicyst structure (Figs 1, 3, 5, 23, 25, 28-34, 42, 43). Cortex very flexible, contains about five rows of minute granules approximately 0.2 µm across between each two ciliary rows. Cytoplasm colourless, usually contains many lipid droplets 0.5-5 µm across; rarely specimens with a large food vacuole containing massive prey, likely a ciliate, were observed. Movement conspicuously slow and worm-like, but glides rather rapidly on microscope slide and between soil particles, showing great flexibility.

Cilia about 8 µm long in vivo, arranged in an average of 10 equidistant, straight, rather loosely ciliated rows abutting on circumoral kinety in acute angles more distinct at right than left side, while basal bodies are rather widely spaced, producing an intermediate Arcuospathidium-Spathidium pattern (Figs 13-16, 39-41, and Discussion). Dorsal brush not as stable as in many congeners, but often with small irregularities, such as minute breaks within rows, supernumerary dikinetids outside rows, or even some extra bristles forming a short fourth row; basically, however, three-rowed and inconspicuous because shorter than oral bulge, occupying only 17% of body length and bristles merely up to 4 µm long, decreasing to 2 µm at end of rows; all rows have one or few ordinary cilia anteriorly and continue as somatic kineties posteriorly; in vivo, bristles slightly inflated distally, and anterior bristles of dikinetids shorter than posterior. Brush rows 1 and 3 of similar length and with rather widely spaced dikinetids; row 2 distinctly longer than rows 1 and 3 and with dikinetids so narrowly spaced (~ 1 µm) that they are difficult to illustrate; row 3 with some minute, about 1 µm long, monokinetidal bristles forming short tail extending to second body third (Figs 1, 8-10, 12-16, 27, 39, 41; Table 1).

Oral bulge very conspicuous due to the large and highly refractive extrusomes contained and the strongly oblique orientation almost in parallel with main body axis (Figs 1, 6, 7, 12, 13, 15, 23-25, 38, 39, 41; Table 1); basically, however, of ordinary size and shape, that is, about twice as long as widest trunk region, moderately convex, and dorsally slightly higher than ventrally; oblong to narrowly cuneate and studded with extrusomes in frontal view (Fig. 4). Circumoral kinety of same shape as oral bulge, composed of comparatively widely spaced and frequently slightly irregularly arranged dikinetids, each associated with a cilium and a fine basket rod recognizable only in over-impregnated specimens (Figs 8, 12-16, 24, 39, 40, 42, 43).

**Distribution and ecology:** as yet found only at type location, that is, 10-20 m inshore the beach of Choroni (67°45'W 10°15'N), Henry Pittier National Park, north coast of Venezuela. The sample consisted of very sandy coastal soil up to 10 cm depth and the mouldy top leave from shrubs, Cactaceae, and grasses. The rewetted mixture had 10‰ salinity and pH 6.7. The species become abundant one week after rewetting the sample. Prey is obviously digested rapidly because only few specimens with food vacuoles containing prey remnants were found in the protargol slides.

**Redescription of Spathidium faurefremieti nom. n.** (Figs 44-59, 65-70; Table 2)

**Nomenclature:** Tucolesco (1962) named a new species Spathidium faurei in honour of the great French protozoologist Fauré-Fremiet (1883-1971). Unfortunately, this name is preoccupied by Spathidium faurëi Kahl, 1930a. Thus, a new name is required: Spathidium faurefremieti nom. n. Accordingly, in future, Tucolesco’s species must be referenced as: Spathidium faurefremieti Foissner, 2003.

**Material:** from 3 sites, as described in the distribution and ecology section, but only the Kenyan population was fully investigated. The Brazilian specimens were also studied rather carefully, while the Australian popu-
Description of Kenyan population: size 170-330 x 13-22 µm in vivo, usually near 240 x 17 µm, as calculated from some in vivo measurements and the morphometric data (Table 2). Very slenderly spatulate or vase-shaped with an average length:width ratio of about 14:1, widest in or slightly underneath mid-body, rather distinctly narrowed subapically producing a slender neck bearing the slightly widened and flattened oral area; posterior end narrowly rounded, in preparations sometimes bulbous due to the contractile vacuole (Figs 44, 48; Table 2). Macronucleus extending in central body quarters, basically a long, irregularly nodulated rod with more or less tortuous, coiled ends; nucleoli small, globular, and numerous. On average 8.5 globular, spongy micronuclei near or attached to macronucleus (Figs 44, 48, 51, 69; Table 2). Invariably two contractile vacuoles without collecting canals: one, as usual, in rear end with scattered excretory pores in pole area; and a second slightly above mid-body with 2-7 serially arranged excretory pores attached to the kinety bearing the middle row of the dorsal brush (Figs 44, 48, 52, 70; Table 2). Extrusomes inconspicuous, accumulated in both sides of oral bulge and scattered in cytoplasm, where a certain, fusiform developmental stage impregnates with protargol; mature oral bulge extrusomes in vivo rod-shaped with rounded ends and slightly curved, about 6 µm long, do not impregnate with the protargol method used (Figs 44, 46, 52). Cortex very flexible, contains rows of inconspicuous granules less than 1 µm across. Cytoplasm colourless, contains few to many lipid globules, depending on state of nutrition, mainly in middle body third. Movement conspicuously slow and worm-like.

Cilia about 8 µm long in vivo, arranged in an average of 12 equidistant, straight, moderately densely ciliated rows abutting on circumoral kinety in typical Spathidium pattern (Foissner 1984), that is, in acute angles at right side and nearly at right angles at left with rows still attached to circumoral kinety fragments (Figs 44, 47-50, 65-67; Table 2). Dorsal brush of usual location and structure, inconspicuous because occupying only 20% of body length on average and bristles merely 3 µm long in vivo; all rows of similar length, an unusual feature; dikinetids narrowly spaced in rows 1 and 2, while widely spaced in row 3 which has, as usual, a monokinetal bristle tail extending to second body third; specimens with a short, fourth row of bristles rarely occur (Figs 44, 49, 50, 52, 68; Table 2).

Oral bulge inconspicuous because only slightly longer than widest trunk region and ordinarily slanted (~ 45°); slightly convex and higher dorsally than ventrally; obovate and studded with extrusomes in frontal view (Figs 44, 45, 47-52, 65-68; Table 2). Circumoral kinety not obovate as oral bulge, but distinctly cuneate, composed of more or less perfectly aligned, dikinetidal kinetofragments frequently still attached to the somatic ciliary rows from which they were produced, especially at left side; dikinetids narrowly spaced, each associated with a cilium and a fine basket rod hardly recognizable in vivo; oral basket, however, fairly distinct in protargol-impregnated specimens (Figs 48-52, 65-68).

Observations on Brazilian and Australian specimens (Figs 53, 55-58; Table 2)

The Brazilian population matches the Kenyan specimens in many main features, for instance, the two contractile vacuoles and the dorsal brush, while the number of ciliary rows (17 vs. 11) and body width (27 µm vs. 15 µm) are conspicuously different (Table 2). Furthermore, the extrusomes are longer, viz. 9-12 µm and thus extend into the somatic cytoplasm, while the short (6 µm) extrusomes of the Kenyan specimens just fill the oral bulge; the macronucleus is more distinctly nodulated and thicker; body shape is cylindroidal rather than spatulate (Figs 56-58); and the oral bulge is elliptical, not obovate in frontal view (Fig. 55). These are rather distinct differences causing doubt on conspecificity (see Discussion).

The Australian specimens were identified in vivo, where they showed the same features as those from Kenya. Extrusomes, however, were 9-10 µm long and thus in between those of specimens from Kenya (6 µm) and Brazil (9-12 µm).

Distribution and ecology: Tucolesco (1962) discovered S. faurefremieti in subterranean cave water in Rumania. I found it, as yet, only in soils from Gondwanan areas, viz., Africa, South America, and Australia. These data indicate a cosmopolitan distribution of
Soil spathidiids

S. faurefremieti, but this is not definite because conspecificity of all populations is doubtful (see Discussion).

In Kenya (Africa), S. faurefremieti occurred in a sample collected by Emmerich Petz (Upper Austria) in the surroundings of the Taita Hills Lodge in the Shimba Hills Nature Reserve (39°25'E 5°S), about 40 km south of the town of Mombassa. This sample was composed of red soil mixed with some grass and shrub litter. The Brazilian (South America) population occurred in a soil sample, kindly provided by Dr. L. F. Machado Velho, from the floodplain of the Paraná River near the town of Maringá (53°15'W 22°40'S). The dark, humic soil was mixed with much partially decomposed leaf litter and had pH 5.1 (in water). The Australian population of S. faurefremieti was found in soil from the Murray

Table 2. Morphometric data on Spathidium faurefremieti from Kenya (upper line) and Brazil (lower line).

<table>
<thead>
<tr>
<th>Characteristics</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>Body, length</td>
<td>213.8</td>
<td>202.5</td>
<td>41.6</td>
<td>12.0</td>
<td>19.4</td>
<td>160.0</td>
<td>305.0</td>
<td>12</td>
</tr>
<tr>
<td>Body, width</td>
<td>255.9</td>
<td>240.5</td>
<td>43.4</td>
<td>14.5</td>
<td>16.9</td>
<td>186.0</td>
<td>330.0</td>
<td>9</td>
</tr>
<tr>
<td>Body length:width, ratio</td>
<td>15.6</td>
<td>15.0</td>
<td>2.2</td>
<td>0.7</td>
<td>14.3</td>
<td>13.0</td>
<td>20.0</td>
<td>12</td>
</tr>
<tr>
<td>Body length:oral bulge length, ratio</td>
<td>9.6</td>
<td>10.9</td>
<td>2.8</td>
<td>0.9</td>
<td>28.9</td>
<td>19.4</td>
<td>47.9</td>
<td>9</td>
</tr>
<tr>
<td>Oral bulge, length</td>
<td>10.0</td>
<td>9.4</td>
<td>1.1</td>
<td>0.7</td>
<td>11.2</td>
<td>8.6</td>
<td>11.8</td>
<td>12</td>
</tr>
<tr>
<td>Circumoral kinety to end of brush row 1, distance</td>
<td>38.4</td>
<td>36.5</td>
<td>5.8</td>
<td>1.7</td>
<td>15.0</td>
<td>30.0</td>
<td>48.0</td>
<td>12</td>
</tr>
<tr>
<td>Circumoral kinety to end of brush row 2, distance</td>
<td>76.2</td>
<td>50.0</td>
<td>9.7</td>
<td>3.2</td>
<td>20.3</td>
<td>30.6</td>
<td>62.9</td>
<td>12</td>
</tr>
<tr>
<td>Circumoral kinety to end of brush row 3, distance</td>
<td>37.7</td>
<td>37.5</td>
<td>7.2</td>
<td>2.1</td>
<td>19.1</td>
<td>30.0</td>
<td>50.0</td>
<td>12</td>
</tr>
<tr>
<td>Anterior body end to first excretory pore, distance</td>
<td>35.6</td>
<td>36.0</td>
<td>9.2</td>
<td>3.1</td>
<td>23.8</td>
<td>20.0</td>
<td>47.0</td>
<td>9</td>
</tr>
<tr>
<td>Macronucleus figure, length</td>
<td>91.7</td>
<td>90.0</td>
<td>16.5</td>
<td>4.8</td>
<td>18.0</td>
<td>60.0</td>
<td>122.0</td>
<td>12</td>
</tr>
<tr>
<td>Macronucleus, width in middle</td>
<td>105.6</td>
<td>96.5</td>
<td>28.8</td>
<td>8.3</td>
<td>27.3</td>
<td>66.0</td>
<td>165.0</td>
<td>12</td>
</tr>
<tr>
<td>Micronuclei, largest diameter</td>
<td>115.0</td>
<td>105.0</td>
<td>26.0</td>
<td>8.7</td>
<td>22.6</td>
<td>82.0</td>
<td>150.0</td>
<td>12</td>
</tr>
<tr>
<td>Micronuclei, number</td>
<td>3.6</td>
<td>4.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.0</td>
<td>4.0</td>
<td>12</td>
</tr>
<tr>
<td>Somatic kinetics, number (including brush)</td>
<td>6.0</td>
<td>6.0</td>
<td>1.0</td>
<td>0.3</td>
<td>16.7</td>
<td>5.0</td>
<td>8.0</td>
<td>9</td>
</tr>
<tr>
<td>Ciliated kinetids in a lateral kinety, number</td>
<td>3.1</td>
<td>3.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.0</td>
<td>3.5</td>
<td>12</td>
</tr>
<tr>
<td>Dorsal brush rows, number</td>
<td>4.9</td>
<td>5.0</td>
<td>0.9</td>
<td>0.3</td>
<td>18.4</td>
<td>4.0</td>
<td>6.0</td>
<td>7</td>
</tr>
<tr>
<td>Dorsal brush rows, numberb</td>
<td>8.5</td>
<td>8.5</td>
<td>1.0</td>
<td>0.3</td>
<td>11.8</td>
<td>7.0</td>
<td>10.0</td>
<td>12</td>
</tr>
<tr>
<td>Dorsal brush rows, numberb</td>
<td>9.5</td>
<td>9.5</td>
<td>1.5</td>
<td>0.6</td>
<td>15.8</td>
<td>8.0</td>
<td>12.0</td>
<td>6</td>
</tr>
<tr>
<td>Dorsal brush rows, numberb</td>
<td>11.7</td>
<td>11.0</td>
<td>0.9</td>
<td>0.7</td>
<td>7.4</td>
<td>11.0</td>
<td>13.0</td>
<td>6</td>
</tr>
<tr>
<td>Dorsal brush rows, numberb</td>
<td>17.1</td>
<td>17.0</td>
<td>1.3</td>
<td>0.4</td>
<td>4.7</td>
<td>15.0</td>
<td>19.0</td>
<td>9</td>
</tr>
<tr>
<td>Excretory pores of anterior contractile vacuole, number</td>
<td>90.4</td>
<td>82.5</td>
<td>24.6</td>
<td>7.1</td>
<td>27.2</td>
<td>64.0</td>
<td>152.0</td>
<td>9</td>
</tr>
<tr>
<td>Excretory pores of anterior contractile vacuole, number</td>
<td>62.1</td>
<td>63.0</td>
<td>7.2</td>
<td>2.7</td>
<td>11.6</td>
<td>50.0</td>
<td>70.0</td>
<td>9</td>
</tr>
<tr>
<td>Excretory pores of anterior contractile vacuole, number</td>
<td>3.0</td>
<td>3.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.0</td>
<td>3.0</td>
<td>12</td>
</tr>
<tr>
<td>Excretory pores of anterior contractile vacuole, number</td>
<td>3.0</td>
<td>3.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.0</td>
<td>3.0</td>
<td>9</td>
</tr>
<tr>
<td>Excretory pores of anterior contractile vacuole, number</td>
<td>23.3</td>
<td>23.0</td>
<td>5.5</td>
<td>1.6</td>
<td>23.6</td>
<td>15.0</td>
<td>32.0</td>
<td>12</td>
</tr>
<tr>
<td>Excretory pores of anterior contractile vacuole, number</td>
<td>24.0</td>
<td>25.0</td>
<td>3.6</td>
<td>1.2</td>
<td>15.0</td>
<td>15.0</td>
<td>27.0</td>
<td>9</td>
</tr>
<tr>
<td>Excretory pores of anterior contractile vacuole, number</td>
<td>29.5</td>
<td>29.5</td>
<td>6.6</td>
<td>1.9</td>
<td>22.4</td>
<td>20.0</td>
<td>40.0</td>
<td>12</td>
</tr>
<tr>
<td>Excretory pores of anterior contractile vacuole, number</td>
<td>34.8</td>
<td>34.0</td>
<td>4.7</td>
<td>1.6</td>
<td>13.6</td>
<td>25.0</td>
<td>42.0</td>
<td>9</td>
</tr>
<tr>
<td>Excretory pores of anterior contractile vacuole, number</td>
<td>19.2</td>
<td>18.5</td>
<td>3.1</td>
<td>0.9</td>
<td>16.0</td>
<td>14.0</td>
<td>23.0</td>
<td>12</td>
</tr>
<tr>
<td>Excretory pores of anterior contractile vacuole, number</td>
<td>23.1</td>
<td>23.0</td>
<td>3.8</td>
<td>1.3</td>
<td>16.4</td>
<td>17.0</td>
<td>27.0</td>
<td>9</td>
</tr>
<tr>
<td>Excretory pores of anterior contractile vacuole, number</td>
<td>3.3</td>
<td>3.0</td>
<td>1.5</td>
<td>0.4</td>
<td>45.7</td>
<td>2.0</td>
<td>7.0</td>
<td>12</td>
</tr>
</tbody>
</table>

aData based on mounted, protargol-impregnated, randomly selected specimens from non-flooded Petri dish cultures. bOnly full rows counted.

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 arithmetic mean, x - arithmetic mean.
Figs 44-54. *Spathidium faurefremietii*, Kenyan (44-52, 54) and Brazilian (53) specimens from life (44-46, 53, 54) and after protargol impregnation (47-52). 44 - left side view of a representative specimen, i.e., “constructed” from live observations and morphometric data shown in table 2. Note the slender shape and the second contractile vacuole above mid-body, a main feature of this species; 45 - frontal view of oral bulge studded with extrusomes; 46 - oral bulge extrusome, length 6 µm; 47 - left side view of anterior body portion, showing circumoral kinety and somatic ciliary rows arranged in typical *Spathidium* pattern; 48-50 - ciliary and contractile vacuole pattern and nuclear apparatus of main voucher specimen. Note the second contractile vacuole above mid-body and the dorsal brush rows, which terminate at almost same level, another unusual feature. The circumoral kinety and the ciliary rows are arranged in the typical *Spathidium* pattern (50); 51, 52 - ventral and dorsal view of anterior body half of another specimen. Note the cuneate circumoral kinety, the widely spaced dikinetids of dorsal brush row 3, and the three excretory pores (arrowhead) of the anterior contractile vacuole; 53 - middle portion of dorsal brush, longest bristles 5 µm; 54 - surface view showing cortical granulation. B - dorsal brush, B1-3 - dorsal brush rows, BA - oral basket, CK - circumoral kinety, CV - contractile vacuoles, E - extrusomes, MA - macronucleus, MI - micronucleus, OB - oral bulge. Scale bars 20 µm (47, 50); 50 µm (44, 48, 49, 51, 52).
River floodplain near the town of Albury (37°S 147°E). The sample, kindly provided by Dipl.-Biol. Hubert Blatterer, was a mixture of leaf litter and light brown soil with pH 5.2 (in water).

The records from cave water (Tucolesco 1962) and two floodplain soils indicate that *S. faurefremieti* occurs in both soil and freshwater, while the slender shape suggests a preference for soil or muddy environments (Foissner 1987). Abundances were low at all sites, and Tucolesco (1962) possibly observed only a single specimen, as indicated by the range-less size value. None of the prepared cells contained definite food inclusions, indicating that prey is digested rapidly and/or dissolves during ingestion, as observed in several members of the group (Foissner, unpubl.).

**DISCUSSION**

**Arcuospathidium pachyoplites**

The boundaries between the spathiidiid genera distinguished by Foissner (1984) and Foissner *et al.* (2002) are not very sharp, but greatly aid in distinguishing...
species groups within this highly diverse assemblage (for detailed discussion, see Foissner 1984 and Foissner et al. 2002). The present species, at first glance, appears as a typical Arcuospathidium because the anterior ends of the kineties seemingly are curved dorsally at both sides of the oral bulge, a diagnostic character for the genus. However, closer investigation shows that most left side kineties have a basal body very near to the circumoral dikinetids, giving the kineties a Spathidium-like pattern (Figs 14, 16). On the other hand, the knife-shaped body and the steep, oblong oral bulge argue for a classification in Arcuospathidium, as does a compari-

Figs 65-70. Spathidium faurefremieti, Kenyan specimens after protargol impregnation. 65, 66 - ciliary pattern of right and left side in anterior body portion of same specimen. This species has a typical Spathidium ciliary pattern, that is, the somatic ciliary rows are attached to the circumoral kinety and curved dorsally at right side and ventrally at left, forming ~90° angles with the circumoral kinety; 67 - left side view of another specimen showing the right angles formed by the circumoral kinety and the curved anterior end of the somatic ciliary rows; 68 - dorsolateral view showing the widely spaced dikinetids of dorsal brush row 3; 69 - nodulated and tortuous macronucleus; 70 - the anterior contractile vacuole has three excretory pores (arrowhead). B2, 3 - dorsal brush rows, CK - circumoral kinety, N - nematodesmata, OB - oral bulge.
son with the supposed nearest relative, *A. vlassaki*, where the *Arcuospathidium* ciliary pattern is more distinct (Figs 20-22).

There are few species with a close resemblance to *A. pachyoplites*, viz., *A. vlassaki* Foissner, 2000; *A. etoschense* Foissner et al., 2002; and *Spathidium etoschense* Foissner et al., 2002. At first glance, *A. pachyoplites* is indistinguishable from *A. vlassaki* because all obvious features are rather similar (Figs 17-22). However, a more detailed comparison reveals differences significant at species level, most related to the extrusomes emphasizing the need of thorough *in vivo* data: lanceolate with narrow end attached to the oral bulge vs. oblanceolate attached with broad end (Figs 1, 5, 17, 19); scattered in entire oral bulge vs. forming a single row in left bulge half (Figs 4, 18); 7 x 1.4 µm and thus highly conspicuous vs. 5 x 1 µm and of ordinary appearance (Figs 32-36). Although there is some variation in these features, the differences are obvious and conspicuous. In this context, it should be mentioned that I found a second population of *A. vlassaki* in Saudi Arabia having the same extrusome characteristics as the type population from Namibia, especially the unusual broad end-attachment. The second main distinguishing feature concerns the relative lengths of the oral bulge and dorsal brush: in *A. pachyoplites* the longest brush row (2) is of same length or shorter than the oral bulge (Figs 8, 9, 12, 14, 16, 41), while in *A. vlassaki* the longest brush row (2) is of same length or longer than the oral bulge (Foissner 2000 and Figs 20-22); the average values are 34.4 µm and 27.2 µm vs. 21.8 µm and 25 µm. Further, minor differences in sum supporting separation of the South American population: dikinetids very narrowly spaced (~1 µm) only in brush row 2 vs. rows 2 and 3; body width:oral bulge length ratio 0.56 vs. 0.75, that is, bulge relatively longer in *A. pachyoplites* than in *A. vlassaki*; *Arcuospathidium* ciliary pattern less distinct in the former than the latter, as proved by the reinvestigation of the African type population (Figs 20-22). Observations on other populations are needed to prove whether these minor differences are stable or within the range of natural variability of the taxa concerned.

*Arcuospathidium pachyoplites* differs from *A. etoschense* mainly by the macronucleus, which is tortuous in the former and nodular in the latter. Furthermore, *A. pachyoplites* is considerably stouter than *A. etoschense* (9.0:1 vs. 13.7:1), and its extrusomes are much longer and thicker (7 x 1.4 µm vs. 3-4 x < 1 µm). *Arcuospathidium pachyoplites* differs from *Spathidium etoschense* by the ciliary pattern, the much longer and tortuous macronucleus, and the thicker (1.4 µm vs. 1 µm), and thus highly conspicuous extrusomes.

**Spathidium faurefremieti**

This species poses the opportunity to discuss some basic problems, especially of recognizing the genus *Supraspathidium* and of species in general. I shall discuss these and other questions in a loose sequence showing my views and ways to solve them.

(1) The genus *Supraspathidium* and generic assignment of *Spathidium faurefremieti*: Foissner and Didier (1981) diagnosed *Supraspathidium* as follows: “Spathidiidae with several to many, serially arranged contractile vacuoles and the oral bulge indistinctly set off from body proper”. They assigned to *Supraspathidium* all spathidiids with more than the ordinary posterior contractile vacuole and, unfortunately, fixed as a type species *Spathidium teres* Stokes, 1886, a poorly known species that awaits redescription. Nonetheless, three “typical” *Supraspathidium* species have been described meanwhile: *S. multistriatum* Foissner and Didier, 1981; *S. etoschense* Foissner et al., 2002; and *S. armatum* Foissner et al., 2002. All have a clear identity and are massive, densely ciliated organisms with one or two rows of contractile vacuoles, each comprising five or more individual vacuoles with several excretory pores. The ciliary pattern is *Epispathidium*-like, with ciliary rows and cilia within rows even more densely spaced, especially at the anterior end of the kineties, where a kinetofragment-like polymerization occurs. Clearly, such spathidiids represent a type of its own and can be classified in the genus *Supraspathidium*, for which I suggest the following refined diagnosis: Massive, densely ciliated Spathidiidae with *Epispathidium*-like ciliary pattern and many (>5) contractile vacuoles, each having several excretory pores, in one or two rows.

Another group of spathidiids has the ordinary posterior contractile vacuole and a second one in the mid-body region. The general organization and the ciliary pattern of these bivacuolate species look like those of classical spathidiids, as shown by *Arcuospathidium bulli* Foissner, 2000 (Figs 62-64) and *Spathidium faurefremieti* re-described here. Thus, generic separation would appear premature given the present state of knowledge, though the second contractile vacuole is a distinct feature. On the other hand, it is now obvious that an increased number of contractile vacuoles evolved independently in

Soil spathidiids 157
several evolutionary lines of the Spathidiidae, viz., in Spathidium, Arcuospathidium and Supraspathidium. Each of these lines can be considered as a distinct evolutionary branch, requiring generic or subgeneric separation, especially when further such species are discovered differing also in other features, for instance, an Arcuospathidium with two contractile vacuoles (like A. bulli), but scattered macronuclear nodules (unlike A. bulli, which has a single, long strand).

(2) Conspecificity of the African and Brazilian population: Several main features, especially the two contractile vacuoles and the shape of the extrusomes agree well, while two other main characteristics do not even overlap, viz., the number of ciliary rows and body width (Table 2). While the latter might be considered as less important because both populations are still slender (body length:width ratio 13.9:1 and 9.6:1, respectively) and body width is known to depend on nutritional state, the former is an important difference because kinety number is rather stable within and between populations in many haptorids (Foissner 1984, Foissner et al. 2002). Furthermore, extrusome length also does not overlap (6 µm vs. 12 µm). This, however, is probably a minor difference because extrusome length is rather variable in general and the Australian specimens are intermediate (9-10 µm).

Basically, the differences argue to consider the Brazilian population as a distinct subspecies because two main morphometric features do not overlap, of which one is very important. Furthermore, geographic distance would support such a separation (Foissner et al. 2002). On the other hand, only one population each has been studied in detail so that the “true” variability of the species is insufficiently known. Furthermore, if such differences are rated too high at the present state of knowledge, it would hardly be possible to identify the populations with the European S. faurefremieti, whose description is much more incomplete and thus requires a broad species concept.

In this situation, the most pragmatic solution is to consider all populations as belonging to a single species, but to avoid neotypification, as suggested by Foissner (2002) and Foissner et al. (2002), because conspecificity is not beyond reasonable doubt and detailed data on the supposed European population (= S. faurefremieti) are lacking. However, if the detailed investigation of an European population shows similar differences as those found between the African and Brazilian populations (Table 2), all should obtain species or subspecies status. Ideally, such data should be supplemented by gene sequences.

(3) Identification: Identification largely depends on the species concept and the treatment of literature data. Our concept is thoroughly discussed in Foissner et al. (2002) and thus needs not to be explained here. Briefly, we apply a simple, population-based, morphological concept and identify populations with previously described species, even if data are poor, whenever it is feasible; specifically, at least the general appearance and one main feature must agree.

Tucolesco (1962) provided the following description of Spathidium faurei (now S. faurefremieti) and a single illustration, reproduced here as figure 59: “Taille 400 µ. Cellule de couleur jaunâtre, de forme allongée et très étroite, à bords parallèles. Pôle postérieur largement arrondi. Partie antérieure de la cellule fortement déviée. Troncature antérieure presque droite, légèrement inclinée sur l’axe longitudinal. Noyau très long (un peu plus long que la moitié de la cellule) et très mince. Striation serrée. Cils fins, épais et courts. Trouvé dans les eaux souterraines de la grotte Pestera Ialomicioara, en août 1958”.

Obviously, my populations agree with Tucolesco’s species in several main features, suggesting conspecificity: size, slender shape; short, moderately oblique oral bulge; macronuclear and contractile vacuole pattern. Unfortunately, Tucolesco did not provide information on two other main features: extrusomes and the number of ciliary rows, which is possibly considerably higher than in my populations because Tucolesco’s illustration appears to indicate a narrow striation pattern (Fig. 59). Further, details of the shape are different because my specimens are definitely not parallel-sided, especially those from Africa have a distinctly narrowed neck. Possibly, of even greater importance is the habitat difference, viz., soil vs. cave water. However, the Australian and the Brazilian populations are from floodplain soils, indicating that my species might occur also in freshwater.

In summary, uncertainties and differences are too pronounced to be entirely confident about conspecificity. Thus, neither the Kenyan nor Brazilian population can serve as a neotype given the present state of knowledge, as explained above.

(4) Comparison with related species: Unfortunately, Tucolesco (1962) did not compare Spathidium faurefremieti with any described species, especially S. vermiforme Penard, 1922. It is unclear whether he did not know of S. vermiforme or considered his
population as sufficiently different to classify it as a new species. In my opinion, *S. vermiforme* and *S. faurefremieti* agree in most main features, especially size, slender shape, and the nuclear and contractile vacuole pattern (Figs 59-61). However, there is an important difference in the habitat: Penard discovered *S. vermiforme* in a sapropelic environment, according to Kahl (1930b), while Tucolesco found *S. faurefremieti* in clean cave water. Sapropelic spathidiids are poorly known, but most are probably different from those living in ordinary freshwaters and soils (Kahl 1926, 1930b; Foissner 1998, and unpublished). Accordingly, synonymy of Penard’s and Tucolesco’s species is questionable given the present state of knowledge. This is emphasized by slight shape differences (Penard emphasized a dorsal convexity, while Tucolesco mentioned that his species is parallel-sided) and the remark of Penard that one of his populations has symbiotic algae, indicating that he might have mixed two species.

Both populations of *Spathidium faurefremieti* are very similar to *S. procerum* Kahl, 1930a, as redescribed by Foissner (1984), except that the latter has only one ordinary, posterior contractile vacuole in over one hundred populations checked. At first glance, *Arcuospathidium bulli* Foissner, 2000 is also similar to *S. faurefremieti*, as already discussed by Foissner (2000), because it has two contractile vacuoles. However, the oral bulge is distinctly longer and much more oblique in *A. bulli*, and the ciliary patterns are conspicuously different (Figs 62-64). Thus, *Spathidium faurefremieti*, as redescribed here, is a very distinct species easily recognizable, even *in vivo*, by the long, slender body; the short oral bulge; the long macronucleus; and, especially, the two contractile vacuoles.

Acknowledgements. The technical assistance of Dr. B. Moser, Dr. E. Herzog and Mag. E. Strobl is gratefully acknowledged. Special thanks to Dr. Kuidong Xu and Mag. Yanli Lei for discussion. Financial support was provided by the Austrian Science Foundation (FWF project P-15017).

REFERENCES


Foissner W., Agatha S., Berger H. (2002) Soil ciliates (Protozoa, Ciliophora) from Namibia (Southwest Africa), with emphasis on two contrasting environments, the Etosha region and the Namib Desert, *Denisia* 5: 1-149


Received on 25th June, 2002; revised version on 2nd February, 2003; accepted on 5th February, 2003
An Improved Silver Carbonate Impregnation for Marine Ciliated Protozoa

Hongwei Ma1, Joong Ki Choi1 and Weibo Song2

1Regional Research Center for Coastal Environments of Yellow Sea, CCEYS, Inha University, Incheon, Korea; 2Laboratory of Protozoology, Ocean University of China, Qingdao, P. R. China

Summary. An improved silver carbonate impregnation method for marine ciliates is described. Compared to conventional methods, the new method of fixing and staining is easy to learn and yields easily reproducible results. Structures that are impregnated with silver carbonate such as nuclear apparatus, infraciliature as well as cortical and cytoplasmic microtubules and even contractile vacuole pore are stained evenly and with high contrast in all cells on the slides, therefore yielding perfect photographs. This method has the characteristic of simplicity, quickness and good reproducibility, so it is especially suitable for ecologists and ontogenetists. The slides with mounted cells can also be stored for months without loss in quality or staining capability.

Key words: marine ciliate, silver carbonate impregnation, staining method.

INTRODUCTION

In order to identify ciliates for taxonomic evaluation, silver impregnation techniques are indispensable. Even though there are many methods for ciliated protozoa (Klein 1926, Chatton and Lwoff 1930, Dragesco 1962, Deroux and Tuffrau 1965, Wilbert 1975, Fernandez-Galiano 1976, Montagnes and Lynn 1987, Foissner 1992), marine protozoologists and ecologists are still perplexed about the staining of the marine ciliated protozoa. Along with the development of marine ecological research, many ciliated protozoa are being more closely examined, so a simpler kind of and more user-friendly staining method for ciliates is necessary for marine ecologists and biologists. The method of silver carbonate impregnation described in this paper was improved so it is easy to learn and consistently yields easily reproducible results for marine ciliated protozoa.

DESCRIPTION OF THE METHOD

Some of the ciliate species that were used in this work were either laboratory cultures or freshly collected and cultivated in Incheon, Korea or Qingdao, China. The ciliates were cultivated either in normal or in deep Petri dishes and kept at 20-25°C according to their special requirements, using either rice or organisms as food source. The following ciliate species were tested:
**Staining Procedure (reagents according to Fernandez-Galiano 1976)**

1. Place 2-3 drops of a rich ciliate culture or even a single specimen in an embryonic dish or in a concave slide.

2. Add 2-3 drops of 10% formalin quickly to the embryonic dish if the organisms are freshwater species (final concentration is 5%), or add 2-3 drops of 10% (or even 50% depending on the kind of species) seawater formalin if the organisms are seawater species and fix for 1-3 min. Shake and try to concentrate the cells to the middle of the dish or concave slide.

3. Carefully wash out the water with micropipette and add distilled water three times. Leave about two or three drops of water and organisms.

4. Add 2 or 3 drops of formalin (10%) and refix for about 1 to 2 min (the final concentration is 5%).

5. Add 2-3 drops of Fernandez-Galiana’s fluid to the refixed ciliates, and mix one minute.

6. Place the embryonic dish on a pre-heated (60°C) hot-plate for staining. Shake the dish in the process of heating. Immediately add 2-3 ml of 5% sodium thiosulfate solution as soon as the color turns golden brown. The process usually needs 5-7 min.

7. Check the impregnation under microscope, take pictures with digital camera or draw figure if the impregnation is perfect.

8. After stabilization with 5% sodium thiosulfate solution for about 8-15 min, wash the organisms very thoroughly in distilled water at least 3 times. For morphogenesis or genus identification, steps 1-8 are enough. For mounting slide, according to the following steps.

9. Add a small drop of well impregnated ciliate to a clean slide and add an equal sized drop of albumen-glycerol, mix thoroughly but gently with a mounted needle and spread mixture in a moderately thin layer in the middle third of the slide and remove the redundant fluid with a thin micropipette.

10. Allow to dry in the oven at 55-60°C for 10-30 min.

11. Dehydrate in alcohol series (70-80-90-95-100%) for 5 min in each.

12. Transfer the slide through 1:1 alcohol and xylene, then through xylene 2 times for 5 min in each.

13. Mount the slide with Permount and cover with a coverslip. Ensure that no air is trapped under the coverslip and put the slide into the oven at 55-60°C for 24 h.

Remarks concerning the steps of the staining procedure

1. This method is especially suitable for species with a firm pellicle. It is necessary to adjust the concentration of formalin and the solvent, if the organisms are freshwater species, use 10% distilled water formalin; seawater species, add 10% filtered seawater formalin. Some species cannot be fixed well with formalin and cells may even burst.

2. Washing out the redundant liquid is also a most important step. Since the chemical component of the original liquid in which the organisms live are different, these chemicals can affect the staining result, so the original liquid should be washed out thoroughly with distilled water at least 3 times.

3. The ratio of Fernandez-Galiano’s fluid usually need not change, but the amount needed depends on many unpredictable factors, e.g., amount and concentration of fixative, kind of organisms. The trial and error method must be frequently used to obtain best results.

4. The correct impregnation time depends on many factors that are difficult to control. The amount of pyridine and silver carbonate in the Fernandez-Galiano’s fluid is especially important. Add another drop of pyridine and/or silver carbonate solution to the Fernandez-Galiano’s fluid if impregnation is repeatedly too faint. Some species can be impregnated well before the fluid turn brown, while others even very faint after the fluid becomes dark brown, so try several times to get good results.

5. Immediately add 2-3 ml 5% sodium thiosulfate solution as soon as the color turns golden brown. Because impregnation can develop well within 1-2 seconds, it is not advisable to check the impregnation results under the microscope. The quickly changing golden brown color is the best opportunity to stop the staining with the 5% sodium thiosulfate solution. Stabilization should occur in no less than 10 min, and only in this way can the mounting slide stay perfect in quality.

6. Before preparing a permanent slide, the sodium thiosulfate solution must be washed out thoroughly with distilled water at least 3 times, or it will affect the staining quality.
Figs 1A-L. Overview of slides stained by the silver carbonate impregnation method. A, B - Dexiotricha granulose, a scuticociliatid, arrow in A shows the caudal cilium complex, arrow in B indicates the cytoplasmic microtubules, arrowhead in B means the contractile vacuole pore; C, D and G - Euplotes vanus, a hypotrichid, arrows in C point to the new adoral membrane anlagen, arrowheads in C indicate the anlage of ventral cirri, arrows in D mean the dorsal kineties, arrowhead in D points to micronucleus, arrows in G display the dorsal kineties anlagen; E, K - Paramecium caudatum, a hymenostomatid, arrows in E indicates the buccal membranelles, arrows in K show the post suture; F, L - Pelagostrobilidium simile, an oligotrichid, arrows in F point to the new built buccal field, arrows in L show the kineties; H - Halteria grandinella, an oligotrichid, arrow shows the micronucleus, arrowhead indicates the external adoral membrane; I - Coleps hirus, a prostomatid; J - Fabrea salina, a heterotrichid. Ma - macronucleus.
Galiano’s fluid is different, while two modifications can improve Foissner’s (1992) modification, the content of Fernandez-Galiano’s fluid and affect the mounting result. Compared to the original method as Fernandez-Galiano used, because in that way cells can easily burst and immingle with other organisms and also avoids the chemical interference of the solution. This ensures the high staining quality and reproducibility, so we fixed seawater sample with filtered seawater formalin and changed the salinity according to species. This is very useful, because most of species even that of thin pellicle can also get perfect staining quality with high concentration of Formalin. For some hypersalinitic species such as Fabrea salina, which grows better at the salinity of 90 psu, hypersalinitic seawater formalin can remedy the deficiency of distilled water formalin.

Acknowledgement. This work was supported by the Regional Research Center for Coastal Environments of Yellow Sea (KOSEF- RRC to Professor J. K. Choi) and The Natural Science Foundation of China (No. 40206021). We would like to thank Mr. H. P. Hong and M. H. Yoo, Department of Oceanography, Inha University, Korea, Mr. H. Ma and J. Gong, Laboratory of Protozoology, Ocean University of China, for collecting samples or culturing organisms, and Ms. Anne Radowick, Department of English Language and Literature, Inha University for improving the English draft.

REFERENCES


Received on 16th December 2002; received version on 4th March, 2003; accepted on 6th March 2003
Extracellular Calcium Changes the Morphology of Induced Pinocytosis in *Amoeba proteus*

Wanda KŁOPOCKA, Anna WASIK and Lucyna GRĘBECKA
Department of Cell Biology, Nencki Institute of Experimental Biology, Warszawa, Poland

**Summary.** The morphology of pinocytosing *Amoeba proteus* induces by two monovalent cations: Na⁺ and K⁺ were examined at different calcium concentration. It was demonstrated that pinocytotic response of amoeba (number, size and shape of pinocytotic pseudopodia) was related to the amount of Ca²⁺ accumulated on the cell surface.

**Key words:** amoebae, [Ca²⁺]e, endocytosis.

**INTRODUCTION**

In *Amoeba proteus* a typical induced pinocytosis is manifested after external application of different agents: aminoacides, proteins, monovalent cations. This pinocytosis is a kind of fluid-phase endocytosis driven by cortical cytoskeleton and may be compared to macropinocytosis in *Dictyostelium discoideum* in which large fluid-filled vesicles are formed (Maniak 2002). During induced pinocytosis *A. proteus* attains a rosette form with specialized pinocytotic pseudopodia containing channel-like invaginations. The morphology of pinocytosing amoebae: number, shape, and size of pinocytotic pseudopodia and polarity of their distribution on the cell surface vary depending on the kind of inducer, as it was demonstrated for two monovalent cations: Na⁺ and K⁺ (Grębecka and Kłopocka 1986). Since Na⁺ and K⁺ can also exert variable effects on the pattern of Ca²⁺ shifting between the surface of amoeba and the medium during induction of pinocytosis (Kłopocka et al. 2000), it seems possible that there is a relationship between the type of pinocytotic reaction and the behaviour of calcium at the cell surface.

**MATERIALS AND METHODS**

*Amoeba proteus* was cultured in Pringsheim medium with standard concentration of Ca²⁺ (0.85 mM) and fed twice a week on *Tetrahymena pyriformis*. Before experiments cells were starved for 2 days. All experiments were carried out at room temperature.

The course of pinocytosis was compared in amoebae exposed to different concentrations of usually used pinocytotic inducers: KCl and NaCl, in the standard Pringsheim medium, and in Pringsheim solution with modified [Ca²⁺]. Pinocytosis was induced by 30 mM KCl, 125 mM KCl and 125 mM NaCl in standard concentration of...
Ca\(^{2+}\) (0.85 mM), by 125 mM KCl in [Ca\(^{2+}\)]\(_{e}\) increased up to 4.85 mM, and by 30 mM KCl and 125 mM NaCl in the presence of 10 mM EGTA. Calcium concentration either was changed before application of the inducer or during pinocytosis.

Changes in the amount of cell-associated Ca\(^{2+}\) during pinocytotic induction were assessed by adding “Ca\(^{2+}\)” (0.1 mCi/ml, Amersham Life Science, Little Chalfont, Buckinghamshire, England) according to the procedure described earlier (Klopopka et al. 2000). Calcium shifts between the cell surface and the surrounding medium are shown as per cent increase or decrease of cell-associated radioactivity during the experiment. Pinocytosis was controlled 7, 12 and 17 min after application of the inducer.

Observations were carried out in the differential interference contrast (Pluta system, PZO Warsaw) and recorded on the tape with a Panasonic wv BL 600 camera and NV-8051 Panasonic time-lapse video recorder. Selected frames of various pinocytotic forms were stored in an IBM PC compatible computer memory. The morphology of pinocytosis is shown by scanning electron microscopy (SEM) of amoebae fixed and processed as it was previously described elsewhere (Grębecki et al. 2001).

**RESULTS AND DISCUSSION**

It was the purpose of present investigations to reveal whether there is a relationship between the decrease or increase of calcium amount on the cell surface and the pinocytotic response induced by two different monovalent cations: K\(^{+}\) and Na\(^{+}\).

Pinocytotic reaction of amoeba was graduated and characterised by a distinct polarity (the first pinocytotic structures appeared in the uroidal region, next ones at the former fronts) and by the development of numerous small pseudopodia with thin channels, after application of 125 mM NaCl (Fig. 1) or 30 mM KCl (Fig. 2) under standard medium conditions, and during pinocytosis induced by 125 mM KCl in the medium with [Ca\(^{2+}\)]\(_{e}\) increased up to 4.85 mM (not shown). It means that this kind of pinocytosis occurred, when Ca\(^{2+}\) was binding to amoeba during stimulation, more or less similar as to the control cells (Fig. 5).

Stimulation by 125 mM K\(^{+}\) applied in standard Pringsheim medium caused immediate cell contraction, the first pinocytotic pseudopodia were formed in any place of strongly deformed cells and, as a result, amoebae produced few large pseudopodia with very wide channels (Fig. 3). This was accompanied with a decrease of [Ca\(^{2+}\)] associated to the surface of amoeba (Fig. 5). Similar coincidence between the alteration of the morphological features of pinocytosis and the displacement of calcium from the cell surface could be produced as well during pinocytosis induced by 125 mM NaCl and 30 mM KCl by Ca\(^{2+}\), chelating by EGTA (not shown).

On the contrary, addition of 4 mM CaCl\(_{2}\) during pinocytosis induced by 125 mM K\(^{+}\), that is increasing [Ca\(^{2+}\)] from 0.85 mM up to 4.85 mM, caused immediate association of more calcium with the cell surface (Fig. 5) accompanied by changes in the morphology of pinocytosis. Within 30s after application of calcium, the pinocytosing cells with few and large pseudopodia (such as shown in Fig. 3) became rosettes with numerous, small pseudopodia with thin channels (Fig. 4).

In general, it may be concluded that changes in the concentration of extracellular calcium associated during pinocytosis with the cell membrane outer surface and/or with glycocalyx caused immediate alterations of size and number of existing pinocytotic structures.

One can suggest that the course of events during macropinocytosis in *Amoeba proteus* depends on the direction of extracellular calcium shifting between amoeba and the medium during cells stimulation. It was postulated that extracellular calcium plays an important role in controlling the physiological state of the plasma membrane in amoebae (Brandt and Freeman 1967, Brandt and Hendil 1972, Kukulis et al. 1986). Low concentration of Ca\(^{2+}\) in the presence of other cations can induce such cellular activities as endocytotic membrane internalisation (Marshall and Nachmias 1965, Hendil 1971, Braatz-Schade and Haberey 1975, Josefsson 1975, Stockem 1977), whereas high concentration of Ca\(^{2+}\) has a stabilising effect on the membrane (Gingell 1972). In our experiments neither 125 mM Na\(^{+}\) nor 30 mM K\(^{+}\) could not induce pinocytosis in *A. proteus* in [Ca\(^{2+}\)]\(_{e}\) increased up to 4.85 mM.

The polarity of pinocytosis is probably related to stability of the cell membrane. When externally bound Ca\(^{2+}\) is substituted by the inducer, as it takes place in 125 mM K\(^{+}\) in standard medium, the membrane potential immediately decreases around the whole cell (Braatz-Schade and Haberey 1975, Josefsson et al. 1975) and the first pinocytotic pseudopodia can develop in any place of the surface of amoeba. If calcium is not displaced from the cell surface by the inducing agent, it is in 125 mM Na\(^{+}\) or 30 mM K\(^{+}\) in the standard medium and 125 mM K\(^{+}\) in the increased [Ca\(^{2+}\)]\(_{e}\) the first channels are formed at the uroid where suitable conditions for a permanent pinocytosis exist (Wohlfarth-Bottermann and Stockem 1966), because of the strong membrane folding (Czarska and Grębecki 1966) and its low stability in this area (Batueva 1965a, b; Bingley...
Calcium effect on *A. proteus* pinocytosis

167

...next channels are formed at the front when it begins to contract (Grębecka and Klopocka 1985, Grębecka 1988) and similar conditions as in uroid are established.

Calcium also controls cortical cytoskeleton functions that are responsible for cell shape changes and pseudopodia formation: reorganisation of cortical cytoskeleton in amoebae (Hellewel and Taylor 1979, Taylor and Fechheimer 1982, Bray 1992) and interactions of actin microfilaments with the plasma membrane (Taylor *et al.* 1980, Kawakatsu *et al.* 2000), and thus it may influence the course of pinocytosis. According to the view of Maeda and Kawamoto (1986) a low [Ca\textsuperscript{2+}] activates pinocytosis in *Dictyostelium discoideum* because under such conditions the content of actin in the cell cortex and consequently the number of membrane-associated microfilaments are reduced, which may restrict the plasma membrane flexibility, necessary for pinocytotic structures formation. Our results seem to confirm this hypothesis and indicate that membrane flexibility in *A. proteus* may influence the number and size of pinocytotic structures.

According to Klein *et al.* (1988) the size of pinocytotic pseudopodia and the diameter of channels are...
regulated by the mode of membrane-microfilament interaction. Pinocytic pseudopodia are developed due to the circular detachment of the microfilament layer from the plasma membrane with the exception of a central region of fast contact (Stockem et al. 1983, Grębecki 1991). The diameter of the channels depends on the extent of the areas of contact between the microfilament layer and the internal face of the plasma membrane (Klein et al. 1988). The size of pseudopodia is related to the area of detachment and probably the contraction degree. It seems possible that Ca$^{2+}$ can control, via the membrane of amoeba, the rearrangement of actin cortical network and its attachment to the cell surface.

Conclusions:

1. The pinocytic response of Amoeba proteus is related to the amount of Ca$^{2+}$ accumulated on the cell surface during the induction of this phenomenon.
2. The direction of calcium shifting during the initiation of pinocytosis depends on the strength of inducer, its concentration, and the concentration of extracellular calcium.

Acknowledgement. Authors wish to express their sincere gratitude to Prof. A. Grębecki for his constructive suggestions for improvement and modification of the manuscript. Authors also thank to Dr. P. Pomorski for his help in preparation of figures.

REFERENCES

Batueva I. V. (1965a) The electrical parameter of Amoeba proteus I. Motionless amoebae. Cytologia 7: 188-196

Batueva I. V. (1965b) The electrical parameters of Amoeba proteus. II. The motile amoebae. Cytologia 7: 553-561


Received on 28th March, 2003; accepted on 31st March, 2003
INSTRUCTIONS FOR AUTHORS

_Acta Protozoologica_ publishes original papers, short communications, as well as longer review articles on comprehensive and updated basic, experimental, and theoretical contributions to the broad aspects of protistology and cell biology of lower Eukaryota including: behavior, biochemistry and molecular biology, development, ecology, genetics, parasitology, physiology, photobiology, systematics and phylogeny, and ultrastructure. Contributions should be written in English. Submission of a manuscript to _Acta Protozoologica_ implies that the contents are original and have not been published previously, and are not under consideration or accepted for publication elsewhere. There are no page charges except colour illustration. Names and addresses of suggested reviewers will be appreciated. In case of any question please do not hesitate to contact Editor.

Mrs Małgorzata Woronowicz-Rymaszewska  
Managing Editor of ACTA PROTOZOOLOGICA  
Nencki Institute of Experimental Biology,  
ul. Pasteura 3  
02-093 Warszawa, Poland  
Fax: (4822) 822 53 42  
E-mail: jurek@ameba.nencki.gov.pl

Extensive information on ACTA PROTOZOOLOGICA is now available via internet. The address is: [http://www.nencki.gov.pl/ap.htm](http://www.nencki.gov.pl/ap.htm)

Organization of Manuscripts

Submissions

Please enclose three copies of the text, one set of original line drawings (without lettering!) and three sets of copies with lettering, four sets of photographs (one without lettering). In case of photographs arranged in the form of plate, please submit one set of original photographs unmounted and without lettering, and three sets of plates with lettering. 

The ACTA PROTOZOOLOGICA prefers to use the author’s word-processor disks (format IBM or IBM compatible, and Macintosh 6 or 7 system on 3.5" 1.44 MB disk only) of the manuscripts instead of rekeying articles. If available, please send a copy of the disk with your manuscript. Preferable programs are Word or WordPerfect for Windows. Disks will be returned with galley proof of accepted article at the same time. Please observe the following instructions:

1. Label the disk with your name: the word processor/computer used, e.g. IBM; the printer used, e.g. Laserwriter; the name of the program, e.g. Word for Windows.
2. Send the manuscript as a single file; do not split it into smaller files.
3. Give the file a name which is no longer than 8 characters.
4. If necessary, use only italic, bold, underline, subscript and superscript. Multiple font, style or ruler changes, or graphics inserted into the text, reduce the usefulness of the disc.
5. Do not right-justify and use of hyphen at the end of line.
6. Avoid the use of footnotes.
7. Distinguish the numerals 0 and 1 from the letters O and I.

Text (three copies)

The text must be typewritten, double-spaced, with numbered pages. The manuscript should be organized into Summary, Key words, Abbreviations used, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables and

Figure Legends. The Title Page should include the full title of the article, first name(s) in full and surname(s) of author(s), the address(es) where the work was carried out, page heading of up to 40 characters. The present address for correspondence, Fax, and E-mail should also be given.

Each table must be on a separate page. Figure legends must be in a single series at the end of the manuscript. References must be listed alphabetically, abbreviated according to the World List of Scientific Periodicals, 4th ed. (1963). Nomenclature of genera and species names must agree with the International Code of Zoological Nomenclature, third edition, London (1985) or International Code of Botanical Nomenclature, adopted by XIV International Botanical Congress, Berlin, 1987. SI units are preferred.

Examples for bibliographic arrangement of references:

_Journals:_


_Books:_


_Articles from books:_


_Illustrations:_

All line drawings and photographs should be labelled, with the first author's name written on the back. The figures should be numbered in the text as Arabic numerals (e.g. Fig. 1). Illustrations must fit within either one column (86 x 231 mm) or the full width and length of the page (177 x 231 mm). Figures and legends should fit on the same page. Lettering will be inserted by the printers and should be indicated on a tracing-paper overlay or a duplicate copy.

Line drawings (three copies + one copy without lettering)

Photographs (three copies + one copy without lettering)

Photographs at final size should be sharp, with a glossy finish, bromide prints. Photographs grouped as plates (in size not exceeding 177 x 231 mm including legend) must be trimmed at right angles accurately mounted and with edges touching and mounted on firm board. The engraver will then cut a fine line of separation between figures. Magnification should be indicated. Colour illustration (charged) on positive media (slides 60 x 45 mm, 60 x 60 mm, transparency or photographs) is preferred.

_Proof sheets and offprints:_

Authors will receive one set of page proofs for correction and are asked to return these to the Editor within 48-hours. Fifty reprints will be furnished free of charge. Orders for additional reprints have to be submitted with the proofs.

ORIGINAL ARTICLES

Z. Chen, W. Song and A. Warren: Species separation and identification of Uronychia spp. (Hypotrichia: Ciliophora) using RAPD fingerprinting and ARDRA riboprinting ................................................................. 83

L. Kőhidai, C. Bánya and G. Csaba: Comparison of lectin induced chemotactic selection and chemical imprinting in Tetrahymena pyriformis ......................... 91


M. Todorov and V. Golemansky: Morphology, biometry and ecology of Arcella excavata Cunningham, 1919 (Rhizopoda: Arcellinida) .................................................. 105

W. Foissner: The Myriokaryonidae fam n., a new family of spathidiid ciliates (Ciliophora: Gymnostomatea) .... 113

W. Foissner: Two remarkable soil spathidiids (Ciliophora: Haptorida), Arcuospathidium pachyplites sp. n. and Spathidium faurefremieti nom. n. ......................... 145

SHORT COMMUNICATIONS

H. Ma, J. K. Choi and W. Song: An improved silvercarbonate impregnation for marine ciliated protozoa ........................................................................... 161

W. Klopocka, A. Wasik and L. Grębeka: Extracellular calcium changes the morphology of induced pinocytosis in Amoeba proteus ........................................ 165