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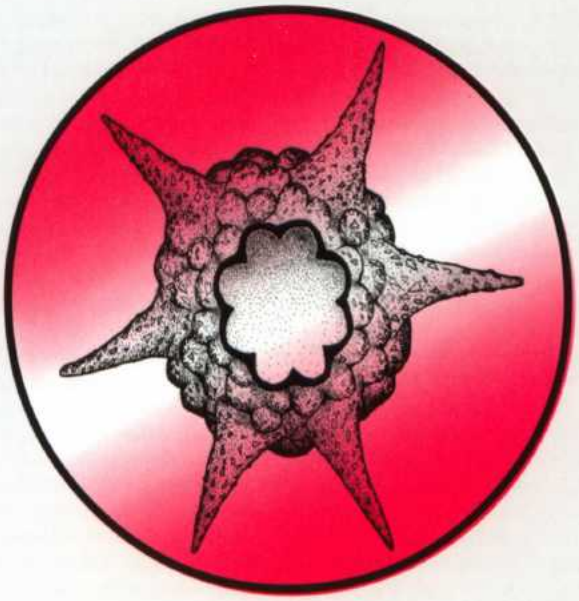

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Changes in Testate Amoebae (Protists) Communities in a Small Raised Bog. A 40-year Study

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Summary. We analyzed the testate amoebae communities from two sets of moss samples taken forty years apart (1961 and 2001) in the same locations of a peat bog of the Swiss Jura Mountains. Peat cutting and lateral drainage of Le Cachot bog have caused a clear increase in tree cover, especially near the edges. Changes affecting herbaceous plants, mosses, or soil organisms can be subtle, and may easily be overlooked. We hypothesized that we would see changes in the dominant *Sphagnum* species and the structure of testate amoebae communities living in the mosses. More specifically, we hypothesized that the frequency of bryophyte and testate amoebae species indicative for dry conditions would increase and that the frequency of species indicative for wet conditions would decrease. The mean testate amoebae species richness per sample decreased from 11.9 to 9.6, but the overall species richness was identical (33 species) in both years. Three species increased significantly in mean relative abundance: *Nebela tincta s. l.* (+97%), *Bullinularia indica* (+ 810%), and *Cyclopyxis eurystoma* (+ 100%; absent in 1961), while two species decreased significantly: *Assulina muscorum* (- 63%) and *Euglypha compressa* (-93%). The testate amoebae communities clearly differed among microhabitat types (hummocks, lawns, hollows), but no overall significant change in the community was detectable between the two sampling dates (Mantel test). These results could signify that changes at the microscopic level had already taken place by 1961 and no further overall significant changes in micro-environmental conditions took place during the 40-year period of this study. This would agree with the faster response time usually attributed to microorganisms and would also imply that the bushes and trees may be poor predictors of the response of microorganisms if they are themselves in a non-equilibrium stage. Other possible causes for the lack of overall differences are discussed.

Key words: autogenic succession, drainage, long-term changes, Protozoa, *Sphagnum*-dominated peatland, testaceans.

INTRODUCTION

In Central Europe many peatlands have been damaged or destroyed, mainly through peat extraction, drainage for cultivating crops or for forestry. Today, in many European countries, the conservation of the remaining peatlands has become a priority. The two main aims of such conservation measures are the preservation of biodiversity and of the carbon pool and sequestration functions of peatlands (Grünig 1994, Raeymaekers 2000, Joosten and Clarke 2002).

An important challenge for the management of the remaining peatlands is the effect human activities have had and continue to have on their hydrology. The surface of most remaining peatlands is significantly smaller than it was originally resulting in a lowering of the water table starting in the periphery and extending towards the central part of the mires (Freléchoux *et al.* 2000a). Such effects are especially dramatic in the case of small

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raised bogs, which are quite common in mountainous areas of Central Europe. The drainage of peatlands causes changes in the vegetation and biogeochemical functioning. Peatlands often become overgrown with trees (pine, spruce, or birch) and from C sinks become C sources as decomposition rates increase. Although peatlands in some cases are capable of recovering from the damage, this process takes long time (Chapman *et al.* 2003). In a conservation perspective, although small wetlands can play a vital role in the persistence of local population of wetland-associated organisms (Gibbs 1993), even preserving the remaining peatlands may not suffice to stop the loss of unique species related to the wettest habitats because of the gradual modification of small raised bogs through autogenic vegetation succession due to change in the hydrology (Freléchoux *et al.* 2000b). The challenge here is that such changes happen slowly and can easily go unnoticed unless, as is rarely the case, a good monitoring program is established. For protistologists, a further challenge is that there is much less available data than for other taxonomic groups, especially plants and animals.

A number of peatlands have been studied around the middle of the 20th century. In some cases, especially when the author of these early studies is still active, it is possible to go back and collect new data to study the changes that have taken place. Several such studies have recently been published, but none of them have included microorganisms. Gunnarsson and others observed changes in pH, conductivity and plant communities in two Swedish mires. The observed changes were interpreted either as autogenic succession, or indirect human impact through acid precipitation and increased N availability (Gunnarsson *et al.* 2000, 2002). Chapman and Rose investigated changes in the vegetation of a peatland in the UK over a period of 28 years and found important shifts in the vegetation that were interpreted as being due to afforestation in the periphery of the bog as well as changes in the use of the bog itself (Chapman and Rose 1991).

We analyzed the changes in the testate amoebae communities from a small raised bog of the Swiss Jura Mountains, Le Cachot bog, over a period of forty years, between 1961 and 2001. Generations of scientists from the Neuchâtel region have studied the ecology, paleoecology, vegetation, arthropod and protist communities of peatlands of the Jura Mountains since the mid 19th century (Lesquereux 1844). See Matthey and Borcard (1996) for a list of some of these studies. One of these scientists, Dr Willy Matthey had kept *Sphagnum* samples he had collected in 1961 and was able to lead the second author through the mire to the same spots to sample a second series of samples in 2001. This made it possible for us to assess how much testate amoebae communities have changed in this site over a period of forty years.

Like most peatlands of the region, as a consequence of peat cutting, the surface of Le Cachot peatland has been strongly reduced over the years and its edges are constituted of more or less eroded peat extraction walls. The resulting lateral drainage causes changes in the vegetation on the bog with a strong increase in tree cover, especially near the edges of the peatland. Vegetation changes however are not instantaneous and, although the site has not suffered from peat extraction in recent decades and is now protected by law, recent comparative studies have shown a significant increase in tree cover and reduction of small water bodies - many of which were the result of peat extraction activities - over the second part of the 20th century (Matthey 1998, 2000, 2001). Changes in tree cover are relatively easy to see and less likely to be overlooked than more subtle changes such as those affecting herbaceous plants, mosses, or soil organisms. *Sphagnum* species have relatively well-defined preferences along the water table depth gradient (Vitt and Slack 1984; Rydin and McDonald 1985; Rydin 1986, 1993; Vitt 2000). Testate amoebae also respond well to this gradient (Tolonen 1986; Charman and Warner 1992, 1997; Tolonen *et al.* 1992; Warner and Chmielewski 1992; Charman 1997; Bobrov *et al.* 1999; Mitchell *et al.* 1999; Booth 2001, 2002). Hence we hypothesized that we would see changes in the dominant *Sphagnum* species and the structure of testate amoebae communities living in the mosses. More specifically, we hypothesized that the frequency of bryophyte and testate amoebae species indicative for dry conditions would increase and that the frequency of species indicative for wet conditions would decrease.

MATERIALS AND METHODS

Site description, site history, and field sampling

La Cachot bog is located in the Jura Mountains, northwest Switzerland (47.5°N, 6.4°E), at an altitude of 1050 m a. s. l. The climate of the region is favorable for peatland development: The annual mean precipitation is1446 mm and 36% of the precipitation falls between June and September. The mean annual temperature is 4.7°C and ranges between 9.7 and 13.3°C between June and September although it may fall below 0°C at any time of the year. The relative humidity is

usually almost 100% in the evening so the area is often foggy in the morning. Winter temperatures can be extremely cold during windless nights (the record is almost - 40°C) due to the frequent inversion of air masses caused by the closed basin topography of the valley (karst landscape).

Traditional peat harvest for heating was practiced until the end of World War II and caused the loss of approximately 90% of the original surface of raised bogs in the region (Grünig 1994). Peat extraction has now stopped and in places natural regeneration is taking place (Matthey 1996). At present, Le Cachot bog is delimited in several places by 1 to 2 meter-high vertical peat walls that are slowly eroding. The bog surface located near these walls is dry because of the lateral drainage due to the modified topography. The peat extraction process had also created some pools, many of which are now being re-colonized by the vegetation. As a result, their number and surface area has been decreasing substantially (Matthey 1998, 2000, 2001). The lateral drainage has clearly been beneficial to trees, manly pine (*Pinus rotundata*) and birch (*Betula pubescens*), and to ericaceous shrubs (e.g. *Vaccinium uliginosum, V. myrtillus, V. vitisidaea*, and *Calluna vulgaris*). This site, like other comparable ones in the region, nevertheless has a high conservation value for the many rare species of plants and animals that still thrive on it, as well as for other animals, not necessarily linked to peatlands, but that may benefit from the food, water or protection it offers (Matthey and Borcard 1996).

Dr Willy Matthey collected a number of *Sphagnum* samples in 1961 in different microhabitats of Le Cachot bog. A total of 23 samples were available for this study. In September 2001, Dr Willy Matthey lead the second author to the study site to collect *Sphagnum* samples from, as close as possible to the same spots where the 1961 samples had been taken. The general characteristic of the sites were recorded at the same time (Table 1). All mosses were air-dried and preserved in envelopes until extractions could be done. The relative proportion of different bryophytes was estimated in each sample to the nearest 5%. All bryophytes were identified to the lowest taxonomic level. Taxonomy follows Tutin *et al.* (1964-1980) for vascular plants, Isoviita (1967) for *Sphagnum*, and Smith (1980) for other mosses.

Testate amoebae extraction and analysis

The top 3 cm of the mosses was used for testate amoebae extraction. The mosses and about 75 ml of water were boiled for 10 min to detach the tests from the mosses. The samples were filtered through a tea strainer first then through a 300 µm mesh, then through a 150 µm mesh and finally over a 20 µm filter. The fraction between 150 µm and 20 µm was then back sieved and preserved in 5 ml vials with glycerol. Microscopic slides were prepared and amoebae tests were identified and counted at 200× and 400× magnification. The following taxonomic references were used for identification (Deflandre 1929, 1936; Grospietsch 1958; Ogden and Hedley 1980; Ogden 1983; Lüftenegger *et al.* 1988; Charman *et al.* 2000). For each sample a minimum total of 150 individuals were counted. One or more entire slides were counted for each sample. The rotifer *Habrotrocha angusticollis* was also counted but treated separately in the numerical analyses. Both living and dead amoebae are included in the counts. Given that only the top 3 cm were counted and that *Sphagnum* mosses usually grow at least 1cm a year, we consider the counts to be representative of the mean testate amoebae community over a 3-year period.

Data analyses

The following grouping of species were used: (1) *Nebela tincta s. l.*, includes *Nebela tincta*, *Nebela tincta* var. *major*, *N. collaris*, *N. bohemica* and *N. parvula*; (2) *Centropyxis aerophila s. l.* includes *Centropyxis aerophila* and *Centropyxis cassis*; (3) *Phryganella acropodia s. l.* includes *P. acropodia* and *Pseudodifflgia gracilis;* (4) *Phryganella* sp. includes *Phryganella paradoxa* and similar taxa such as some small *Difflugia* species. In addition, *Heleopera sylvatica* was excluded from the data because it appeared that this species might in some cases have been mistaken for other similar species (at least in lateral view) such as some *Difflugia* species. For six samples from 1961 the available material was very limited and the total count was less than 100. These samples were therefore excluded from both the 1961 and 2001 data sets, leaving 17 samples for each year and 34 in total. As a consequence of this, three rare species, *Centropyxis cassis* var. *spinifera*, *Trinema complanatum*, and *Difflugia bacillifera* were lost from the data. Because entire slides were counted, in some 2001 samples the total number of individual counted was much higher than the target of 150 to 200 individuals. As a result there was a significant difference between the mean total count between the 2001 and 1961 samples. Such a difference could cause a bias in the data if very rare species were found in the samples with a higher count. As the limited amount of material available from 1961 did not allow us to increase the counts for these samples we randomly reduced the count of four samples from 2001 (samples 8, 9, 14 and 23) to bring the total below 300 individuals. This was sufficient to reduce the mean total count difference between the two sets of samples and make it no longer significant.

We compared the species richness, Shannon-Wiener diversity and evenness indices between the two data sets using t-tests after checking for normality of the data. The multivariate community data of bryophytes and testate amoebae were analyzed using Mantel tests to assess if there was a significant difference in community structure between the 1961 and 2001 samples (Legendre and Legendre 1998). For each of these analyses we used two similarity matrices: (1) a species × samples matrix and (2) a binary variable represented the two sampling dates. A Steinhaus similarity coefficient was used for the species percentage data and a simple matching coefficient was used for the binary variable (Legendre and Legendre 1998). The possible influence of rare testate amoebae species on the result was assessed by removing successively the species that occurred (1) only once (5 species), (2) once or twice (9 species), or (3) up to three times (13 species) in the overall data set. These tests were performed using the R package free software (Casgrain and Legendre 2004). To illustrate in reduced space the structure of the multidimensional testate amoebae data we performed a detrended correspondence analysis (DCA) using the software CANOCO (Ter Braak 1988-1992).

RESULTS

Micro-habitat types

A brief description sampling sites is given in Table 1. We identified four micro-habitat types: hummocks, lawns, hollows, and non-*Sphagnum* mosses (usually growing on very dry hummocks or in closed pine forests). At

Table 1. Description of the 17 sampling sites from Le Cachot bog used in the study.

Sample Nr.	Site descriptions 1961	2001
1	Small hollow	Lawn on the edge of a *Pinus rotundata* forest
4	Hollow near a *Sphagnum fuscum* hummock	Lawn approximately 20 m from the edge of a *Pinus rotundata* forest
5	Hummock, not far from sample 4	Hummock in an open *Pinus rotundata* forest
6	Hollow *Pinus rotundata* forest	Small hollow in an open
7	Lawn of an open *Pinus rotundata* forest	Lawn with tussocks on the edge
8	Small hollow not far from the *Pinus rotundata* forest edge	Lawn with low bushes on the edge of an open *Pinus rotundata* forest
9	Lawn with *Andromeda polifolia* on the side of a hollow	Hollow in an open *Pinus rotundata* forest
10	Lawn	Wet lawn
11	Small hollow in an open *Pinus rotundata* forest	Low, wet hummock in a pool
12	Hummock near a *Pinus rotundata* tree	Lawn in open *Pinus rotundata* forest
13	Small cushion with *Polytrichum strictum* in heath vegetation	Lawn, no *Sphagnum*
14	Hummock in *Vaccinium* heath a *Pinus rotundata* forest, no *Sphagnum*	Opening with low bushes in
15	Lawn	Lawn in an open *Pinus rotundata* forest
17	Hummock at the edge between the pine forest and the open *Sphagnetum magellanici* open bog community	Lawn with tussocks on the edge of an open *Pinus rotundata* forest
21	*Pinus rotundata* forest	Closed *Pinus rotundata* forest
22	*Pinus rotundata* forest with *Polytrichum strictum*	Closed *Pinus rotundata* forest
23	*Pinus rotundata* forest	Closed *Pinus rotundata* forest

Fig. 1. Changes in the dominant moss type and microtopography of 17 sampling sites in Le Cachot bog between 1961 and 2001. Hollows, lawns and hummocks all were dominated by *Sphagnum* mosses.

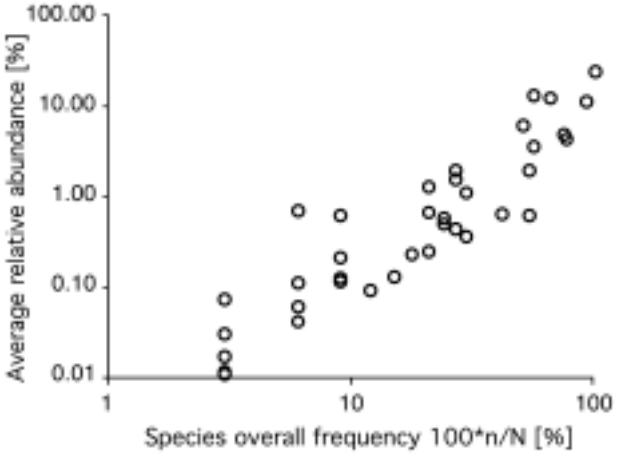

Fig. 2. Relationship between the frequency of testate amoebae species and their overall mean relative abundance.

most sites the microtopography either did not change or seemed to have evolved towards drier conditions between 1961 and 2001. As all the sites sampled in1961 were dominated by *Sphagnum* mosses, the total number of possible transitions was 12. Of these 12 possible transitions, only seven were actually observed (Fig. 1). The microhabitat status of seven sites did not change. Of those that changed, seven became drier (four hollows became lawns and three hummock lost their *Sphagnum* cover) while only three became wetter (one lawn became a hollow and two hummocks became lawns). However, this trend towards drying was not significant (paired sign test, P = 0.3438).

Bryophytes

Overall our samples contained 14 species of bryophytes in 1961 and 12 species in 2001. There was no significant difference in the species richness of individual samples between the two sampling periods (2.12 ± S.E. 0.30 in 1961; 2.06 ± S.E. 0.23 in 2001). Three species increased both in terms of frequency and relative abun-

Table 2. Number of presences and average relative abundance of 14 bryophyte species in 17 samples from Le Cachot. Swiss Jura Mountains, in 1961 and 2001.

	Number of presences			Relative abundance [%]					
	1961	2001	Change	1961		2001		Change 1961-2001	
	n	n		Average	SE	Average	SE	Absolute	[%]
Aulacomnium palustre	3	2	-1	0.9	0.5	0.6	0.1	-0.3	-33
Mylia anomala	1	1	0	0.3	0.3	0.3	0.1	0.0	0
Pleurozium schreberi	2	5	3	0.6	0.4	9.4	2.3	8.8	1500
Polytrichum strictum	5	3	-2	3.8	2.6	12.4	3.0	8.5	223
S. angustifolium	1	5	4	4.1	4.1	22.9	5.6	18.8	457
S. capillifolium s. l.	3	3	0	12.6	7.7	2.9	0.7	-9.7	-77
S. contortum	2	0	-2	7.6	6.0	0.0	0.0	-7.6	-100
S. cuspidatum	2	1	-1	11.8	8.1	4.7	1.1	-7.1	-60
S. fallax	3	2	-1	10.9	7.3	9.7	2.4	-1.2	-11
S. fuscum	3	3	0	10.9	6.5	13.5	3.3	2.6	24
S. magellanicum	5	8	3	11.8	6.6	17.6	4.3	5.9	50
S. quinquefarium	1	0	-1	5.6	5.6	0.0	0.0	-5.6	-100
S. rubellum	2	1	-1	7.1	5.5	0.6	0.1	-6.5	-92
S. subsecundum	3	1	-2	12.1	7.5	5.3	1.3	-6.8	-56

dance between 1961 and 2001: *Pleurozium schreberi*, *Sphagnum angustifolium*, and *S. magellanicum*. In addition to these, *Polytrichum strictum*, and *S. fuscum* increased in relative abundance but either decreased or did not change in frequency. Seven species decreased in relative abundance between 1961 and 2001: *S. capillifolium, S. cuspidatum, S. fallax, S. rubellum, S. subsecundum, S. quinquefarium* and *S. contortum*, the latter two being totally absent from the 2001 samples (Table 2). There was no overall significant difference in bryophyte community structure between the two years as assessed by a Mantel test between a similarity matrix of a binary site variable and a similarity matrix of bryophyte relative abundance (P = 0.491; 999 permutations).

Testate amoebae

A total of 6795 testate amoebae were identified and counted in this study (3641 in 2001 and 3154 in 1961). The mean count per sample was 200 (SE: 8.3). Overall a total of 37 taxa were recorded. The overall mean species richness per sample was 10.8 (SE: 0.5). The overall mean relative abundance of testate amoebae taxa was positively correlated with their frequency in the samples (Fig. 2). The four most abundant taxa *Nebela tincta s. l.*, *Hyalosphenia papilio*, *Amphitrema flavum*, and *Assulina muscorum* together made up 64.5% of the total count. The eight most abundant taxa accounted for 84.7% the total count (Table 3). The rotifer *Habrotrocha angusticollis* was observed in 18 samples and its mean relative density was 1.2 % (SE: 0.3) of the total testate amoebae count (Table 3).

The total species richness of individual sampling periods was identical (33 species) in 1961 and 2001, although four of the 37 taxa recorded were absent from each set of samples. The mean species richness per sample however was significantly lower in 2001 than 1961 (means and SE in 1961 and 2001 respectively 11.9 ± 0.7 and 9.6 ± 0.7, paired t-test $p = 0.042$, unpaired t-test $p = 0.022$; Table 3). However, the diversity and evenness indices were not significantly different between 1961 and 2001 (Table 4).

Overall the relative abundance of 21 taxa increased between 1961 and 2001 while that of 16 taxa decreased. However, due to the variability in the data few of these differences were significant. Three taxa, *Nebela tincta* s. l. (+ 97%), *Bullinularia indica* (+ 810%), and *Cyclopyxis eurystoma* (+ 100%; absent in 1961) increased significantly between 1961 and 2001, while two other species significantly decreased: *Assulina muscorum* (- 63%) and *Euglypha compressa* (- 93%). Despite these differences, neither the changes in relative abundance of the species nor the change in rank of relative abundance could be related to their known preferences for water table depth or pH in the region (Mitchell *et al.* 1999) (data not shown). The rotifer *Habrotrocha angusticollis* was more frequent in 2001 (n=13) than 1961 (n=5). The relative abundance (% of the total testate amoebae count) of this species increased significantly from 0.43 (SE 0.20) in 1961 to 1.92 (SE 0.49) in

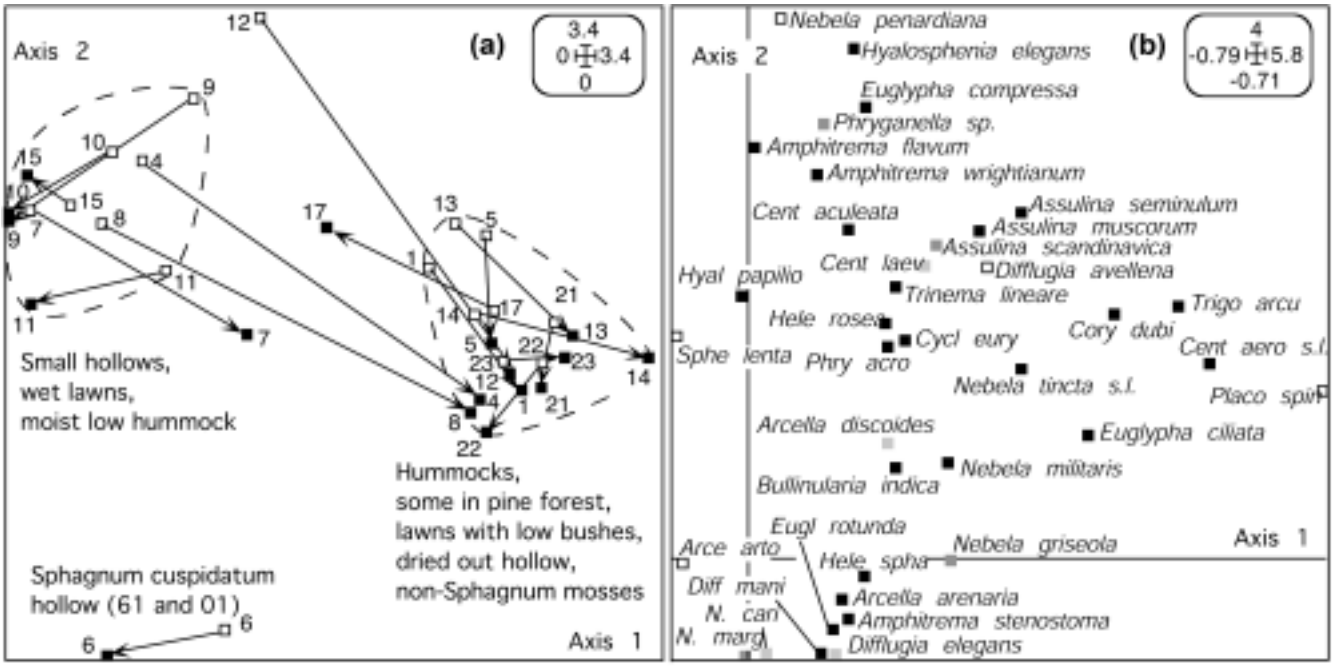

Fig. 3. Scatter diagram of a DCA of testate amoebae data with representation of samples **(a)** and species **(b)**. Axes 1 and 2 explain 21.5 and 12.8% of the total variation in the species data. Samples from 1961 are represented by open squares and samples from 2001 by black squares. Arrows connect each pair of samples. Groups of samples are outlined in dashed lines and the types of micro-habitats sampled are summarized. Rare species that occurred in a single sample, two and three samples are represented respectively by open squares, light gray squares and dark gray squares.

2001 (paired t-test p = 0.013, unpaired t-test p = 0.009; Table 3).

The position of samples in the first two axes of the DCA revealed which samples shared similar testate amoebae communities and how similar or dissimilar pairs of samples were between 1961 and 2001 (Fig. 3). Most samples were clustered into two major groups corresponding to (1) samples taken mostly in moist lawns and (2) samples taken mostly in hummocks, forests and non-*Sphagnum* mosses. In addition some samples were projected in between these two groups and the pair of samples no 6, representing a wet *Sphagnum cuspidatum* hollow in 1961 and 2001 stood out as being a group of their own. For each sampling sites arrows linking pair of samples illustrate the changes in testate amoebae communities over the 40-year period. In most cases the 1961 and 2001 samples were quite similar but some pairs of samples underwent important changes (e.g. samples 4, 7, 8, 9, 12, and 17). Interestingly, while some of these suggest drier conditions in 2001 than in 1961 (samples 4, 7, and 8), others seem to have become wetter (samples 9, 12, and 17).

The position of testate amoebae in the ordination shows which species were associated with the different groups of samples and the known ecology of the species in the region (Mitchell *et al.* 1999) can be used to interpret the ordination space. Species indicative for low water table, such as *Corythion dubium*, *Nebela tincta*, *Nebela militaris*, *Trigonopyxis arcula*, and *Euglypha ciliata* have high scores on the first axis of the ordination. Species characteristic of bog pools such as *Nebela carinata*, *Nebela marginata*, and *Amphitrema stenostoma* have low scores on both axis 1 and 2, in agreement with the position of samples 6 (*Sphagnum cuspidatum* hollow). Other species, such as *Amphitrema flavum*, *Amphitrema wrightianum*, and *Hyalosphenia papilio*, usually found in bog hollows but also abundant in moist lawns had low scores on axis 1 but higher scores on axis 2.

Despite the changes observed for some species between 1961 and 2001, no overall significant difference was found between the two data sets: The Mantel tests did not reveal any overall significant differences between the two data sets (999 permutations

Table 3. Relative abundance of testate amoebae and the rotifer *Habrotrochoa angusticollis* in moss samples from Le Cachot bog collected in 1961 and 2001.

	Relative abundance									Relative abundance rank				n			
	Overall		1961		2001		Change	t-tests	P-value	Overall	1961	2001	Change	Overall	1961	2001	Change
	Average	SE	Average	SE	Average	SE	[%]*	Paired	Unpaired					N=34	N=17	N=17	
Amphitrema flavum	12.70	3.18	16.08	4.79	9.31	4.18	-42	0.106	0.294	3	3	3	0	22	13	9	-4
Amphitrema stenostoma	1.67	0.65	1.13	0.86	2.21	0.97	95	0.322	0.414	11	13	9	-4	9	3	6	3
Amphitrema wrightianum	1.38	0.95	2.28	1.88	0.47	0.33	-80	0.362	0.347	12	10	20	10	7	4	3	-1
Arcella arenaria	0.68	0.26	0.23	0.14	1.13	0.49	381	0.098	0.088	16	23	14	-9	14	4	10	6
Arcella artocrea	0.01	0.01	0.00	0.00	0.02	0.02	100	0.332	0.325	36	34	32	-2	1	0	1	1
Arcella discoides	0.04	0.03	0.00	0.00	0.09	0.06	100	0.203	0.194	33	34	26	-8	2	0	2	2
Assulina muscorum	11.82	2.35	17.25	3.84	6.39	2.07	-63	0.015	0.018	4	2	5	3	31	17	14	-3
Assulina scandinavica	0.13	0.09	0.22	0.18	0.04	0.04	-82	0.342	0.327	27	24	31	7	3	2	1	-1
Assulina seminulum	3.78	1.29	5.45	2.37	2.12	0.96	-61	0.131	0.202	8	6	10	4	19	13	6	-7
Bullinularia indica	0.47	0.18	0.09	0.07	0.85	0.34	810	0.050	0.039	21	28	16	-12	9	2	7	5
Centropyxis aculeata	0.27	0.12	0.37	0.21	0.16	0.14	-57	0.419	0.395	23	20	23	3	7	5	2	-3
Centropyxis aerophila s. l.	1.15	0.70	0.48	0.22	1.83	1.39	283	0.360	0.344	13	17	11	-6	10	5	5	0
Centropyxis laevigata	0.12	0.10	0.04	0.04	0.20	0.20	403	0.449	0.438	29	30	21	-9	2	1	1	0
Corythion dubium	4.59	1.18	3.95	1.43	5.23	1.91	32	0.620	0.595	7	7	6	-1	26	16	10	-6
Cyclopyxis eurystoma s. l.	0.10	0.05	0.00	0.00	0.20	0.09	100	0.052	0.044	30	34	22	-12	4	0	4	4
Difflugia avellena	0.02	0.02	0.04	0.04	0.00	0.00	-100	0.332	0.325	35	31	34	3	1	1	0	-1
Difflugia elegans	0.73	0.69	1.46	1.39	0.00	0.00	-100	0.309	0.301	14	12	34	22	2	2	0	-2
Difflugia manicata	2.06	1.23	1.91	1.60	2.21	1.93	15	0.507	0.907	10	11	8	-3	9	6	3	-3
Euglypha ciliata	5.26	1.13	3.57	1.03	6.95	1.97	95	0.055	0.137	6	8	4	-4	25	13	12	-1
Euglypha compressa	0.38	0.15	0.71	0.27	0.05	0.03	-93	0.030	0.020	22	14	30	16	10	8	2	-6
Euglypha rotunda	0.51	0.29	0.37	0.17	0.65	0.56	76	0.644	0.634	20	21	19	-2	8	5	3	-2
Heleopera rosea	0.70	0.43	0.57	0.30	0.83	0.83	45	0.783	0.773	15	16	17	1	7	6	1	-5
Heleopera sphagni	0.61	0.27	0.27	0.24	0.94	0.49	249	0.250	0.225	19	22	15	-7	8	2	6	4
Hyalosphenia elegans	6.50	2.67	9.60	4.68	3.40	2.52	-65	0.287	0.252	5	5	7	2	17	9	8	-1
Hyalosphenia papilio	14.14	3.74	11.65	4.43	16.64	6.10	43	0.282	0.512	2	4	2	-2	19	12	7	-5
Nebela carinata	0.06	0.05	0.02	0.02	0.11	0.11	360	0.461	0.450	32	32	25	-7	2	1	1	0
Nebela griseola	0.12	0.08	0.09	0.06	0.15	0.15	69	0.719	0.708	28	29	24	-5	3	2	1	-1
Nebela marginata	0.65	0.52	0.13	0.13	1.17	1.02	805	0.333	0.321	18	27	13	-14	3	1	2	1
Nebela militaris	2.08	0.62	2.75	1.01	1.41	0.69	-49	0.338	0.282	9	9	12	3	18	10	8	-2
Nebela pennadiana	0.01	0.01	0.02	0.02	0.00	0.00	-100	0.332	0.325	37	32	34	2	1	1	0	-1
Nebela tincta s. l.	25.88	4.27	17.44	4.37	34.31	6.88	97	0.026	0.047	1	1	1	0	34	17	17	0
Phryganella acropodia s. l.	0.65	0.14	0.60	0.16	0.71	0.24	18	0.703	0.708	17	15	18	3	18	10	8	-2
Phryganella sp.	0.23	0.20	0.43	0.39	0.02	0.02	-95	0.318	0.309	25	18	33	15	3	2	1	-1
Placocista spinosa	0.03	0.03	0.00	0.00	0.06	0.06	100	0.332	0.325	34	34	29	-5	1	0	1	1
Sphenodeia lenta	0.08	0.08	0.15	0.15	0.00	0.00	-100	0.332	0.325	31	26	34	8	1	1	0	-1
Trigonopyxis arcula	0.24	0.10	0.41	0.18	0.08	0.08	-81	0.133	0.106	24	19	27	8	6	5	1	-4
Trinema lineare	0.14	0.07	0.21	0.12	0.07	0.07	-67	0.356	0.328	26	25	28	3	5	4	1	-3
Average total count	200	8.3	186	8.0	214	14.0	15	0.103	0.085								
Total species richness	37		33		33		0										
Average sp richness	10.8	0.5	11.9	0.7	9.6	0.7	-19	0.042	0.022								
Habrotrochoa angusticollis #	1.2	0.3	0.43	0.20	1.92	0.49	350	0.013	0.009					18	5	13	8

- percentage of the total testate amoebae count; * - calculated as 100 × a/b (where a = 2001, and b = 1961 relative abundance).

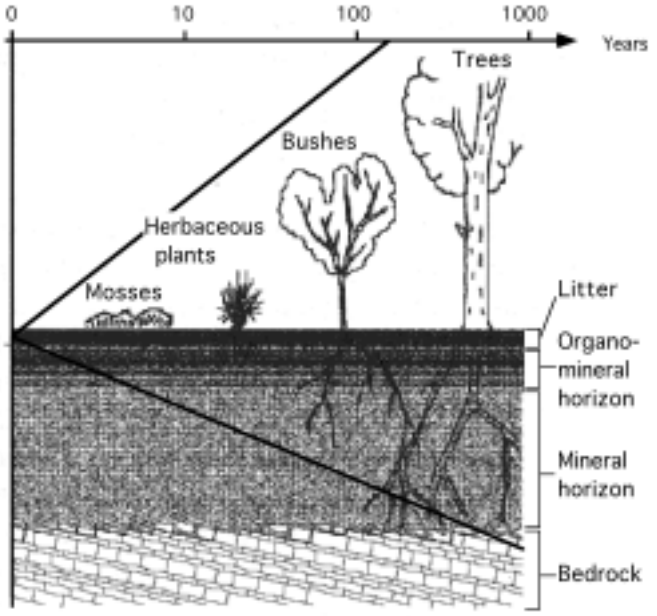

Fig. 4. Comparative response time of different plant strata and soil horizons to changes in the environment. (Modified from "Le Sol vivant", Jean-Michel Gobat, Michel Aragno and Willy Matthey, Presses polytechniques et universitaires romandes, copyright 2003). By analogy, the response time of different soil organisms is expected to increase with size.

p-value = 0.096). Removing rare species occurring in a single, two or three samples did not influence the result.

DISCUSSION

The most significant changes that can be observed at the landscape level are the growth of trees, mainly pines (*Pinus rotundata*) but also locally birch (*Betula pubescens*), at the periphery of the bog and the infilling through natural succession of many small water bodies. Indeed, Matthey (1998, 2000, 2001) recorded a decrease in the number of open water bodies from 43 to 24 and a reduction of the overall surface from 309 m^2 to 176 m^2 over the same time period. With such dramatic changes at the macroscopic level we expected to see major changes in the bryophyte communities and also, at the microscopic level, in the testate amoebae communities, but only few significant changes were detected and therefore our data provided little support to our hypothesis.

No overall significant change was found between 1961 and 2001 in the bryophyte communities despite some changes in absolute and relative abundance of some species. For example, the dramatic increase in *Pleurozium schreberi* could be seen as a clear indication that in several sampling sites conditions were drier in 2001 than in 1961. Furthermore, species such as *Sphagnum cuspidatum* and *S. subsecundum*, typically associated with wet hollows were among those that decreased most dramatically. One of the species that decreased, *S. capillifolium*, is usually found in dry conditions, but this species was replaced by *Polytrichum strictum*, *Pleurozium shreberi*, or *S. angustifolium* three species that are often associated with equally or more dry conditions. However, in some cases the changes may suggest shift to wetter conditions, for example for sample number 22 where *Polytrichum strictum* disappeared and was replaced by *S. magellanicum*.

Our results show a significant reduction in testate amoebae diversity, but no changes in the Shannon-Wiener diversity or equitability. Although the overall species richness was exactly the same (33 species in both years), the mean value per sample decreased significantly from 11.9 to 9.6. Furthermore five species either increased or decreased significantly. However no clear interpretation can be drawn from these effects. For example, while *Nebela tincta s. l.* increased *Assulina muscorum* decreased, but as both species are indicators of dry conditions in peatland of the region (Mitchell *et al.* 1999) these contradictory responses cannot be interpreted as an indication of changes in hydrology. A better taxonomic resolution for the *Nebela tincta s. l.* species complex might have allowed further interpretation. Furthermore, despite the significant response of some of the dominant species, the Mantel test revealed no overall significant difference was found between 1961 and 2001 at the community level. Several tentative explanations can be given for the overall low level of response of testate amoebae.

The first possible explanation related to the sampling protocol: As the sampling sites were not permanently marked, it may be that, for some sites at least, the 2001 sampling spot did not correspond exactly to the one of 1961. However, it must be pointed out that Dr Willy Matthey knows the Le Cachot bog very well, having studied it in detail continuously since the early 1960s. Nevertheless, although for many sampling sites he was able to point to the exact spot where the moss sample had been taken in 1961, in a few cases he was not able to locate the spot within less than 1 m and, in these cases, a representative sampling site for the general area was selected. For this reason, both paired and unpaired test

Table 4. Diversity indices calculated from the testate amoebae data from Le Cachot bog.

Sample No	Species richness			Shannon-Wiener diversity			Shannon-Wiener equitability		
	1961	2001	Change	1961	2001	Change	1961	2001	Change
1	17	6	-11	0.71	0.18	-0.53	0.25	0.10	-0.15
4	14	12	-2	0.63	0.76	0.13	0.24	0.31	0.07
5	8	14	6	0.53	0.89	0.37	0.25	0.34	0.08
6	15	13	-2	0.91	0.87	-0.05	0.34	0.34	0.00
7	11	10	-1	0.56	0.48	-0.08	0.23	0.21	-0.02
8	15	12	-3	0.76	0.71	-0.04	0.28	0.29	0.01
9	12	8	-4	0.70	0.39	-0.31	0.28	0.19	-0.09
10	13	5	-8	0.69	0.37	-0.32	0.27	0.23	-0.04
11	11	13	2	0.70	0.50	-0.20	0.29	0.20	-0.10
12	9	11	2	0.48	0.61	0.13	0.22	0.25	0.04
13	10	7	-3	0.76	0.65	-0.10	0.33	0.34	0.01
14	13	7	-6	0.84	0.73	-0.11	0.33	0.38	0.05
15	13	12	-1	0.61	0.48	-0.13	0.24	0.19	-0.05
17	11	7	-4	0.79	0.58	-0.21	0.33	0.30	-0.03
21	8	8	0	0.63	0.38	-0.25	0.30	0.18	-0.12
22	8	11	3	0.48	0.78	0.30	0.23	0.33	0.10
23	15	8	-7	0.82	0.61	-0.21	0.30	0.29	-0.01
Mean	11.9	9.6	-2.3	0.68	0.59	-0.09	0.28	0.26	-0.02
SE	0.7	0.7	0.7	0.03	0.05	0.67	0.01	0.02	0.67
t-test P-values									
Paired			0.042			0.105			0.389
Unpaired			0.022			0.102			0.463

Table 5. Comparison of meteorological measurements from Neuchâtel, Switzerland between a three and a five year period preceeding the 1961 and 2001 sampling of *Sphagnum* mosses in Le Cachot bog, detail for the main growing period (May to September).

	Five year period					Three year period				
	5.1957-9.1961		5.1997-9.2001		t-test	5.1959-9.1961		5.1999-9.2001		t-test
	Mean	SD	Mean	SD	P-value	Mean	SD	Mean	SD	P-value
Minimum temperature [°C]	11.8	2.1	13.0	2.0	0.045	11.9	2.1	13.1	2.0	0.109
Maximum temperature [°C]	21.5	2.5	22.0	2.5	0.487	21.8	2.6	22.1	2.4	0.719
Precipitation [mm]	85	38	93	31	0.368	85	47	91	27	0.644
Mid-day relative humidity [%]	69.6	4.5	69.8	4.6	0.882	68.3	4.7	69.8	3.1	0.296

results are given. As testate amoebae show heterogeneous horizontal distribution patterns even in apparently homogeneous surfaces in response to micro-environmental gradients (Mitchell *et al.* 2000), and any uncertainty in the location of the 1961 sampling sites can be expected to have an important effect on the data. In this respect this study can be viewed as a test for the limit of using testate amoebae as biomonitors in *Sphagnum* peatlands. Their micro-distribution patterns may make it impossible to simply return to a given sampling spot years or decades after an initial study unless the initial sampling sites were marked permanently. A paleoecological approach on a series of short cores taken in different parts of the peatland, or the same sampling spots would eliminate this problem and also offer the opportunity to see how testate amoebae communities changed between 1961 and 2001. However, one challenge of this approach will be to obtain exact dates for the different sampling levels.

The second possible explanation is that the climate changed over the 40-year period in a way that counteracted the effect of drainage and vegetation succession.

We used to nearest reliable meteorological survey station, in Neuchâtel (487 m a.s. l. - n. b. approximately 600 m lower than the studied peatland which is located at 1050 m a.s. l.) to determine if the climate (parameters analyzed: monthly average of minimum and maximum temperatures, precipitation and relative humidity) had changed between a three and a five-year period preceding the two sampling periods. Three to five years corresponds to the approximate time covered by mosses sampled for testate amoebae. The climate record suggests that for the growing period (May through September) of the five-year period preceding the 1961 sampling the average minimum temperature was warmer than for the same period preceding the 2001 sampling. These observations are in agreement with the long-term climatic observations for Switzerland where a clear general warming is due mostly to an increase in minimum (i.e. night) temperature while a slight but significant reduction in maximum temperatures is observed (Rebetez 2001, Rebetez *et al.* 2003). However, the trend for warmer minimum temperatures was not significant when only the three-year preceding the samplings were compared (Table 5). By contrast no significant difference was observed for the same time periods for precipitation and relative humidity (Table 5). If we accept that the trends in climatic data measured at Neuchâtel, at a much lower elevation than Le Cachot bog (487 m *vs.* 1050 m), are representative for the studied site, we consider it unlikely that changes in climate could have compensated for the effects of drainage and vegetation changes at the bog surface.

The third possible explanation is that if indeed major changes in micro-environmental conditions did occur, the testate amoebae did not respond to them. This would be in contradiction with (a) results from multiple studies on the ecology of testate amoebae (Tolonen 1986; Warner 1990; Charman and Warner 1992, 1997; Tolonen *et al.* 1992, 1994; Warner and Chmielewski 1992; Charman 1997; Mitchell *et al.* 1999; Booth 2001, 2002), (b) their fine-scale distribution patterns in relation to micro-topography (Mitchell *et al.* 2000), and (c) the observed rapid changes in testate amoebae communities during peatland regeneration (Buttler *et al.* 1996).

The fourth possible explanation, and the one we consider most likely, is that most of the changes at the microscopic level had already taken place when the 1961 samples were taken and no overall significant changes in micro-environmental conditions took place during the 40-year period covered by this study. This would agree with the faster response time usually attributed to microorganisms (Foissner 1987, 1999). This would also agree with the observed differential response times of different plant strata and soil horizons to environmental change (Gobat *et al.* 2003) (Fig. 4). This interpretation also implies that the bushes and trees are poor predictors of the response of microorganisms if they are themselves in a non-equilibrium stage. A series of short peat cores in this or comparable bogs would probably be the best way to test this hypothesis.

Despite the lack of overall clear change between the two sampling dates, the testate amoebae clearly differed among the microhabitat types as attested by the position of wet pool, lawn and hummock samples in the ordination space (Fig. 3). Furthermore, most of the sample pairs for which marked differences between 1961 and 2001 appeared in the DCA (i.e. samples 4, 7, 8, 12, and 17) indeed corresponded to observed changes in the microhabitat and *Sphagnum* species (Table 1). These observations confirm the well-established responsiveness of testate amoebae to the major ecological gradients that exist in *Sphagnum*-dominated peatlands.

Opportunities to carry out studies such as this one are few, owing to the small number of ecological studies that were done several decades ago. An invaluable asset to alleviate some of the uncertainties of such studies would be to have permanently marked sampling sites, but these do not always exist.

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Cell Surface Glycoproteins in *Crithidia deanei*: Influence of the Endosymbiont

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Summary. *Crithidia deanei*, a protozoan of the family Trypanosomatidae harboring an endosymbiont bacterium in its cytoplasm and cured strain (endosymbiont-free) were compared as to glycoprotein composition. The wild strain of *C. deanei* showed a double band with molecular mass of 70/74 kDa. These bands were absent from symbiont-free cells. Indirect immunofluorescence microscopy using specific antibodies against the 70/74 kDa glycoprotein isolated from the symbiont-containing cells revealed intense labeling of the cell surface in the symbiont-harboring *C. deanei*. However, no such labeling was observed in symbiont-free cells. These observations suggest that the endosymbiont influences the composition of the glycoprotein on the cell surface of *C. deanei.*

Key words: *Crithidia deanei*, endosymbiont, glycoproteins, immunofluorescence.

INTRODUCTION

The large family Trypanosomatidae includes some species associated with human, animal, or plant diseases. Most of the studies involving this family have focused on the human-pathogenic species, and few biochemical analyses have been attempted on the so-called non-pathogenic trypanosomatids. Some trypanosomatids: *Crithidia deanei*, *C. oncopelti*, *C. desouzai*, *Blastocrithidia culicis*, and *Herpetomonas roitmani* harbor endosymbiotic bacteria (De Souza and Motta 1999). The ability of high doses of antibiotics to eliminate the endosymbiont has increased interest in the study of endosymbiont-harboring species, because several bacteria-protozoa interactions can be analyzed by comparing "cured" (endosymbiont-free) and wild strains. It is recognized that these intracellular symbionts are considerably integrated into the physiology of the host cell (McGhee and Cosgrove 1980). Furthermore, the presence of symbionts induces several morphological alterations in the host cells, such as the rearrangement of kinetoplast DNA fibers and the disappearance of the paraxial rod structure observed in *C. deanei*, *C. oncopelti*, and *Blastocrithidia culicis* (Freymüller and Camargo 1981). The endosymbiont causes addi-

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tional modifications, such as: a different carbohydrate composition of glycocalyx in *C. fasciculata*, a reduced surface charge and influence on the heme-synthesis pathway in *C. deanei*, the urea cycle in *B. culicis*, the enzyme threonine deaminase, nutritional requirements in *B. culicis*, *C. oncopelti*, and *C. deanei*, and the secretion of proteinases in *C. desouzai*, *C. deanei*, and *C. oncopelti* (De Souza and Motta 1999; Esteves *et al.* 1982; Oda *et al.* 1984; d'Avila-Levy *et al.* 2001, 2003). For all these reasons, endosymbiont-bearing trypanosomatids constitute excellent models for studies on symbiosis, which may contribute to better understanding of the origin of organelles in eukaryotic cells.

Carbohydrates are only a minor fraction of cell components, but they play an important role in the regulation of cell growth, antigenicity, and cell recognition (Nicolson 1974, 1976). Most of the carbohydrates exposed on the cell surface are bound to proteins, and therefore many studies have focused on membrane glycoproteins. Previous studies have reported that trypanosomatid protozoans do not contain cytoplasmic storage polysaccharides, so that carbohydrates are associated with cell membranes (De Souza 1989). In general, *C. deanei* and other trypanosomatids such as species of *Trypanosoma*, *Leishmania*, and *Herpetomonas* show similar profiles of cell-surface carbohydrates (Dwyer 1977, Chiari *et al.* 1978, Sixel *et al.* 1978).

The present investigation demonstrated the influence of the endosymbiont on the composition of the glycoproteins present on the cell surface of *C. deanei* grown in a chemically defined medium, through the use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)/indirect immunofluorescence assays.

MATERIALS AND METHODS

Microorganism. Cultures of symbiont-bearing *Crithidia deanei* (ATCC 30255) were maintained by weekly transfers into a chemically defined medium (Mundim *et al.* 1974), added in 5-ml volumes to screw-capped tubes. The symbiont-free strain of *C. deanei* was maintained in the same defined medium with 0.030 g/l of nicotinamide (Sigma Chemical Company, St. Louis, USA) (Mundim and Roitman 1977). Cells were grown at 28°C for 48 h and stored at 4°C.

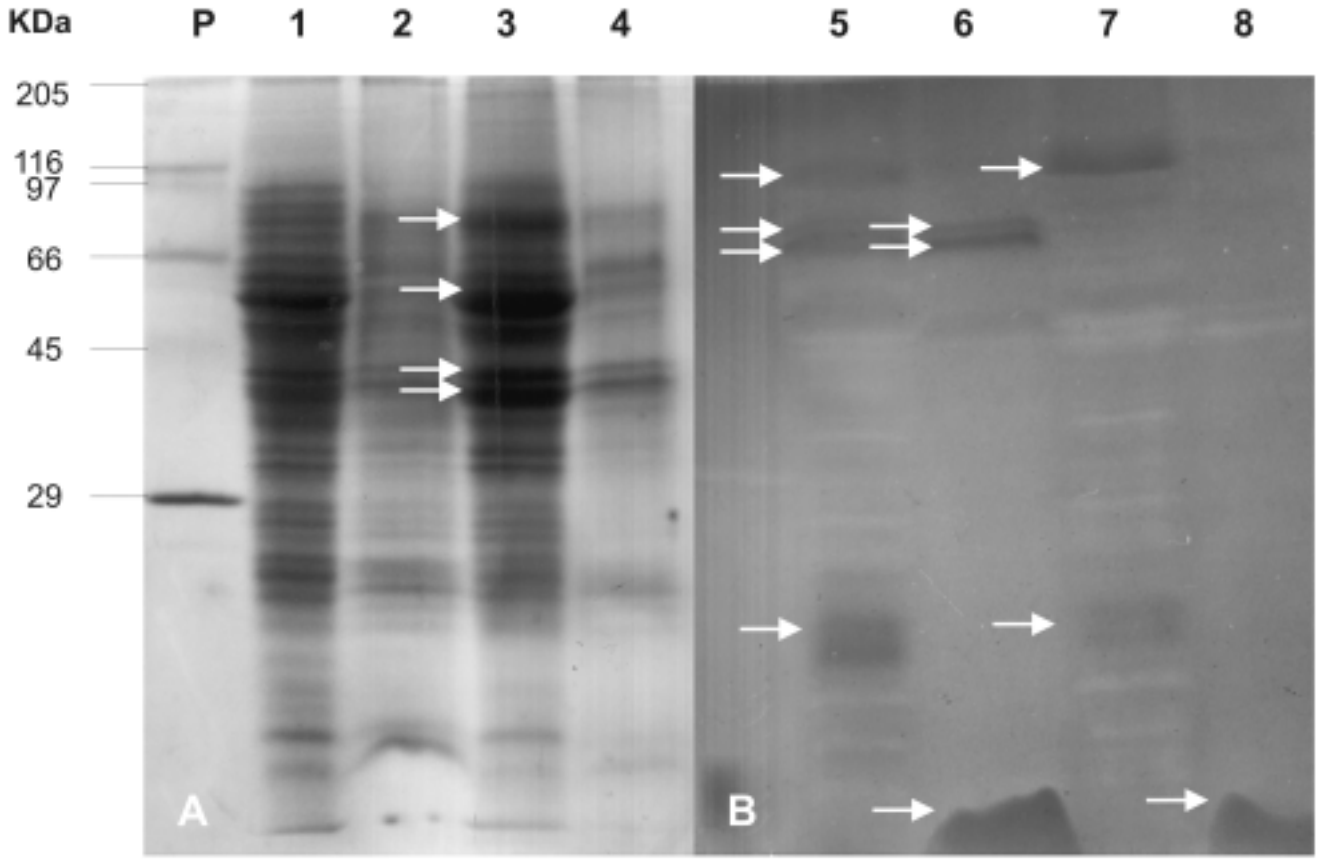
Cells cultivated in 1.5 l of defined medium (7×10^7 cells/ml) were harvested at the exponential phase (48 h) by centrifugation for 10 min at 2,000 g at 4°C and washed 4 times with cold 0.01M phosphate-buffered saline (PBS) pH 7.2.

Triton X-114 extraction. Symbiont-containing and symbiont-free cells (1×10^8 cells/ml) were solubilized in 2% Triton X-114 (Sigma) pre-condensed in Tris-saline buffer (TSB) (10 mM Tris - Invitrogen Life Technologies, 150 mM NaCl, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma) for 30-40 min at 0-4°C. Insoluble material was removed from the lysate by centrifugation at 20,000 g for 30 min at 0-4°C. The hydrophobic and hydrophilic phases were separated using a 6% (w/v) sucrose (Sigma) cushion (1:1.5, v/v) as described by Bordier (1981). Separation of the detergent from the proteins was effected by addition of zinc chloride (Mallinckrodt) to the samples, to obtain a final concentration of 0.05 M. The glycoproteins were precipitated by addition of 5 volumes of cold acetone (Merck). The pellet was collected by centrifugation, and the supernatant solution containing Triton X-114 was discarded. The proteins were resuspended in water and precipitated again with 5 volumes of cold acetone. After centrifugation, the pellet was washed with 50% cold acetone, dried at room temperature, and stored at -20°C until use.

SDS-PAGE electrophoresis. Samples of proteins and glycoproteins (hydrophobic and hydrophilic phases) precipitated with acetone were solubilized in the same volume of hot sample buffer (10 mM Tris-HCl, pH 8.0 1 mM EDTA, 1.0% SDS and 0.5% β-mercaptoethanol) and further boiled for 3 min. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (12% SDS-PAGE - Gibco Invitrogen Corporation, New York, USA) was run in duplicate (Laemmli 1970). Proteins were revealed by soaking in 0.25% (w/v) Coomassie Brilliant Blue R-250 (Sigma), in 50% (v/v) methanol and 10% (v/v) acetic acid. Destaining was achieved in the stain diluent. Glycoproteins were detected by staining the gel by the modified periodic acid-Schiff (PAS) method proposed by Doerner and White (1990). Briefly, gels were fixed overnight in 7.5% (v/v) acetic acid (Vetec) for 30 min, followed by addition of periodic acid (Sigma) for 1 h at 4°C and then incubated with Schiff's reagent for 1 h at 4°C. Reddish-pink bands of stained glycoprotein would then be visible. Reduction with 2% (w/v) sodium metabisulfite (Mallinckrodt) in 7.5% (v/v) acetic acid was performed overnight and subsequently stored in water.

Extraction and purification of 70/74 kDa glycoprotein. The cells were subjected to one cycle of freezing-thawing, cold water was added, and the mixture centrifuged at 3,000 g for 30 min. The resulting pellet was dispersed in cold water and the suspension once more centrifuged. The combined supernatants were heated at 100°C and lyophilized. The pellet remaining after centrifugation was extracted with aqueous phenol at 75°C (Mendonça-Previato *et al.* 1983), the aqueous layer dialyzed 3 times against 0.05 M phosphate buffer, pH 7.0, for 48 h, and applied to a Sephadex G-100 (Pharmacia Biotech) equilibrated column and run (1 ml/min) with a 0.1 M phosphate buffer, pH 7.0, at 4°C. The column was eluted with the same buffer at a flow rate of 1 ml/min, and 1 ml fractions were collected. The 70/74 kDa glycoprotein from endosymbiont-containing *C. deanei* was obtained. The elution profile was calibrated with known standards (Blue-dextran = 2,000,000 Da, Yellow-dextran = 2,000 Da, and Vitamin B_{12} = 125 Da) (Amersham Biosciences).

Production and purification of antibody. Approximately 2 mg/ml of 70/74 kDa glycoprotein was mixed with an equal volume of complete Freund's adjuvant for the first injection, and with incomplete Freund's adjuvant for the subsequent injections. A rabbit was immunized once a week for six weeks, and once a month thereafter. Pooled sera from four different bleeds were used in the experiments (Dias Filho *et al.* 1999). Purification of the crude antibody was affected by adjusting to pH 8.0 by adding 1/10 volume of 1.0 M Tris (pH 8.0), and then passing through a Protein A bed column (Amersham Biosciences). After the bed had been washed with 10 column volumes



Figs 1 A, B. Triton X-114 fractionation of endosymbiont-containing and endosymbiont-free *Crithidia deanei* analyzed by SDS-PAGE stained by Coomassie brilliant blue R-250 (**A**) and stained by periodic acid-Schiff (PAS) (**B**). **Lanes 1, 2, 5, and 6** endosymbiont-harboring *C. deanei*, **Lanes 3, 4, 7, and 8** aposymbiotic strain of *C. deanei*, **Lane P** molecular mass markers, **Lanes 1, 3, 5, and 7** hydrophilic phase, **Lanes 2, 4, 6, and 8** hydrophobic phase, arrows major bands as described in Results. Amount of protein loaded on the gel = 30 µg in each slot.

of 100 mM Tris (pH 8.0) and 10 column volumes of 10 mM Tris (pH 8.0), the column was eluted with 100 mM glycine (Sigma) (pH 3.0). The eluate was collected in 1.5-ml conical tubes containing 50 µl of 1 M Tris (pH 8.0). The tubes were then mixed gently to return the pH to neutral. The immunoglobulin-containing fractions were identified by absorbance at 280 nm (Kessler 1975).

Immunofluorescence staining. Cells were fixed for 30 min at room temperature in a solution containing 4% freshly prepared paraformaldehyde in PBS at pH 7.2, washed in same buffer, and allowed to adhere for 10 min to coverslips previously coated with 0.1% poly-L-lysine. Subsequently, they were incubated for 30 min in the presence of 50 mM NH_4Cl to block free aldehyde groups, washed in PBS, and incubated for 60 min in the presence of the polyclonal antibody recognizing 70/74 kDa glycoprotein (1:100 dilution in PBS) of endosymbiont-bearing *C. deanei*. Afterward they were washed in PBS-3% bovine serum albumin and incubated in the presence of fluorescein-labeled goat anti-rabbit IgG (EY Laboratories, San Mateo, California, USA) for 60 min (diluted 1:100 in PBS). Next, the specimens were mounted with N-propyl gallate and observed in a Zeiss microscope equipped for fluorescence. Control preparations were incubated without the primary antibody.

Analytical methods. Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Neutral sugars were determined with the phenol-sulfuric method, with glucose as a standard (Dubois *et al.* 1956).

RESULTS

Symbiont-containing and symbiont-free strains of *C. deanei* showed very similar SDS-PAGE protein profiles (Fig. 1A). Both strains displayed a large number of bands ranging from 6 to 96 kDa stained with Coomassie Blue. Most of the intensely stained bands were present in the hydrophilic phase. A double band with molecular mass of 40/44 kDa, one major band from 50 to 63 kDa, and one band from 90 to 95 kDa were more evident in endosymbiont-free cells (Fig. 1A, lane 3). Poorly stained proteins were seen in the hydrophobic phases of sym-

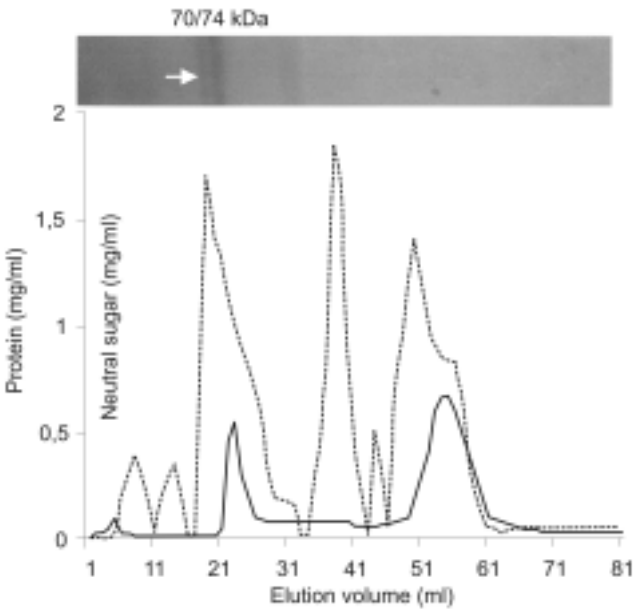
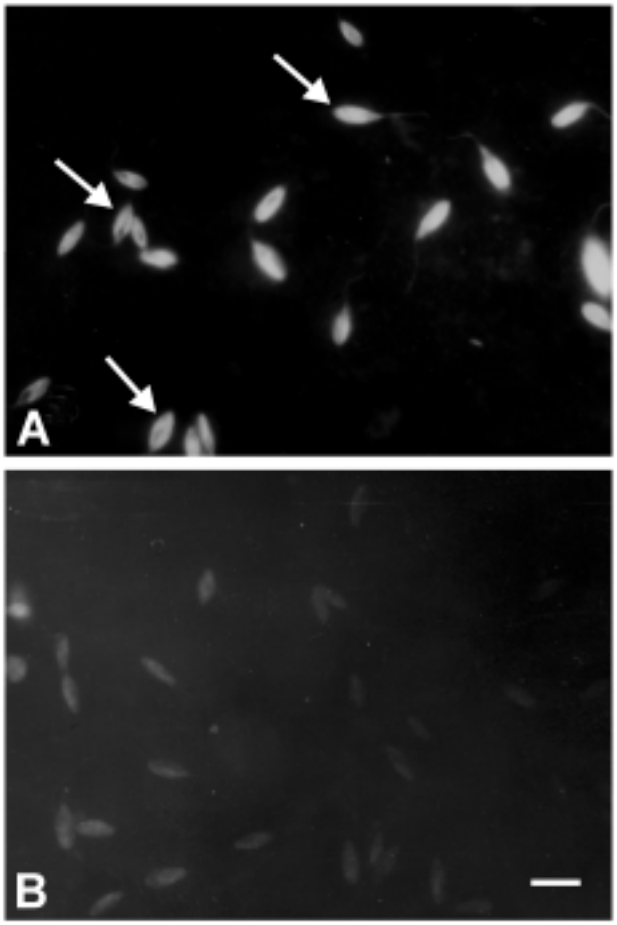


Fig. 2. Gel filtration on a 2 × 120 cm column of Sephadex G-100 of aqueous extracts of symbiont-containing *Crithidia deanei*. Peak I was eluted between 17 to 23 ml at a position of molecular mass from 70-80 kDa, and when examined by polyacrylamide gel electrophoresis (top) showed a double band with a molecular mass of 70/74 kDa, *arrow* major band as described in Results. Fraction size = 5.0 ml.

biont-containing and symbiont-free strains (Fig. 1A, lanes 2 and 4).

A double band with molecular mass of 70/74 kDa was present in the hydrophilic and hydrophobic phases obtained from symbiont-containing *C. deanei* (Fig. 1B, lanes 5 and 6), but absent from symbiont-free cells (Fig. 1B, lanes 7 and 8). Both symbiont-containing and symbiont-free strains exhibited two distinct glycoproteins with masses of approximately 97 and 20 kDa in the hydrophilic phase (Fig. 1B, lanes 5 and 7). Moreover, both strains showed a broad and intensely stained conjugate (less than 20 kDa) near the bottom of the gel in the hydrophobic phase (Fig. 1B, lanes 6 and 8).

Treatment of *C. deanei* with Triton X-114 non-ionic detergent at 0°C, followed by low-speed centrifugation, generated a detergent-insoluble pellet and a detergent-soluble supernatant. The supernatant was further fractionated by phase separation at 37°C into a detergent-rich phase and a detergent-depleted or aqueous phase. The results showed that all the strains of *Crithidia* contained proteins with molecular masses ranging between 6 and 96 kDa. Between the hydrophilic (aqueous) and hydrophobic (detergent-rich) phases, only quantitative differences were observed.



Figs 3A, B. Immunofluorescence microscopy. *Crithidia deanei* were incubated first in the presence of antibodies recognizing 70/74-kDa glycoprotein and subsequently in the presence of FITC-labeled goat anti-rabbit IgG and observed fluorescence microscopy; **A** - intense labeling of the surface of wild strain of *C. deanei* (arrows); **B** - surface labeling was not observed when symbiont-free cells were used. Scale bar 10 µm.

Marked differences in the plasma-membrane glycoproteins between the cured and the symbiont-containing strains of *C. deanei* were observed in the present study, mainly the presence of the double band (70/74 kDa) in both phases obtained from symbiont-harboring *C. deanei*, versus its absence from symbiont-free cells. On the other hand, a glycoconjugate with a low molecular mass (< 20 kDa) was detected in the hydrophobic phases of both symbiont-containing and symbiont-free cells, as shown in Fig. 1B (lanes 6 and 8).

In order to confirm this finding, glycoconjugates were extracted from endosymbiont-bearing *C. deanei* as described in the Material and Methods. The supernatant was lyophilized and the residue fractionated by column

chromatography on Sephadex G-100 (Fig. 2), resulting in the separation of two major peaks monitored for carbohydrate and protein. Peak I was eluted between 17 to 23 ml at a position of molecular mass from 70 to 80, and when examined by polyacrylamide-gel electrophoresis showed a double band with a molecular mass of 70/74 kDa (Fig. 2, top).

Cell localization of 70/74 kDa glycoprotein was performed by using polyclonal antibodies which were detected with FITC-labeled secondary antibodies. Intense labeling of the cell surface of endosymbiont-bearing *C. deanei* was observed in previously fixed cells (Fig. 3A). Cell surface labeling was not observed in symbiont-free cells (Fig. 3B). No labeling was observed when purified glycoprotein was added to the incubation medium, or when cells were incubated only in the presence of FITC-labeled secondary antibodies (data not shown).

DISCUSSION

Evaluation of the total protein profile of trypanosomatid strains by SDS-PAGE is not a particularly useful method, because the strains showed a large number of protein bands of different apparent molecular masses, and they also were very similar among different strains (data not shown). Protein extraction performed with Triton X-114 is a more efficient method to analyze proteins and glycoconjugates in SDS-PAGE, because it requires small amounts of sample and provides clearer profiles, allowing individualization of bands. *C. deanei* displayed a large number of proteins ranging from 6 to 96 kDa. Proteins with molecular masses of 40 kDa, 50/63 kDa, and 90/95 kDa are more evident in endosymbiont-free cells than in endosymbiont-containing cells. d'Avila-Levy *et al*. (2001) demonstrated the absence of the cell-associated cysteine proteinase of 100 kDa and a two-fold enhancement of extracellular proteinases in the cured strain, suggesting that the prokaryote endosymbiont induces alteration in the proteolytic profile in *C. deanei*. Proteinases are enzymes that have been implicated in a number of aspects of host-parasite interactions, including tissue and cell invasion, parasite differentiation, inactivation of deleterious host proteins, and catabolism of exogenous proteins for nutrition purposes (McKerrow *et al*. 1993, Faria e Silva *et al*. 1994, Engel *et al*. 1998, Sajid and McKerrow 2002).

In order to identify and compare the major glycoproteins, the two phases (hydrophobic and hydrophilic) obtained from Triton X-114 extracts of endosymbiont-harboring *C. deanei* and cured with high doses of chloramphenicol were analyzed by SDS-PAGE after staining with Schiff's reagent. Only the wild strain of *C. deanei* contained a glycoprotein with molecular mass of 70/74 kDa. On the other hand, two different glycoproteins with masses of approximately 97 and 20 kDa, were shown by both symbiont-containing and symbiont-free strains, in the hydrophilic phase. Moreover, both strains showed a broad and intensely stained conjugate (less than 20 kDa) near the bottom of the gel in the hydrophobic phase. These results concord with those of previous studies, in which, after analysis of the glycoconjugates of trypanosomatid genera such as *Herpetomonas, Endotrypanum, Leishmania, Trypanosoma* (Branquinha *et al.* 1995), and *Phytomonas* (Abreu Filho *et al.* 2001), glycoconjugates with molecular masses below 20 kDa were observed.

The immunofluorescence assay demonstrated that the glycoproteins of 70/74 kDa, present only in the endosymbiont-harboring *C. deanei*, are located in the plasma membrane. It has been reported that there is a difference in the composition of carbohydrates exposed on the cell surface of endosymbiont-containing and endosymbiont-free strains of *C. deanei* and *Herpetomonas roitmani*, suggesting that the presence of the symbiont bacterium can induce alterations in the surface of the cells (Dwyer and Chang 1976, Esteves *et al.* 1982, Faria e Silva *et al*. 1994). It is interesting that these studies suggest that endosymbiont-bearing *Crithidia* species present fewer surface-exposed carbohydrates than do other species such as *C. fasciculata* and *C. lucillae*, which naturally lack the symbiont. Recently, Fampa *et al*. (2003) showed that an endosymbiont-free strain showed a significant decrease in the interaction with cells and gut tissue from several insect species, compared with the endosymbiont-harboring strain. The reported differences in the cell-surface carbohydrates between endosymbiont-bearing and endosymbiont-free strains imply that these carbohydrates may be involved in the interaction between monoxenous trypanosomatids and cell lines or insect guts. It has been reported that carbohydrates present on the cell surface of *Leishmania* are responsible for the adhesion of the protozoan to the midguts of sandflies (Sacks and Kamhawi 2001). In addition, mouse peritoneal macrophages engulf more endosymbiont-containing than endosymbiont-free protozoans, and the presence of the endosymbiont confers resistance to the macrophage killing mechanisms (Rozental *et al.* 1987).

The major result emerging from the present study was the differences found in the plasma-membrane glycoproteins between the cured and the symbiont-containing strains of *C. deanei*. The presence of the double band (70/74 kDa) in both phases obtained from symbiont-containing *C. deanei*, and its absence from symbiont-free cells, suggests that the symbiont somehow influences the glycoconjugate composition of the plasma membrane in these cells. Further studies using the purified 70/74-kDa glycoprotein may clarify the basic aspects of the role played by the glycoconjugate in the process of the interactions of *C. deanei* with its intracellular symbionts.

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Updating the Ciliate Genus *Chlamydodon* Ehrenberg, 1835, with Redescriptions of Three Species (Ciliophora: Cyrtophorida)

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Summary. The morphology and infraciliature of three marine cyrtophorid ciliates, *Chlamydodon obliquus* Kahl, 1931, *C. mnemosyne* Ehrenberg, 1835 and *C. triquetrus* (Müller, 1786), collected from coastal waters near Qingdao (Tsingtao), China, were investigated *in vivo* and using protargol impregnation. Based on the previous and current studies, the definition for the genus *Chlamydodon* is updated: chilodonellids with a cross-striated band around the periphery of the somatic field; rightmost kineties extending dorso-apically and bending to the left, making a conspicuous suture with left kineties; one preoral and two circumoral kineties obliquely arranged; equatorial fragment with loosely spaced kinetosomes; several to many terminal fragments. A revised diagnosis for the little known species *C. obliquus* is suggested: large-sized *Chlamydodon*, about 120-180 × 50-120 µm *in vivo*, body ellipsoid to triangular in outline; cross-striated band continuous, largest portion of which runs along cell margin, anterior region crossing onto dorsal surface; about 37 right, 4 postoral and 27 left kineties; 11-14 nematodesmal rods; about 7 terminal fragments on dorsal side; *ca*. 10 contractile vacuoles irregularly distributed; marine habitat. Improved diagnoses for *C. mnemosyne* and *C. triquetrus* are also supplied. Comparisons between congeners demonstrate that *C. exocellatus* Ozaki *et* Yagiu, 1941 and *C. kasymovi* Aliev, 1987 are synonyms of *C. obliquus* and *C. triquetrus*, respectively; *C. pedarius* Kaneda, 1953 and *C. apsheronica* Aliev, 1987, should be conspecific with the type species *C. mnemosyne*. Based on the data of morphology and infraciliature, an updated key to 5 *Chlamydodon* species is supplied.

Key words: *Chlamydodon*, Cyrtophorida, infraciliature, key to *Chlamydodon*, marine ciliate, morphology.

INTRODUCTION

Species of the cyrtophorid genus *Chlamydodon* are usually large and often occur in biofilm or in the periphyton of eutrophic biotopes (Sauerbrey 1928; Kahl 1931; Kiesselbach 1936; Borror 1963, 1972; Katter 1970; Hartwig 1973, 1980; Jones 1974; Hartwig and Parker 1977; Agamaliev 1978, 1983; Al-Rasheid 1996, 1997). One of the most recognizable features in this genus is the cross-striated band (CSB), which is located at the perimeter of the flattened cell and separates the dorsal and ventral sides of the cell. Using TEM and SEM methods, Kurth and Bardele (2001) gave a detailed observation of the type species *Chlamydodon mnemosyne*; they examined the CSB and other organelles such as the somatic cortex and oral structure. Morphogenetic processes in *C. mnemosyne* were also

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studied in detail by Fauré-Fremiet (1950) and Bardele and Kurth (2001).

Although the ultrastructural and morphogenetic characters of the genus *Chlamydodon* are well understood, there are many problems of species identification and separation in this genus. The confusion derived from that species have been distinguished by some live features (e.g. the body shape and size) and appearance of CSB, number of nematodesmal rods, and presence of a pigment spot (Kahl 1931, Ozaki and Yagiu 1941), many of which are likely variable or inconspicuous and thus cannot be used as diagnostic characters. Furthermore, only half of the 16 nominal species have been studied using silver impregnation methods (Fauré-Fremiet 1950; Kaneda 1960a; Borror 1963, 1972; Dragesco 1963, 1965, 1966; Katter 1970; Agamaliev 1978; Dragesco and Dragesco-Kernéis 1986; Aliev 1987; Alekperov and Asadullayeva 1997; Kurth and Bardele 2001). Several of these remain poorly described, which renders identification difficult.

Three morphotypes of *Chlamydodon* were isolated from coastal waters off Qingdao. Detailed investigations indicated that one is the species *Chlamydodon obliquus* and the other two are the well-known forms, *Chlamydodon mnemosyne* and *C. triquetrus.* Improved descriptions of these three species are here presented and their synonymy is discussed.

MATERIALS AND METHODS

Chlamydodon obliquus. One population was collected on 15 June 2002 from a rock pool of a tideland near Qingdao (Tsingtao, 36° 08' N; 120°43' E), China, which contained large amounts of seaweed. Temperature was 15°C, salinity was about 28‰, pH was about 7.3.

Chlamydodon mnemosyne. Three isolations were made (10 August 2002; 25 August 2002; 2 July 2003) from shrimp-farming ponds and from a rock pool near Qingdao. Temperature was 17-19°C, salinity was about 25-34‰, pH was about 7.3-7.6.

Chlamydodon triquetrus. Four isolations were made (12 March 2003; 10 April 2003; 10 May 2003; 17 May 2003) from an abalone-culture pond and from scallop-farming waters. Temperature was 13-16°C, salinity was about 26-31‰, pH was about 7.2-7.8.

Isolated specimens were maintained in Petri dishes with seawater (salinity *ca* 30‰) at 18°C. Some seaweeds and diatoms collected from original sites were supplied as food.

Living cells were observed with differential interference microscopy. The infraciliature was revealed by the protargol impregnation method according to Wilbert (1975). Living individuals were examined and measured at 1000× magnification; drawings of stained specimens were performed at 1250× with the aid of a camera lucida. Protargol impregnated voucher slides of three species are deposited in the Laboratory of Protozoology, OUC, China, with the following registration numbers: *Chlamydodon obliquus*, G02071501; *Chlamydodon mnemosyne*, G02081001; *Chlamydodon triquetrus*, G03041001. Terminology is mainly according to Corliss (1979).

RESULTS

To our knowledge, the definition of the genus *Chlamydodon* has not been revised following examination using modern methods. Therefore, we provide an improved diagnosis based on the data obtained.

Improved diagnosis for the genus *Chlamydodon*: Chilodonellids with a cross-striated band around the periphery of the somatic field; rightmost kineties extending apical-dorsally and bending to left, making a conspicuous suture with the left kineties; one preoral and two circumoral kineties obliquely arranged; equatorial fragment with loosely spaced kinetosomes; several to many terminal fragments.

Remarks: Considering the general infraciliature, *Chlamydodon* is similar to *Cyrtophoron* Deroux, 1974 in ciliary pattern (Deroux 1974, Aliev 1991). It differs from the latter, however, by possessing a CSB.

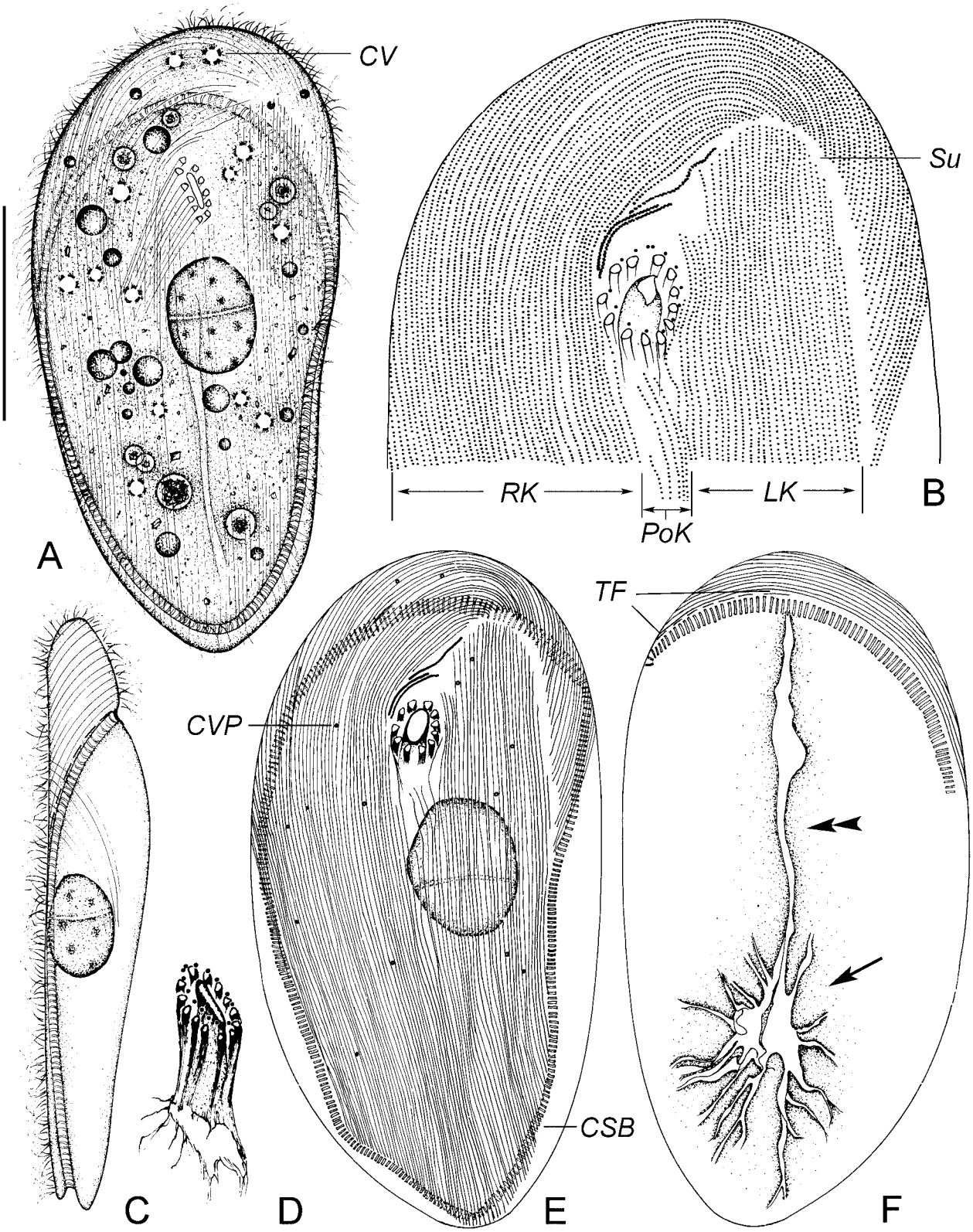
The CSB is a generic feature of another cyrtophorid taxon, *Coeloperix* Deroux in Gong and Song 2004, but *Chlamydodon* can be separated from *Coeloperix* by: (1) somatic kineties in three fields: right, postoral and left (*vs.* in two fields, preoral and postoral, in *Coeloperix*); (2) presence of anterior suture formed by right and left somatic kineties (*vs.* absent in *Coeloperix*); and (3) during morphogenesis, the oral kineties in the opisthe derive from postoral somatic kineties (*vs.* from leftmost ones in *Coeloperix*) (Deroux 1976, Gong and Song 2004).

Chlamydodon obliquus Kahl, 1931 (Figs 1, 2; Tables 1, 2)

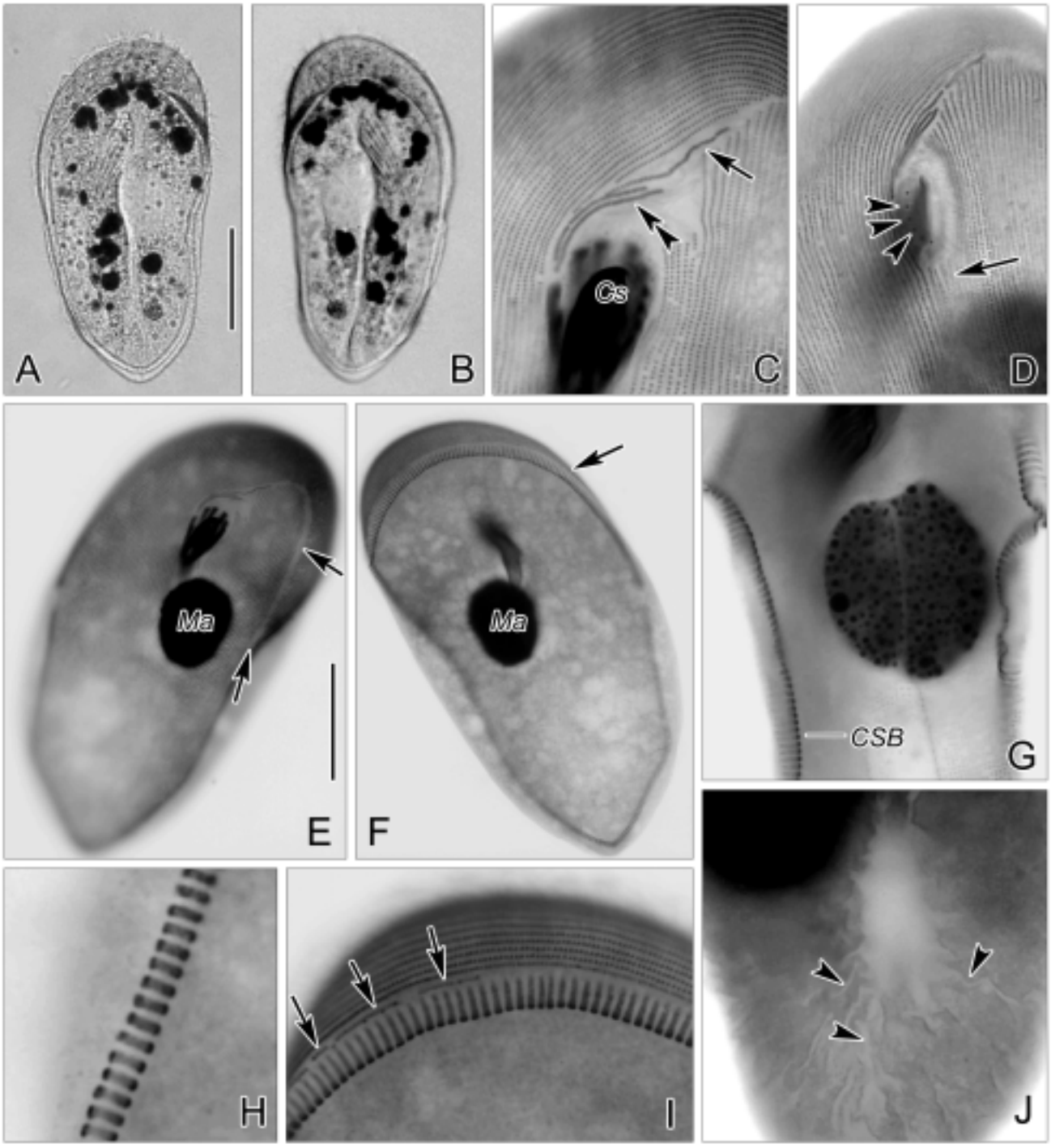
Syn**:** *Chlamydodon exocellatus* Ozaki *et* Yagiu, 1941

Improved diagnosis: About 120-180 × 50-120 μm *in vivo*, cell ellipsoid to triangular in outline; cross-striated band (CSB) continuous, anterior portion crossing to the dorsal surface, ~ 37 right, 4 postoral, and 27 left kineties; 11-14 nematodesmal rods; ~ 7 terminal fragments on dorsal side; ~5-15 irregularly distributed contractile vacuoles; macronucleus ~ 20-40 μm in diameter; marine habitat.

Description of Qingdao population: Size 160 × 90 μm *in vivo*, as calculated from some measurements of live specimens and values shown in Table 1, assuming a



Figs 1A-F. Morphology and infraciliature of *Chlamydodon obliquus* from life (A, C) and after protargol impregnation (B, D-F). **A** - ventral view of a typical individual; **B** - ventral view showing detailed infraciliature of anterior part; **C** - lateral view; **D** - cyrtos; **E, F** - ventral (E) and dorsal (F) views of infraciliature, arrow indicates the dorsal depression and double-arrowheads mark the long canal-like depression extending anteriorly. CSB - cross-striated band; CV - contractile vacuoles; CVP - contractile vacuole pores; LK - left kineties; PoK - postoral kineties; RK - right kineties; Su - suture; TF - terminal fragments. Scale bar 50 µm.



Figs 2A-J. Photomicrographs of *Chlamydodon obliquus* from life (A, B) and after protargol impregnation (C-J). **A, B** - ventral (A) and dorsal (B) views of a typical individual; **C** - detailed infraciliature of oral field, arrow indicates preoral kinety and double-arrowheads indicate circumoral kineties; **D** - focusing on basal body-like granules (arrowheads) and postoral kineties (arrow); **E** - ventral view of infraciliature, showing the anterior suture (arrows); **F** - dorsal view, focusing on the part of cross-striated band (arrow) that extends onto dorsal surface; **G** - macronucleus; **H** - a close-up of a section of cross-striated band; **I** - dorsal view, showing that terminal fragments (arrows) are variable in length; **J** - dorsal view of posterior portion of cell, to show the canal-like depressions. Cs - cytostome; CSB - cross-striated band; Ma - macronucleus. Scale bars 50 μm.

Table 1. Morphometric characteristics of *Chlamydodon mnemosyne* (first line), *C. triquetrus* (second line) and *C. obliquus* (third line). Data from protargol impregnated specimens. All measurements in µm. Abbreviations: Max - maximum, Mean - arithmetic mean, Min - minimum, n - number of individuals examined, SD - standard deviation.

Characters	Min	Max	Mean	SD	n
Body length	54.0	78.0	65.4	6.7	17
	69.0	96.0	84.6	8.5	19
	120.0	164.0	139.6	15.2	11
Body width	31.0	50.0	43.1	5.6	17
	26.0	45.0	36.7	3.9	19
	50.0	84.0	60.2	9.6	11
Total number of somatic kineties	29.0	35.0	31.4	1.4	17
	36.0	48.0	40.4	3.0	19
	66.0	72.0	69.2	2.0	11
Number of right kineties	14.0	18.0	15.3	1.1	17
	16.0	24.0	19.9	2.3	19
	34.0	40.0	37.1	1.7	11
Number of left kineties	11.0	13.0	12.1	0.6	17
	15.0	21.0	16.7	1.8	19
	26.0	29.0	27.7	1.2	11
Number of postoral kineties	4.0	4.0	4.0	0.0	17
	4.0	4.0	4.0	0.0	19
	3.0	5.0	4.0	0.8	11
Number of terminal fragments	2.0	4.0	2.5	1.0	16
	4.0	8.0	5.9	1.0	18
	6.0	10.0	7.0	1.3	11
Number of nematodesmal rods	8.0	11.0	9.4	0.7	16
	9.0	14.0	11.9	1.2	19
	11.0	14.0	12.8	1.0	9
Macronucleus length	22.0	30.0	25.2	2.2	17
	17.0	30.0	23.7	3.3	19
	24.0	36.0	29.5	4.3	11
Macronucleus width	10.0	22.0	13.8	3.5	17
	9.0	18.0	14.1	2.4	19
	24.0	36.0	29.5	4.3	11
Number of C-shaped structures in the whole CSB	80.0	110.0	104.0	-	4
	88.0	151.0	122.6	19.5	19
	255.0	298.0	275.2	17.1	9
Contractile vacuole pores, number	4.0	12.0	10.0	-	4
	3.0	15.0	7.2	3.5	18
	6.0	15.0	12.0	-	3

shrinkage of about 12% due to the preparation procedure. Ventral side flattened with a conspicuous canal-like depression, 3-4 µm in width, extending from postoral area to subcaudal region of cell (Figs 1A, 2A); dorsal side evenly humped, dorso-ventrally flattened, width: thickness ratio ~ 2:1 (Fig. 1C). From ventral view, cell outline reniform; anterior end evenly rounded and projects slightly to left (Figs 1A, 2A). Right ciliary rows cross to anterior region of dorsal surface. Cross-striated band 3-4 µm wide, continuous, with anterior portion lying across the dorsal surface and posterior portion running along cell margin (Figs 1A, C, F; 2B, F, I). Endoplasm with several to many granules (2-3 µm across) and 1-4 large food vacuoles (~ 10 µm in diameter); often containing ingested particles of seaweed. Cytostome oval, inconspicuous *in vivo*, positioned half way between mid-body and anterior end of cell. Cyrtos extends posteriorly and rightward. 5-15 contractile vacuoles, each 2-4 µm in diameter, irregularly distributed beneath ventral cortex (Fig. 1A); contractile vacuole pores recognizable after protargol impregnation (Fig. 1E). Cilia about 8 µm long *in vivo*. Movement by gliding on substratum or swimming. Feeds mainly on seaweed debris and microalgae.

Somatic kineties densely arranged, ~ 66-72. When viewed dorsoventrally, somatic kineties mostly confined to region bounded by cross-striated band. Somatic kineties are grouped: 3-5 postoral kineties (PoK) that terminate anteriorly below cytostome; 34-40 right kineties (RK)

Table 2. Morphometrical comparison between *Chlamydodon obliquus* and related species.

Character	*C. obliquus*	*C. minutus*	*C. roseus*	*C. exocellatus**
Body length in μm	120-180	50-60	80-100	215-230
Body width in μm	50-117	-	-	100-132
Number of somatic kineties (total)	66-72	30-32	54-58	-
Number of right kineties	34-40	14-15	c. 30	-
Number of postoral kineties	4 (3-5)	4	c. 6	-
Number of left kineties	26-29	12-13	c. 21	-
Number of nematodesmal rods	11-16	16-18	8-9	15-16
Pigment spot**	absent	present	present	absent
Number of contractile vacuoles	5-15	3-4	5-6	5
Data source	Original	Dragesco (1965)	Dragesco (1966)	Ozaki and Yagiu (1941)

- data not available; * misidentified; ** pigment spot: a conglomeration of tiny endoplasmic granules, usually positioned at the anterior-left of cell.

extend anteriorly beyond the level of cytostome and bend to left; 26-29 left kineties (LK) extend subapically and form a hook-like suture (Su) with the right kineties (Figs 1B, E; 2D, E; Table 1). Usually the outermost 10-18 rows of the right kineties extend onto dorsal surface; the innermost 2 are interrupted by oral kineties. ~ 7 terminal fragments, each composed of 7-16 basal bodies, arranged in a row adjacent to CBS (Figs 1F, 2I); equatorial fragment difficult to discern. CSB comprising ~ 280 C-shaped structures (Figs 1E, F; 2F arrows). Heteromerous macronucleus rounded to oval, ~ 30 μm in diameter/length after protargol, positioned in body center. Micronucleus not detected. Some canal-like depressions with one extending anteriorly always present in dorsal side of cell after protargol impregnation (Figs 1F, 2J).

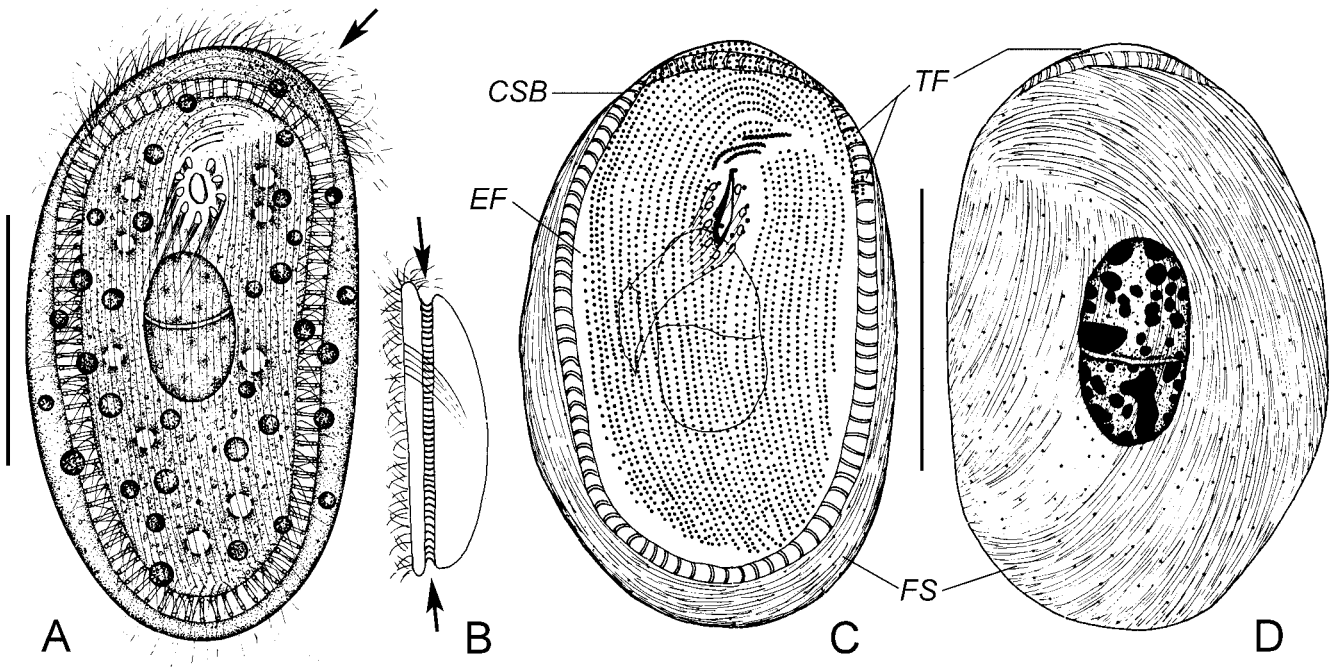
Three oral kineties typical of genus: the anterior preoral kinety overlap the two circumoral kineties, which are parallel and closely arranged; all three kineties obliquely oriented (Figs 1B, E; 2C). Cyrtos (Cy) relatively short (~ 30 μm in length), composed of ~ 13 nematodesmal rods; one circle of kinetosome-like dots always observed around cyrtos in protargol impregnated specimens (Figs 1B, D, 2D).

Remarks: *Chlamydodon obliquus* was originally described by Kahl (1931) with notes of characters observed *in vivo*. Subsequently, Chatton (1936) illustrated the lateral aspect of this species, showing the oblique appearance of its CBS. No redescriptions were published until Borror (1963) described an American population using the Chatton-Lwoff method (Figs 7H, I). Borror, however, did not report on the detailed structure of the somatic and oral kineties, the number and positions of the contractile vacuoles. Despite these, according to the original and the subsequent investigations, this species can be recognized by the combination of the following characters: (1) large size (130-180 μm in length); (2) continuous CSB; (3) ~10 contractile vacuoles; and (4) ~70 ciliary rows (Kahl 1931, Chatton 1936, Borror 1963).

Considering the size, general live appearance, number of kineties, continuous CBS positioned obliquely relative to the body axis when viewed from the side (Kahl 1931, Borror 1963), the Qingdao population resembles previous descriptions reasonably well except for having fewer nematodesmal rods (11-14 *vs.* 15-16) (Borror 1963). We suppose this difference is minor and hence establishment of a new species is not justified.

About 16 nominal species have been assigned to the genus *Chlamydodon*. With reference to the general morphology, the features of CSB, and the marine habitat, at least two species (*C. minutus* Dragesco, 1965 and *C. roseus* Dragesco, 1966) should be compared with *C. obliquus*: *C. minutus* can be distinguished from *C. obliquus* by its smaller size (50-60 *vs.* 120-180 μm in length), fewer somatic ciliary rows (30-32 *vs.* 66-72), and fewer contractile vacuoles (3-4 *vs.* 5-15) (Figs 7A, B; Table 2). Likewise, *C. roseus* differs from *C. obliquus* in its smaller body size (80-100 *vs.* 120-180 μm in length), presence of pigment spots in the anterior-left and posterior regions of the cell (*vs.* none in *C. obliquus*), and in having fewer somatic kineties (54-58 *vs.* 66-72) and nematodesmal rods (8-9 *vs.* 11-14) (Figs 7C, D; Table 2).

Alekperov and Asadullayeva (1997) misidentified a morphotype of *Chlamydodon obliquus* from the Caspian



Figs 3A-D. Morphology and infraciliature of *Chlamydodon mnemosyne* from life (A, B) and after protargol impregnation (C, D). **A** - ventral view of a typical individual, arrow indicates pigment spot located at the anterior-left of cell; **B** - lateral view showing the cross-striated band (arrows) around the perimeter of cell; **C, D** - ventral (C) and dorsal (D) views of infraciliature. CSB - cross-striated band; EF - equatorial fragment; FS - fine stripes; TF - terminal fragments. Scale bars 40 μm.

Sea (Russia) with only 38 somatic kineties (see Fig. 7E). The Russia form most resembles *C. rectus* Ozaki *et* Yagiu, 1941 in terms of numbers of kineties (*ca* 38 *vs. ca* 30) and nematodesmal rods (19 *vs*. 20-22), although it is somehow larger than the Japanese population of *C. rectus* (*vs*. 70-84 × 42-49 μm). Possibly, the Russia form represents a large-sized population of *C. rectus*.

Another nominal species, *Chlamydodon exocellatus* Ozaki *et* Yagiu, 1941, the infraciliature of which remains unknown, was isolated form Japanese coastal waters (Ozaki and Yagiu 1941). Besides the similarities in CSB, it also resembles *C. obliquus* in body shape, absence of pigment spots and numbers of contractile vacuoles and nematodesmal rods (Fig. 7G; Table 2). The only difference is its larger size (215-230 *vs.* 120-180 μm) which, however, could be variable among populations in different food conditions. Hence, we regard *C. exocellatus* as a junior synonym of *C. obliquus*.

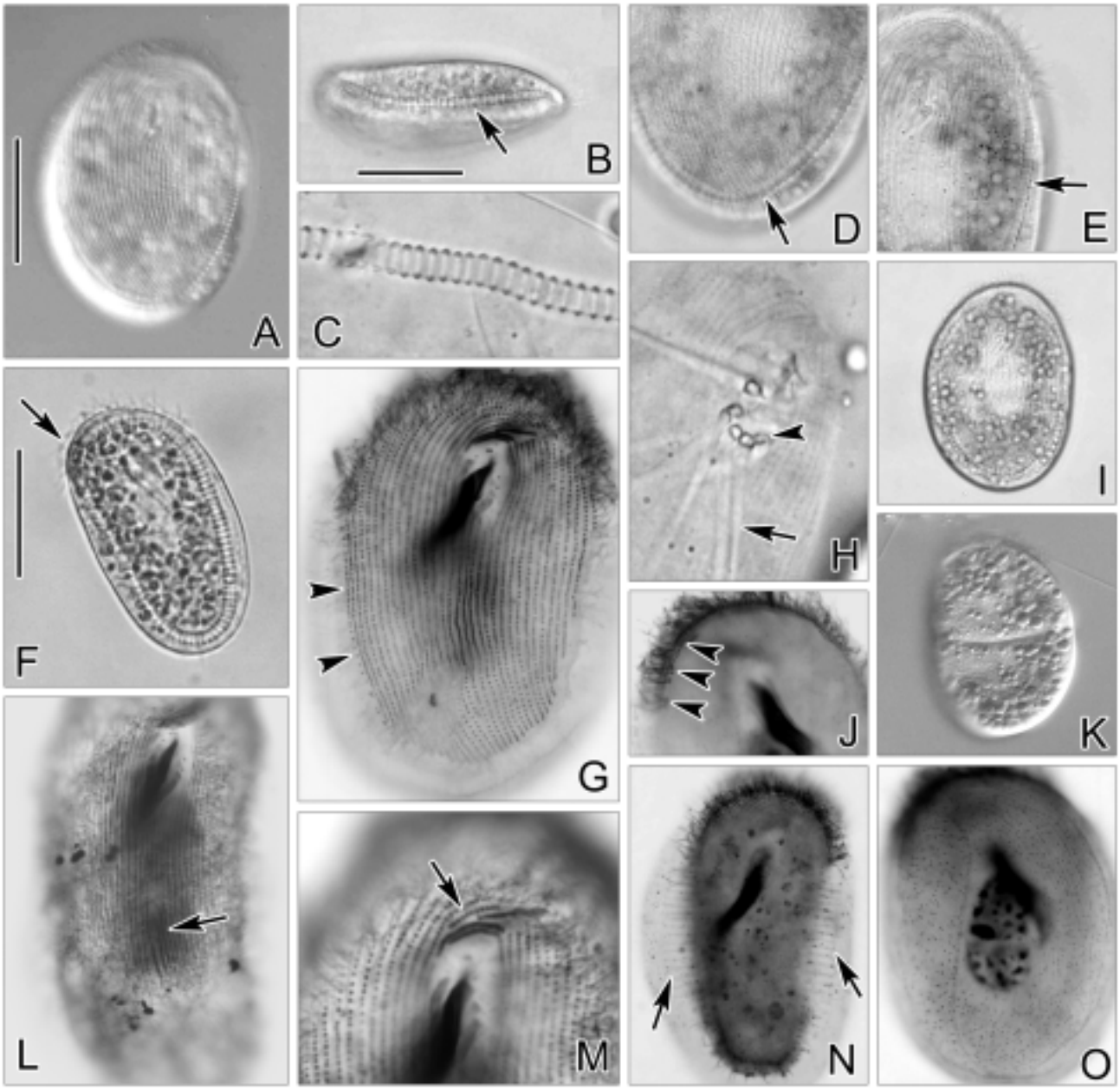
Chlamydodon mnemosyne Ehrenberg, 1835 (Figs 3, 4; Tables 1, 3)

Syn: *Chlamydodon pedarius* Kaneda, 1953
Chlamydodon apsheronica Aliev, 1987

Based on previous and the present studies, an improved diagnosis is suggested:

Improved diagnosis: Size 30-150 × 15-70 μm *in vivo*, body shape oval in outline; cross-striated band continuous around cell margin; 9-20 left, 11-18 right, and 3-4 postoral kineties; ~ 3 terminal fragments; 8-15 nematodesmal rods; macronucleus about 25 × 14 μm in size; marine habitat.

Description: Size usually 70 × 40 μm *in vivo*, as calculated from some measurements of live specimens and values shown in Table 1, assuming a shrinkage of about 7% due to the preparation procedure. From ventral view, cell oval in outline (Figs 3A; 4A, I). Ventral side flattened and dorsal side humped, dorsoventrally flattened, width: thickness ratio ~ 2:1 (Figs 3B, 4B). Both ends evenly rounded; left margin straight, right margin convex. Cross-striated band 2-3 μm wide, completely encircles cell perimeter (Figs 3A, B; 4B-F); a loop of the cross-striated band is positioned equatorially in the early stage of cell division (Fig. 4K). Cytoplasm colorless, with irregularly distributed food granules (3-4 μm across) and yellowish particles (~ 0.2 μm in diameter) which are densely located in the extreme anterior-left of cell to form the pigment spot; note that pigment spot inconspicuous and easily overlooked. Cytostome oval, ventrally located in anterior 1/5 of cell. Nematodesmal rods straight, each about 24 μm long and tipped with one tooth



Figs 4A-O. Photomicrographs of *Chlamydodon mnemosyne* from life (A-F, H, I, K) and after protargol impregnation (G, J, L-O). **A** - ventral view of a typical individual; **B** - lateral view, arrow indicates cross-striated band; **C** - part of cross-striated band at magnification; **D, E** - ventral views, focusing on sections of cross-striated band (arrows) in posterior (D) and anterior-left (E) portions of cell; **F** - dorsal view, arrow marks pigment spot; **G** - ventral view of infraciliature; arrowheads indicate the equatorial fragment; **H** - deformed cyrtos in a squeezed individual, showing the tooth (arrowhead) and rod (arrow); **I** - ventral view of a individual containing many food vacuoles; **J** - to note the terminal fragments (arrowheads) positioned at the anterior-left margin of dorsal surface; **K** - dorsal view of a individual in binary fission; **L** - to note the multiplying of basal bodies; **M** - anterior portion of infraciliature on ventral side, showing that the innermost right kinety (arrow) is interrupted by oral kineties; **N** - to show cross-striated band (arrows) encircling ciliated ventral surface; **O** - dorsal side showing fine stripes. Scale bars 40 µm.

(Fig. 2H). ~ 5-12 contractile vacuoles, irregularly distributed underneath ventral cortex (Fig. 3A). Cilia about 8 µm long *in vivo*. Feed on cyanobacteria and particles of seaweed.

Infraciliature as shown in Figs 3C, D, 4G, L-O. A total of 29-35 somatic kineties, 14-18 rows on right, 11-13 rows on left, and 4 postoral rows. In common with its congeners, three postoral kineties are involved in oral

Table 3. Comparison among populations of *Chlamydodon mnemosyne*, for which infraciliature is described.

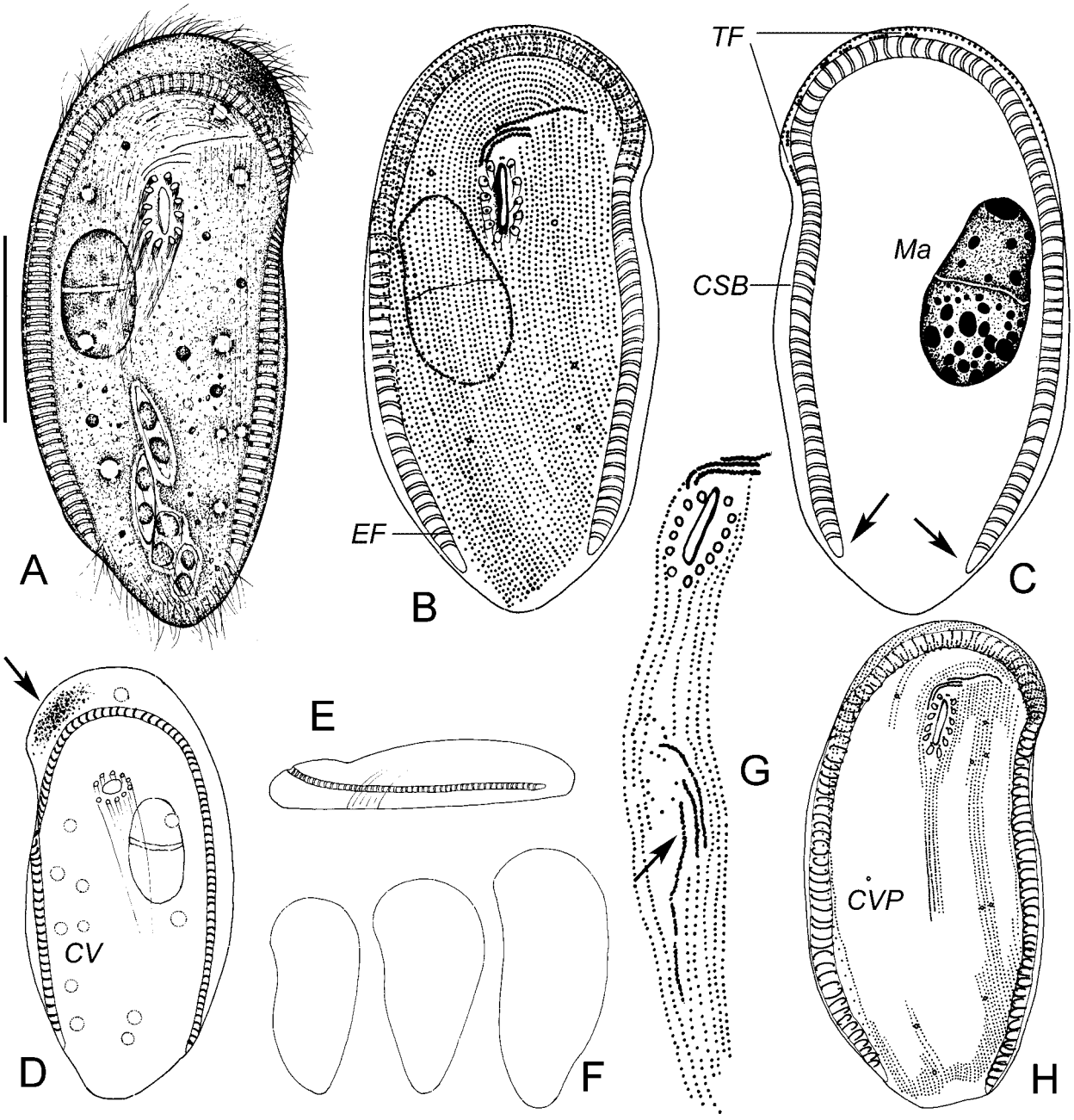
Character	*C. mnemosyne* Qingdao pop.	*C. mnemosyne* German pop.	*C. mnemosyne* African pop.	*C. mnemosyne* French pop.	*C. mnemosyne* Russian pop.	*C. pedarius** Japanese pop.	*C. apsheronica** Russian pop.
Body length in µm	50-100	40-100	30-150	50-110	60-110	110-138	60-70
Body width in µm	30-50	23-65	-	-	-	50-70	45-55
Number of right kineties	14-18	12-15	11-18	11-16	16	16-18	17
Number of postoral kineties	4	4	4	4	-	4	3
Number of left kineties	11-13	9-12	11-20	11-14	14	16-18	13
Number of somatic kineties (total)	29-35	25-31	26-40	26-34	36	35-40	30-35
Number of nematodesmal rods	8-11	9-13	10	-	10	11-13	*ca* 13
Number of contractile vacuoles	5-12	5-11	*ca* 5	*ca* 15	-	5-12	20-26
Data source	Original	Kurth and Bardele (2001); Bardele and Kurth (2001)	Dragesco and Dragesco-Kernéis (1986)	Fauré-Fremiet (1950)	Alekperov and Asadullayeva (1997)	Kaneda (1953, 1960a, b)	Aliev (1987)

* misidentified; - data not available.

Table 4. Comparison among populations of *Chlamydodon triquetrus.*

Character	*C. triquetrus* Qingdao pop.	*C. triquetrus* Brazilian pop.	*C. triquetrus* Russian pop.	*C. triquetrus* French pop.	*C. triquetrus* African pop.	*C. kasymovi* * Russian pop.
Body length in µm	60-100	70-100	120-140	50-90	50-140	220-260
Body width in µm	26-45	28-40	45-50	-	-	85-95
Number of somatic kineties (total)	36-48	40	48-50	38-40	30-50	40-45
Number of right kineties	16-24	-	26-28	20-21	-	c. 20
Number of postoral kineties	4	-	5	5	5	4
Number of left kineties	15-21	-	16-18	14-19	-	*ca* 19
Number of nematodesmal rods	9-14	15	13-15	14-16	*ca* 14	13-15
Data source	Original	Katter (1970)	Agamaliev (1978)	Dragesco (1963)	Dragesco and Dragesco-Kernéis (1986)	Aliev (1987)

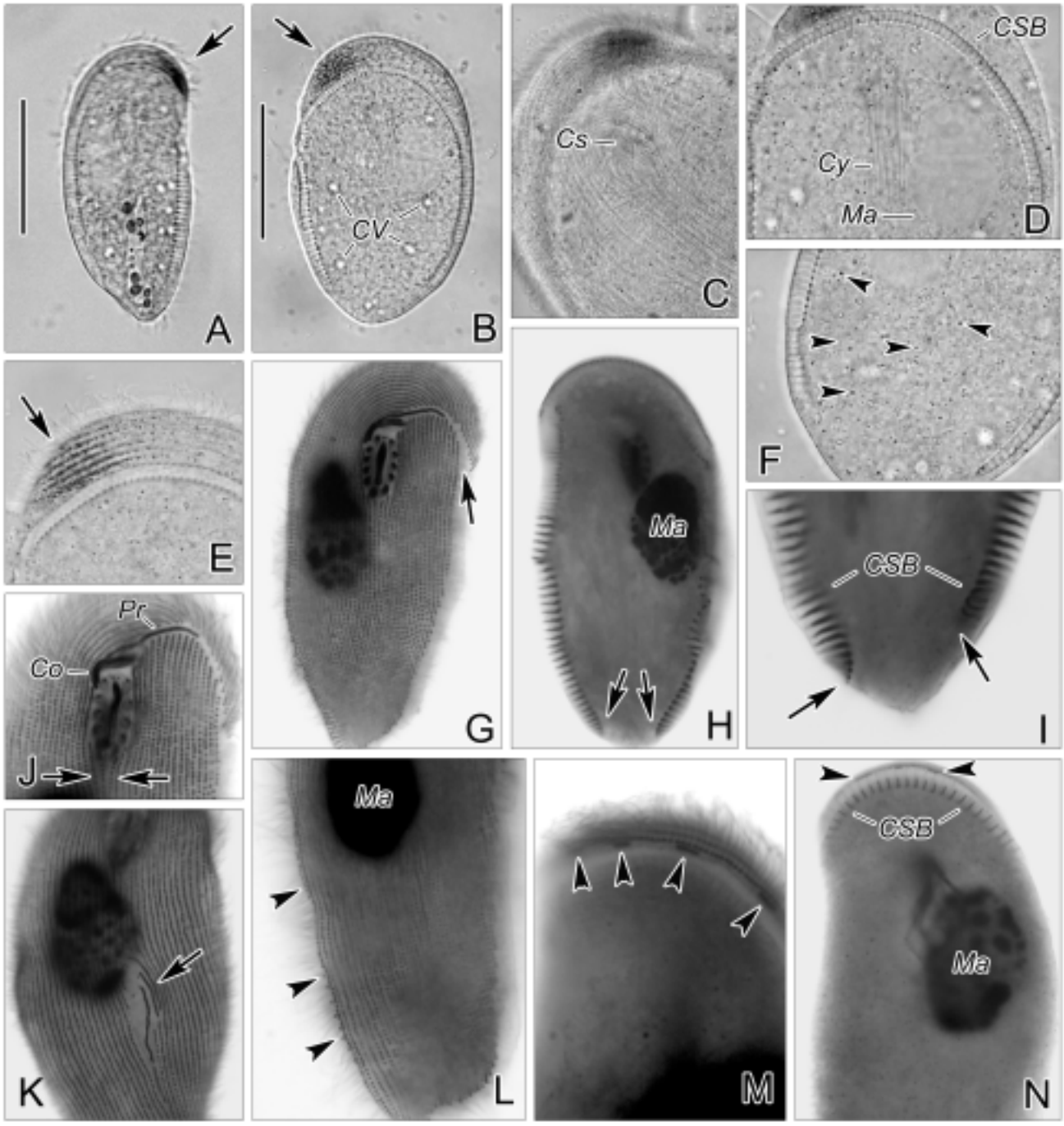
* misidentified; - data not available.



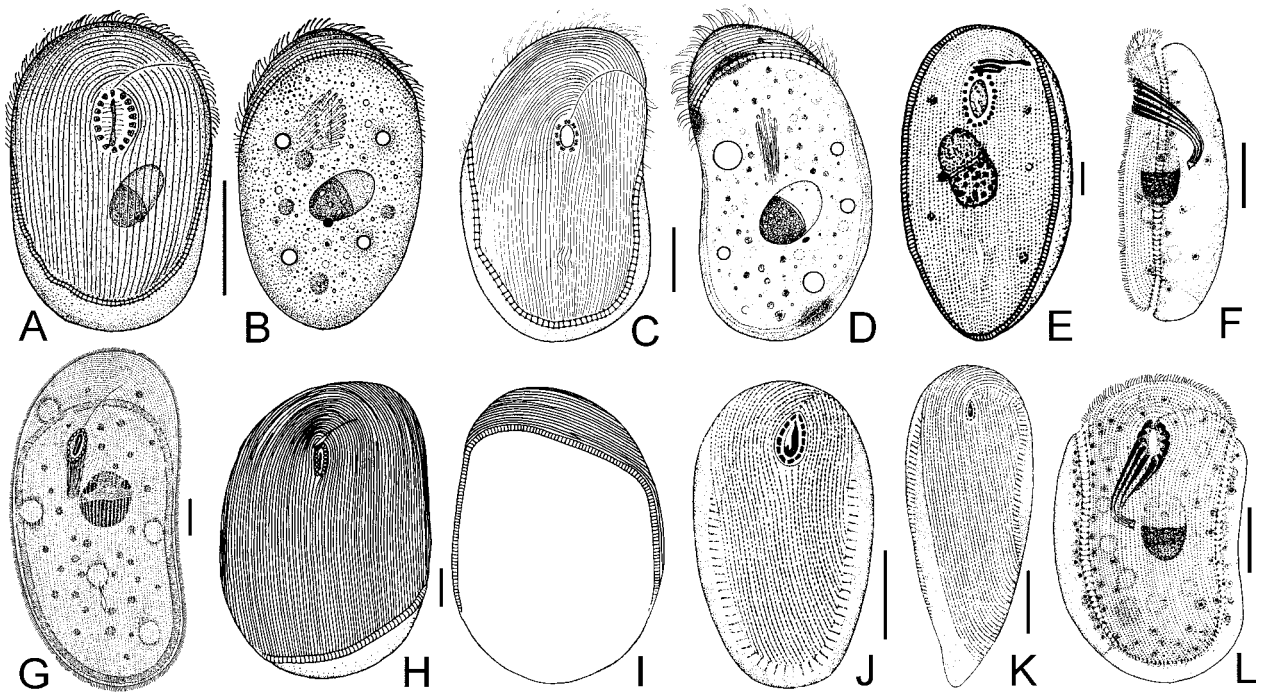
Figs 5A-H. Morphology and infraciliature of *Chlamydodon triquetrus* from life (A, D-F) and after protargol impregnation (B, C, G, H). **A** - ventral view of a typical individual; **B, C, H** - ventral (B, H) and dorsal (C) views of infraciliature, note that the cross-striated band is posteriorly discontinued (arrows); **D** - dorsal view showing pigment spot (arrow); **E** - lateral view; **F** - to show body shapes of different specimens; **G** - showing the multiplying of basal bodies (arrow) in the mid-body field of an individual at an early stage of morphogenesis. CSB - cross-striated band; CVP - contractile vacuole pores; EF - equatorial fragment; Ma - macronucleus; TF - terminal fragments. Scale bars 40 μm.

formation during morphogenesis (Fig. 4L). Anterior portion of the innermost row of right kineties interrupted by oral kineties (Fig. 4M). Equatorial fragment (EF, Fig. 3C; arrowheads in Fig. 4G) composed of about 25 loosely spaced basal bodies. Usually 3 terminal fragments (TF, Figs 3D, 4J), each comprising ~ 3 densely spaced basal bodies. Macronucleus ellipsoidal, heteromerous, ~ 25 × 14 μm after protargol impregnation. Micronucleus not detected.

Three oral kineties almost equal in length: two circumoral kineties (outer and inner) anterior of cytostome and one preoral kinety left of these. Cyrtos obliquely



Figs 6A-N. Photomicrographs of *Chlamydodon triquetrus* from life (A-F) and after protargol impregnation (G-N). **A** - ventral view of a slender specimens, arrows indicate pigment spot; **B** - dorsal view of a well-fed individual, arrow indicates pigment spot; **C** - anterior portion of ventral surface; **D** - focusing on anterior portion of dorsal surface; **E** - pigment spot at high magnification; **F** - showing numerous yellowish granules in endoplasm (arrowheads); **G** - ventral view of infraciliature; arrow indicates the anterior suture; **H, I** - dorsal view, arrows marks two ends of interrupted cross-striated band; **J** - oral field, arrows indicate postoral kineties; **K** - ventral view of a individual in morphogenesis, arrow marks the area where basal bodies of three kineties multiply; **L** - posterior portion of cell, arrowheads indicate sparsely-spaced basal bodies in equatorial fragment; **M, N** - dorsal view of anterior portions, arrowheads marks terminal fragments positioned near the margin of the ciliated field. Co - circumoral kineties; Cs - cytostome; CSB - cross-striated band; CV - contractile vacuole; Cy - cyrtos; Ma - macronucleus; Pr - preoral kinety. Scale bars 40 µm.



Figs 7A-L. Ventral (A, C, E, G, H, J-L), dorsal (B, D, I) and side (F) views of *Chlamydodon* species. **A, B** - *C. minutus* (from Dragesco 1965); **C**, **D** - *C. roseus* (from Dragesco 1966); **E** - *C. obliquus* sensu Alekperov and Asadullayeva, 1997; **F, L** - *C. pedarius* sensu Kaneda, 1953; **G** - *C. exocellatus* sensu Ozaki and Yagiu, 1941; **H, I** - *C. obliquus* (from Borror 1963); **J** - *C. apsheronica* sensu Aliev, 1987; **K** - *C. kasymovi* (from Aliev 1987). Scale bars 20 μm.

oriented, composed of 8-11 nematodesmal rods and associated fibrous membranes, extends posteriorly to about 40% of cell length (Fig. 3C).

Remarks: The type species *Chlamydodon mnemosyne* was originally described by Ehrenberg (1835) from the German Baltic Sea. The first description of its infraciliature was by Fauré-Fremiet (1950). Subsequent redescriptions based on live and silver impregnated cells indicate different populations possess overlapped/variable characters: cell size, numbers of somatic kineties, contractile vacuoles, and nematodesmal rods (Table 3, Dragesco 1960, Dragesco and Dragesco-Kernéis 1986, Alekperov and Asadullayeva 1997, Kurth and Bardele 2001). The Qingdao populations is most similar to those described by Fauré-Fremiet (1950) and Kurth and Bardele (2001) in terms of morphology and ciliature patterns (Table 3).

Chlamydodon pedarius Kaneda, 1953 isolated from Japanese coastal waters was later redescribed using living observations and silver impregnation (Kaneda 1953, 1960a, b). However, *C. pedarius* does not differ from *C. mnemosyne* in any of the main diagnostic features: cell size, configuration of CSB, numbers of somatic kineties, nematodesmal rods, and contractile vacuoles (Figs 7F, L; Table 3). Therefore, *C. pedarius* we regarded it as a junior synonym of *C. mnemosyne*.

Another nominal species *Chlamydodon apsheronica* Aliev, 1987, isolated from hypersaline water reservoirs, Russia (Aliev 1987), is similar to *C. mnemosyne* in cell size, configuration of the CSB, numbers of somatic kineties, and nematodesmal rods (Fig. 7J; Table 3). Considering that some ciliates may exhibit variable living features in waters with different salinities (Esteban and Finlay 2003), we suppose that the only difference of the number of contractile vacuoles (20-26 *vs.* 5-15) is not sufficient to circumscribe a new species, but rather represents a population variation. *Chlamydodon apsheronica* is therefore conspecific with, and a junior synonym of, *C. mnemosyne.*

Chlamydodon triquetrus (Müller, 1786) (Figs 5, 6; Tables 1, 4)

Syn: *Chlamydodon kasymovi* Aliev, 1987

Since there is no definition following examination using modern methods for *Chlamydodon triquetrus*,

we provide an improved diagnosis based on the data obtained.

Improved diagnosis: Yellowish *Chlamydodon*, size 50-140 × 25-60 μm *in vivo*, body elongate ellipsoid to triangle in outline; CSB posteriorly interrupted; one yellow pigment spot in anterior-left of cell; 14-21 left, 16-28 right, and 4-5 postoral kineties; ~ 6 terminal fragments; 9-16 nematodesmal rods; with 5-12 contractile vacuoles; macronucleus ~ 24 × 14 μm; marine habitat.

Description: Size 100 × 50 μm in vivo, as calculated from some measurements of live specimens and values shown in Table 1, assuming a shrinkage of about 15% due to the preparation procedure. Cell shape variable, usually ellipsoid to triangular in outline, with anterior protrusion to left. Anterior end evenly rounded, posteriorly tapering; left margin somewhat sigmoid, right margin convex (Figs 5A, D, F; 6A). Ventral side flattened, dorsal side humped, dorso-ventrally flattened, width: thickness ratio ~ 2:1 (Fig. 5E). Cross-striated band (3 μm wide) generally inverted U-shaped, i.e. encircles the cell perimeter but with a conspicuous gap in the subcaudal region (Figs 5A-D, H; 6A, B, D, F, H, I). Endoplasm colourless, with tiny yellow granules (0.2-0.3 μm across) that renders cell yellowish at lower magnifications (40×); granules irregularly distributed in most parts of the cell (Fig. 6F) except in anterior left region where they are relatively densely spaced forming an amorphous "pigment spot" (area ~ 14 × 7 μm; n = 30; Figs 5A, D, 6E); food vacuoles 4-10 μm across, usually filled with ingested diatoms. Cytostome oval, located at anterior 1/3 of ventral surface. Cyrtos composed of 9-14 nematodesmal rods. ~ 5-12 contractile vacuoles (CV), irregularly distributed underneath ventral cortex (Figs 5A, D; 6B); 3-15 contractile vacuole pores (CVP) recognizable after protargol impregnation (Figs 5B, H). Macronucleus oval, positioned slightly right of body centre. Cilia about 5 μm long *in vivo*. Gliding on substratum or swimming; when swimming, rotates around body axis. Feeds on seaweed debris and diatoms.

Infraciliature as shown in Figs 5B, C, 6J-N. In total 30-50 somatic kineties with 16-28, 14-21 and 4 rows in right, left and postoral fields, respectively (Table 1). Right kinety not interrupted by oral kineties. Three of the postoral kineties are involved in the formation of the oral structure during morphogenesis (Figs 5G, 6K). Left kineties progressively reduced at posterior ends. Equatorial fragment long (EF in Fig. 5B; arrowheads in Fig. 6L) composed of about 40 loosely spaced basal bodies. Usually 6 terminal fragments (TF) almost equal in length, each comprising ~ 5 basal bodies (Figs 5C; 6M, N). Macronucleus ellipsoidal, heteromerous, size ~24 × 14 μm after protargol impregnation. Micronucleus not detected.

Oral structure typical of genus: circumoral kineties (Co) almost equal in length, preoral kinety (Pr) relatively longer, extending leftwards to about mid-point of suture line. A circle of non-ciliated kinetosome always present with nematodesmal rods (Fig. 6J).

Remarks: The Qingdao population corresponds well with previous descriptions of *Chlamydodon triquetrus* in terms of living morphology (body size and shape, presence of yellowish pigment spot, posterior interruption in cross-striated band, number of contractile vacuoles, etc.) and features revealed by silver impregnation (number and pattern of somatic kineties, number of nematodesmal rods, etc.), thus the identification is undoubted (Dragesco 1963, Katter 1970, Hartwig 1973, Agamaliev 1978, Dragesco and Dragesco-Kerneis 1986; Table 4).

Chlamydodon kasymovi Aliev, 1987 is similar to *C. triquetrus* in every respect except it has a larger body size (220-260 *vs.* 50-140 μm; Aliev 1987) (Fig. 7K; Table 4). Considering that cell size may considerably vary between populations of *Chlamydodon* (e.g. *C. mnemosyne* and *C. obliquus*, this paper), we provisionally regard *C. kasymovi* as an extreme form of *C. triquetrus*.

Based on both morphology and infraciliature, a key to the species that have been defined by silver impregnation is supplied:

Key to *Chlamydodon* species whose infraciliature is known

1 CSB without interruption.....2
1' CSB posteriorly interrupted.....*C. triquetrus*
2 Sixteen to 18 nematodesmal rods.....*C. minutus*
2' Less than 16 nematodesmal rods.....3
3 More than 50 somatic kineties.....4
3' Less than 50 somatic kineties.....*C. mnemosyne*
4 More than 60 somatic kineties.....*C. obliquus*
4' Less than 60 somatic kineties.....*C. roseus*

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The Ultrastructural Study of *Nosema artemiae* (Codreanu, 1957) (Microsporidia: Nosematidae)

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Summary. Ultrastructure of microsporidium *Nosema artemiae* from the solar saltern located on the Southern Ukraine was studied. The parasite infects the musculature of brine shrimp *Artemia salina* (L.). All developmental stages of the parasite are diplokaryotic. Merogony occurs through binary division of the tetranucleate merogonial plasmodium. Sporogony is disporoblastic. Unfixed spores are broadly oval measuring 3.0 ± 0.2 (2.7-3.5) × 4.9 ± 0.3 (4.5-5.4) µm. The layered exospore includes a coarse-grained basal layer, and an external coat resembling a double membrane. The polar filament is slightly anisofilar, making 13-16 coils (9-12 + 4). The polaroplast is composed of narrow anterior lamellae and posterior wide lamellae, tapered at the peripheral area. The obtained new data permits the elucidate of features which were incompletely characterized in the previous reports.

Key words: *Artemia salina*, Crustacea, Microsporidia, *Nosema artemiae*, ultrastructure.

INTRODUCTION

Six species of Microsporidia infecting *Artemia* spp. were recorded (Table 1). The descriptions were based on material collected in Romania (Codreanu 1957; Codreanu-Balcescu and Codreanu 1978, 1980), Spain (Martinez *et al.*1989, 1993, 1994) and Brazil (Martinez *et al.* 1992). The ultrastructural data of five of them were obtained, but detailed morphological descriptions were not given. Two species: *Vavraia anostraca* Martinez *et al.*, 1992 and *Endoreticulatus durforti* Martinez, Vivares *et* Bouix, 1993 were completely studied. The ultrastructural data of *Pleistophora myotropha* Codreanu, 1957 include some information concerning exospore construction and the merogony of the parasite. The ultrastructure of *Gurleya dispersa* Codreanu, 1957 is unknown. Three species with binary divided merogonial stages were noticed in *A. salina*. They are: *Unikaryon exiguum* (Codreanu, 1957), *E. durforti* and *Nosema artemiae* (Codreanu, 1957). The ultrastructure of *U. exiguum* was briefly observed, and only few data relating to sporogony and numbers of polar filament coils were presented (Codreanu-Balcescu and Codreanu 1978). *E. durforti* was completely studied by Martinez *et al.* (1993). *N. artemiae* was moderately studied by

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Codreanu-Balcescu and Codreanu (1978) in Romania, and more completely described by Martinez *et al.* (1994) in Spain, but the descriptions of the earliest stages of merogony were not presented.

The microsporidium identified as *N. artemiae* was newly found in South Ukraine. The peculiarities of the ultrastructure of the parasite are completely described, and the new data relating to early merogony and the spore construction are obtained. The systematic position of this microsporidium is discussed.

MATERIALS AND METHODS

The microsporidium was isolated from a brine shrimp, inhabiting the solar saltern located on the territory of Chernomorski Biosphere Reserve (46°35′ N, 32°16′ E). Infected tissues were prepared for light and ultrastructural study. The live spores and Giemsa stained slides were examined under a light microscope (phase contrast microscopy was used for observations of the live spores). For measurements, the software „Analysis Pro 2.11" in combination with Olympus BX50F4 microscope were used. One hundred spores were measured on every slide. For transmission electron microscopy, the infected tissues were fixed in a 2.5 % (v/v) glutaraldehyde in a 0.2 M sodium cacodylate buffer (pH 7.2) for 1-3 days. After washing and postfixation in 2.0 % (w/v) osmium tetroxide in cacodylate buffer for 1 h at 4°C, the pieces were dehydrated and embedded in Epon-Araldite as reported in previous papers (Ovcharenko and Wita 2001a, b). Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a JEM 100B electron microscope.

RESULTS

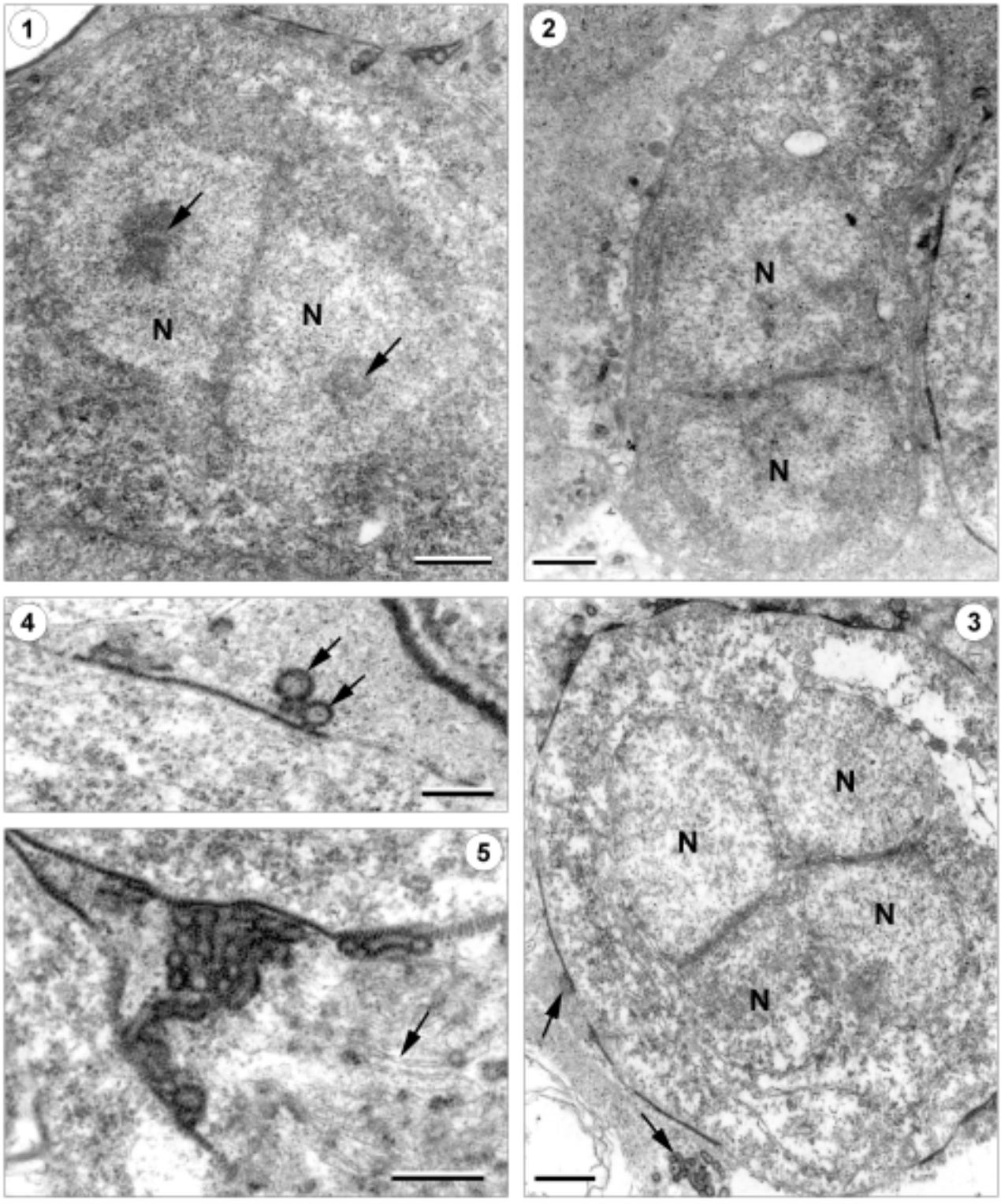
One of the 112 examined specimens of *A. salina* was found to be infected. The primary site of infections was the muscle tissue. Heavily infected muscles were hypertrophied and easily disrupted. All stages of the parasite were in direct contact with the host cell cytoplasm.

The earliest observed stages of investigated microsporidium were rounded and elongated meronts approximately 2.6 × 4.4 µm in size (Figs 1, 2). They possessed two nuclei in diplokaryotic arrangement. The diameter of each nucleus measured 1.5 µm. The cytoplasm of the meronts was homogenously granular. Electron-dense nucleoli were registered in the nucleoplasm of early merogonial stages (Fig. 1). Merogony occurs by binary fission of tetranucleate rounded stages about 4.6 µm in diameter (Fig. 3).

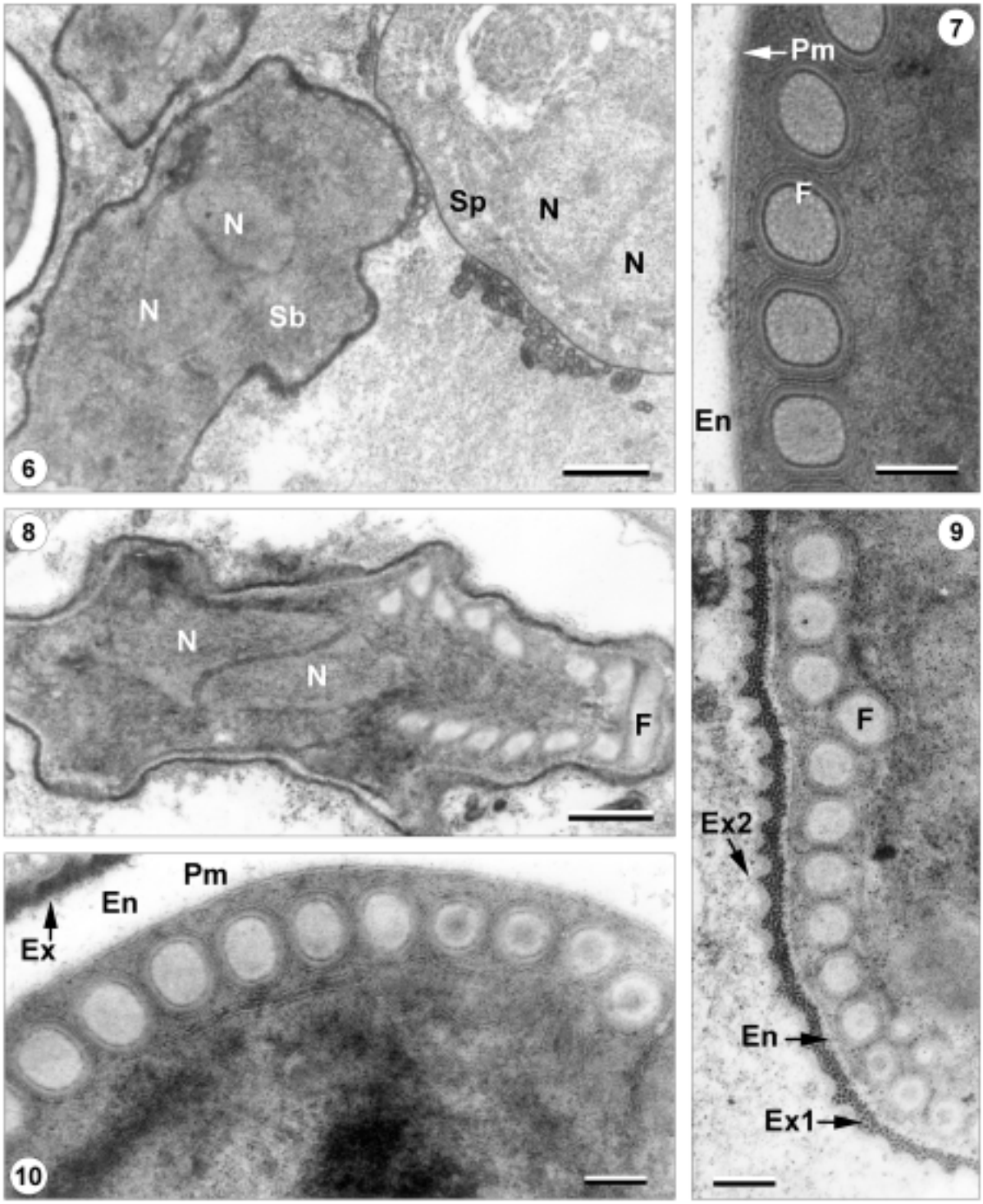
The beginning of the sporogony was marked by the structural transformation of the envelope surrounding the late meronts. The thickening of the late meronts surface was accompanied by appearance of electron-dense granules which was connected with vesiculo-tubular secrets, adhering to the plasma membrane (Figs 3-5). The vesiculo-tubular structures occur between the developing parasite cells. It can be present as clusters of irregularly arranged vesicles to strands of vesicles, covered with electron-dense granules (Fig. 5). Finely the sporont surface is covered with electron-dense granular material about 40 nm thick, connecting with a tubular caverns and ridges (Fig. 6). Few membrane profiles and vacuoles were visible inside of the cytoplasm of the sporont. Diplokaryotic sporonts were rounded to broadly oval. Each sporont gives rise by binary fission to two diplokaryotic sporoblasts. The rough episporontal coat developed into the exospore of the future spore wall (Figs 8, 9).

The elongated sporoblasts were diplokaryotic, measuring about 2.1 × 3.9 µm. Their cytoplasm contained cisternae of the endoplasmic reticulum, free ribosomes and the polar filament primordia (Figs 6, 8). The wall of the future spore consists of electron-transparent endospore about 30 nm thick and double-layered exospore (Figs 8, 9). The electron-dense internal layer of the exospore contains regular granular protrusions which may represent in tangential view as a circular rows (Fig. 9). The outer coat of the exospore resembled double membrane (Fig. 9). During spore maturation the endospore thickness increased, except the apical pole (Figs 12, 14).

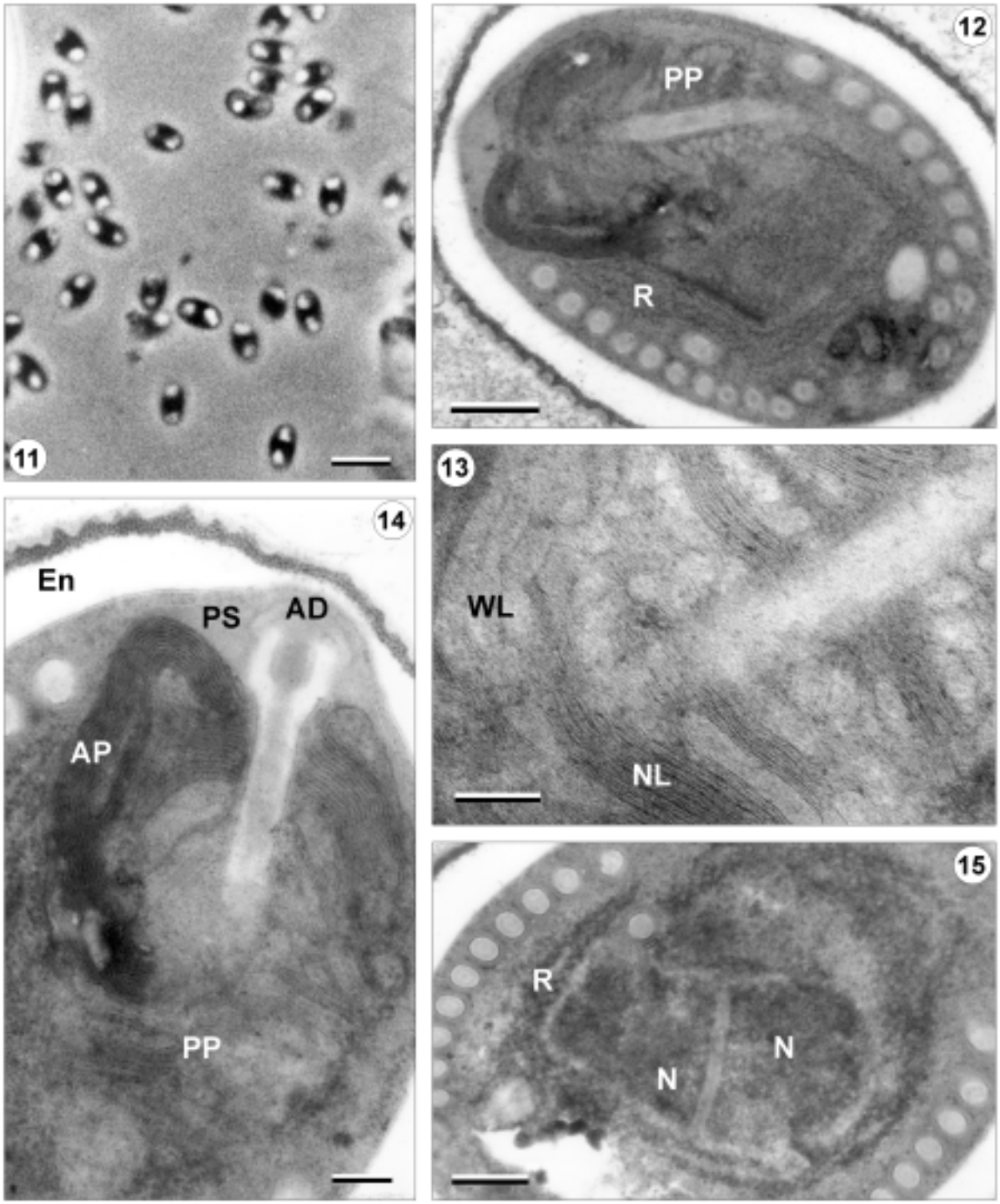
Unfixed spores were broadly oval (Fig. 11) measuring 3.0 ± 0.2 (2.7-3.5) × 4.9 ± 0.3 (4.5-5.4) µm. The Giemsa stained spores were 2.3 ± 0.3 (2.0-3.2) × 3.5 ± 0.2 (3.1-4.6) µm in size. The spore envelope was composed of the 150-200 nm thick electron-transparent endospore, and the electron-dense exospore 75-90 nm wide (Figs 7, 10, 12, 14). The endospore was greatly thinned over the anchoring disc (Fig. 14). The exospore was layered with a coarse-grained basal layer 50-60 nm wide, and a thin external coat resembling a double membrane (Figs. 12, 14). The polar filament was slightly anisofilar, making 13-16 coils of 130-140 nm in diameter. The last four coils were slightly narrower, 120-125 nm wide (Fig. 10). The transversal sectioned polar filament exposed classic ultrastructural organization with 19-20 electron-transparent longitudinal threads (Fig. 7). The polaroplast was composed of two lamellar parts. The anterior polaroplast contained densely packed narrow lamellae (Figs 12, 14). The posterior polaroplast had wide lamellae in central



Figs 1-5. Merogony and early sporogony of *Nosema artemiae*. **1 -** rounded early merogonial stage with the nucleoli (arrows) and diplokaryotic nuclei; **2 -** elongated merogonial stage; **3 -** meront/sporont transitional stage with two diplokarya. Patches of electron-dense granules (arrows) appear outside the membrane of parasite cell; **4 -** structural transformation of the parasite cell surface. Electron-dense conglomerates located on the cisternae (arrows), appearing in the host cytoplasm adjacent to parasite cells forms an outer layer; **5 -** modification of the host cytoplasm adjoining to sporogonial stages of the parasite. Electron-dense secretions on the surface of vesiculo-tubular formations are visible. The host endoplasmic reticulum is arrowed. N - diplokaryotic nuclei. Scale bars 0.5 µm (1-3); 0.2 µm (4, 5).



Figs 6-10. Electron micrographs of the late sporogonial stages and polar filament structure of *Nosema artemiae*. **6 -** sporont and sporoblast with diplokaryotic nuclei. The late sporogonial stages are bounded by a rough coat which develops into the exospore of the future spore; **7 -** part of a longitudinal section of the spore. Endospore, plasma membrane and ultrastructure of transversal sectioned polar filament are visible; **8 -** the late sporoblast with diplokaryon and developed polar filament; **9 -** electron micrographs of a part of longitudinal section of immature spore. The exospore contains basal layer with a regular protrusions and the double membrane like external coat. The narrow electron-transparent endospore and sixteen transversely sectioned polar filament coils are also visible; **10 -** electron micrographs of a part of longitudinal sectioned mature spore. The spore wall and one row of polar filament coils are visible. The diameter of polar filament of four last coils is narrower. The spore wall consists from the plasma membrane, endospore and two layered exospore. En - endospore, Ex - exospore, Ex1 - exospore with a regular protrusions, Ex2 - exospore with double membrane like external coat, F - polar filament, N - diplokaryotic nuclei, Pm - plasma membrane, Sb - sporoblast, Sp - sporonts. Scale bars 1.2 µm (6, 8); 0.1 µm (7, 9, 10).



Figs 11-15. Light and electron micrographs of the spores of *Nosema artemiae*. **11 -** phase contrast image of the live spores; **12 -** diagonal section of the middle part of the mature spore. The structure of the spore wall, the construction of the posterior polaroplast and the rows of ribosomes are observable; **13 -** electron micrograph of the peripheral polaroplast construction. The polaroplast cisternae are formed for the narrow and wide lamellae; **14** - ultrastructure of the apical part of the spore. The construction of the spore wall, anchoring disc, polar sac, anterior and posterior parts of polaroplast are shown. The endospore is hardly thinner over the anchoring disk. The anterior polaroplast was constructed of densely packed narrow lamellae. The posterior polaroplast has wide lamellae in the central parts, transforming into narrow lamellae and tubules where it has been sectioned peripherally; **15 -** diagonal section of the posterior part of the spore. The cytoplasm of the spores contains numerous ribosomes forming chains around the nuclei. AD - anchoring disc, AP - anterior polaroplast, En - endospore, N - nuclei, NL - narrow lamellae, PP - posterior polaroplast, PS - polar sac, R - ribosomes, WL - wide lamellae. Scale bars 5 µm (11); 0.5 µm (12); 0.2 µm (15); 0.1 µm (13, 14).

Table 1. Comparative data of Microsporidia described from *Artemia* spp.

Prominent features	*Nosema artemiae* (Codreanu, 1957)	*Endoreticulatus durforti* Martinez Vivares *et* Bouix, 1993	*Gurleya dispersa* Codreanu, 1957	*Pleistophora myotropha* (Codreanu, 1957)	*Unikaryon exiguum* (Codreanu, 1957)	*Vavraia anostraca* Martinez *et al.*, 1992
1	2	3	4	5	6	7
Distribution	Romania, Spain, Brasil, Ukraine•	Spain	Romania	Romania	Romania	Brasil
Site of infection	Musculature, haemocytes, hypoderm	Intestinal epithelium	Haemocel	Musculature	Intestinal epithelium	Musculature, Intestinal epithelium, haemocel
Infected cells	Hypertrophyed. Giant nuclei are produced. Cytoplasmic organelles are destructed•	Hypertrophyed	Destructed	No data	Not hypertrophyed	Destructed
Parasitophorous vesicle	Absent	Appears during merogony	No data	No data	Appears during sporogony	Absent
Meront size (µm)	3.0-6.0 (Martinez *et al.* 1994); 2.6 × 4.4•	2 in diameter	2.3-3.0	No data	2.0-4.0	No data
Meront nuclei	Diplokaryotic	Isolated	Punctiform	No data	Isolated	Isolated
Merogonial plasmodium	1-2 nucleate (Codreanu 1957), tetranucleate•	2-4 nucleate	No data	No data	No data	2-11 nucleate, with folded coat
Mode of division	Binary fission•	Binary fission, rosette-like fragmentation	No data	No data	Binary fission	Plasmotomy
Sporont shape	Elongate, thick walled, with protuberances	Round	Round	Lobulated (early)	No data	Round
Sporont size (µm)	2.1 × 3.9•	Up to 19	No data	8.6-11.6 µm to 16.0-20.3 µm	No data	No data
Sporont nuclei	Diplokaryotic	Isolated	No data	No data	No data	Isolated
Mode of division	Binary fission	Rosette-like fragmentation	No data	No data	Binary fission	Stepwise and rosette-like fragmentation
Sporophorous vesicle	Absent	Subpersistent	Covered by fine membrane	No data	No data	Merontogenetic
Number of sporoblasts	-	8-128	4, 2 (rare)	4-32, mostly 8, 16	No data	16-128, mostly 32, 64, exceptionally 8
Inclusions	-	No data	No data	Fine granules and tubules	No data	Tubules
Size of sporophorous vesicle (µm)	-	Up to 19	7.5-9.4	No data	No data	11-21

Table 1 (contd.)

Spore shape	Oval (Codreanu 1957), oval with blunt ends (Martinez *et al.* 1994), broadly oval•	Oval	Pyriform	Ovoid	Ovoid	Oval (stained)
Spore size (µm)	4.2-5.7 long (Codreanu 1957); 3.0 (2.8-3.5) × 4.6 (3.9-5.1) (Martinez *et al.* 1994); 3.0 ± 0.2 (2.7-3.5) × 4.9 ± 0.3 (4.5-5.4)•	0.9 ± 0.12 × 1.7 ± 0.15	5.0-5.9 long	5.3-6.9 long	2.6-3.2 long	Macrospores 2.0-3.0 × 3.5-5.0; microspores 1.5-2.0 × 2.8-3.5
Spore nuclei	Diplokaryotic	Single,with additional envelope	Single	Single	Single	Isolated
Spore wall wide (nm)	156-213 (Martinez *et al.* 1994); 215-290•	140-150	No data	No data	No data	94-97
Exospore	Two layered, the surface layer resemble double membrane. Coarse-grained basal layer and double membrane shaped coat•	Uniform	No data	No data	No data	With a dense surface coat
Exospore thickness (nm)	75-90; 57 (Martinez *et al.* 1994)	30	No data	No data	No data	35
Polar sac	Umbrella-shaped, 1/5 of the spore length	Expanded	No data	No data	No data	Laterally prolonged
Polaroplast	Bipartite	Bipartite	No data	Voluminous laminate	No data	Bipartite
Anterior polaroplast	Tightly packed lamellae	Few lamellae	No data	No data	No data	Tightly packed lamellae
Posterior polaroplast	Wide lamellae (Martinez *et al.* 1994); wide lamellae, narrower in peripheral part•	Membraneous saccules	No data	No data	No data	Expanded lamellae
Polar filament	Isofilar, or lightly anisofilar (Martinez *et al.* 1994)	Isofilar	No data	No data	Isofilar	Anisofilar
Polar filament coils	13-16 with more narrow four last coils; 15-17 (Codreanu-Balcescu and Codreanu 1980); 11-13 (Martinez *et al.* 1994); 13-16 with more narrow four last coils•	8-11	No data	11-12, the 3 distal being constricted	6	10-12+5–6; 8-9+3
Polar filament rows	1	2	No data	No data	No data	2; 1
Diameter of polar filament (nm)	110-130 (Martinez *et al.* 1993); 120-140•		No data	No data	No data	120+92; 90+70

(• - authors data)

part, transforming into narrow lamellae where it has been sectioned peripherally (Figs 13, 14). The cytoplasm of the spores contained numerous ribosomes sometimes forming chains around the diplokaryon (Figs 12, 15).

DISCUSSION

Generally, the ultrastructure of the investigated microsporidium was similar to these of *Nosema* and *Brachiola* genera. A few details of the cytology need comments: the ultrathin organization of the exospore, the ultrastructural features of the polar filament and vesiculo-tubular structures along the parasite cell surface.

The exospore construction is the important taxonomic attribute for determination of the generic position of microsporidians (Larsson 1999). Usually the spores of the species of the genus *Nosema* possessed uniformly structured exospore (Sato *et al.* 1982, Sokolova and Lange 2002). The type species *N. bombycis* has "exospore thin, endospore moderately thick" (Sprague *et al.* 1992). Uniform dense exospore has also been reported in *N. granulosis*, infecting *Gammarus duebeni* (Amphipoda) (Terry *et al.* 1999). The layered exospore with a double outer coat is characteristic for the genera of the family Thelohaniidae (Larsson 1988), but an exospore with two distinct strata was also reported among several *Nosema* like microsporidians such as *Schroederella plumatellae* (Morris and Adams 2002) and *N. omaniae* (Diarra and Toguebaye 1995). Generally the layered exospore is not peculiar for representatives of the genus *Nosema*. The first ultrastructural data concerning exospore construction of *N. artemiae* were obtained on the material collected in Spain. The two-layered exospore with the surface layer resembling a double membrane was demonstrated on most of published photographs (Martinez *et al.* 1994). Identically structured exospore has the microsporidium studied (Figs 9, 12, 14), and we are disposed to believe that the exospore with an external double layer is characteristic feature of this species.

The first information about the number of polar filament coils (15-17) of *N. artemiae* was documented by Codreanu-Balcescu and Codreanu (1980). More complete records were presented by Martinez *et al.* (1994). According of them, the coiled part of polar filament of *N. artemiae* is arranged in 13-16 coils, where the last four coils were somewhat narrower, but the spores with 11-13 coils were also observed, mainly in the species coming from South East of Spain. The four coils of polar filament of the spores of microsporidium collected in South Ukraine and Spain were slightly narrower, than the others. The polar filament develops until the spore is perfectly mature, and immature coils are normally slightly narrower than completely mature coils (Larsson 1986). It does not seem that the last coils should be immature, because the number of narrow coils is always four (Fig. 10). A similarly constructed polar filament was described in *Nosema chaetocnemae*, from *Chaetocnema tibialis* (Coleoptera), but the authors defined this polar filament as isofilar (Yaman and Radek 2003).

The vesiculo-tubular secretions observed in the current study appear irregularly along the cell surface of late merogonial and sporogonial stages (Figs 5, 6). Similar structures were described in *Brachiola* spp. and some other microsporidians, but contrary to *N. artemiae* it were observed mostly as a protuberance of the sporont wall but not the sphaerular dense secretions adhering to the plasmalemma of late meront and forming the inner layer od the future exospore. It may be treated rather as episporontal inclusions than as appendages of the sporont wall. In case of *N. artemiae* as "episporontal space" should be defined a part of host cell cytoplasm adjoining to the parasite cells.

A comparison of *N. artemiae* with *Brachiola vesicularum*, *B. algerae* and *B. gambiae* brings some similarities. No multinucleate plasmodia or ribbon-like stages containing more than two diplokarya occur in *N. artemiae* similar to *B. vesicularum* and unlike to other *Nosema* species. The short polar filaments with the more narrower three (*B. vesicularum*), one (*B. algerae*) and four (*N. artemiae*) posterior coils are likewise constructed (Canning and Sinden 1973, Cali *et al.* 1998, Weiser and Žižka 2004). *Brachiola* related species are thermophilic, proliferating and sporulating at temperatures ≥30°C (Cali *et al.*1998). The same and even higher temperatures are characteristic for southern salters, inhabiting *Artemia* spp.

Based on obtained data we are disposed to believe that *N. artemiae* cannot be considered as *Brachiola* or classic *Nosema*-belonging species. More precise definition of taxonomic position of this microsporidium and other *Nosema*-related species with slightly anisofilar polar filament and double-layered exospore becomes possible after the further molecular analysis of these parasites. The achieved data confirms suggestion about heterogeneous character of the genus *Nosema*.

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ACTA
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Cross-infection Experiments Confirm the Host Specificity of *Goussia* spp. (Eimeriidae: Apicomplexa) Parasitizing Cyprinid Fish

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Summary. The host specificity of the coccidian *Goussia carpelli* (Léger *et* Stankovitch, 1921) (Eimeriidae: Apicomplexa) was studied in aquarium experiments. Oocysts were obtained from the gut of 1- to 2-year-old common carp intensively infected with *Goussia carpelli*. These oocysts were mixed into mud containing infection-free oligochaetes (*Tubifex tubifex* and *Limnodrilus hoffmeisteri*). Laboratory-cultured fish demonstrated to be infection free were infected by feeding oligochaetes. The susceptibility of 3-5 cm long fingerlings of 8 fish species [common carp (*Cyprinus carpio*), goldfish (*Carassius auratus*), barbel (*Barbus barbus*), bleak (*Alburnus alburnus*), roach (*Rutilus rutilus*), bream (*Abramis brama*), white bream (*Blicca bjoerkna*) and vimba (*Vimba vimba)*] was experimentally evaluated. In the gut of common carp, intensive infection developed each of eight experiments, and oocysts were consistently detectable in the faeces and gut scrapings on days 11-20 after feeding the fish with the oligochaetes. At the same time, oocyst formation could not be demonstrated in the gut of the other seven cyprinids. In another experiment of similar design, only the goldfish could be infected with oocysts obtained from naturally infected goldfish, and no infection was established in the common carp. The results of these experiments suggest that *G. carpelli* is strictly specific to common carp, while *Carassius* species most closely related to the common carp are parasitised by a distinct species of *Goussia* with which it shares morphological similarity.

Key words: Apicomplexa, coccidia, cyprinid fishes, experimental infection, *Goussia carpelli*, *Goussia* sp., oligochaetes.

INTRODUCTION

Goussia carpelli, described by Léger and Stankovitch (1921) from common carp (*Cyprinus carpio*) and crucian carp (*Carassius carassius*), is one of the longest known and best studied fish coccidians. This parasite is a typical example of gut-parasitic fish coccidia of small size, not exceeding 14 µm in diameter, and having a compact oocyst. Of the closely related known species described from cyprinids, *G. legeri* Stankovitch, 1920, *G. cyprinorum* (Stankovitch, 1921), *G. cylindrospora* (Stankovitch, 1920), *G. cheni* (Chen, 1956), *G. sinensis* (Chen, 1956) and *G. iroquoina* (Molnár *et* Fernando, 1974) have similar intrapiscine location and structure. Although the majority of these coccidian species had been described under the name *Eimeria*, Dyková and Lom (1981) proposed that they should be classified in the genus *Goussia* created by Labbé (1896) because their sporocysts consist of two valves connected by central sutures.

Stankovitch (1920, 1921) had previously suggested that oocysts isolated from different cyprinids repre-

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sented distinct parasite species, a view subsequently advocated by Belova and Krylov (2000) who suggested that the host-specificity of fish coccidia is as strong as that of coccidia parasitising terrestrial vertebrates. Other authors (Musselius and Laptev 1967, Shulman 1984), however, were of the opinion that certain species, such as *G. carpelli*, were able to develop in numerous cyprinid hosts. Moreover, Shulman and Zaika (1964) claimed to have found *G. carpelli* even in a fish species belonging to the Cottidae family. Specificity experiments using *G. iroquoina*, a *G. carpelli* type coccidium species, have been performed by Paterson and Desser (1982). The most reliable experimental results were obtained by Lukeš *et al*. (1991) who described that, with the exception of the goldfish, no infection could be produced in 9 other cyprinid fishes with *G. carpelli* originating from the common carp.

The objective of this experimental work was to determine which cyprinid fish species, other than the common carp, can be infected successfully with *G. carpelli* oocysts originating from common carp.

MATERIALS AND METHODS

Fish and oligochaetes used for experiments. Three- to 5-cm-long fingerlings of cyprinids, hatched in laboratory and reared in parasite-free environment, were used in the experiments. In addition to common carp (*Cyprinus carpio*), fingerlings of goldfish (*Carassius auratus*), barbel (*Barbus barbus*), bleak (*Alburnus alburnus*), roach (*Rutilus rutilus*), bream (*Abramis brama*), white bream (*Blicca bjoerkna*) and vimba (*Vimba vimba*) were studied. For the transmission of infection, laboratory-cultured, parasite-free *Tubifex tubifex* and *Limnodrilus hoffmeisteri* oligochaetes were used.

Coccidians used for experiments. Oocysts of *G. carpelli* were obtained from the gut of common carp fingerlings and two-summer common carp originating from fish farms. These were dissected a day or two after their transfer to the laboratory, after their gut contents had been excreted. Oocysts present in the mucus covering the intestinal mucosa were maintained in tap-water until the infection of oligochaetes. In each experiment, 10,000-20,000 oocysts were poured into plastic dishes containing 100 oligochaetes and sterilised mud. Similar procedures were used in one of the experiments employing *G. carpelli*-like oocysts obtained from goldfish.

Experimental design for infecting fish. A total of 9 experiments were performed. In the first 8 experiments, oligochaetes infected by *G. carpelli* oocysts were fed to fish. Experiments 1 and 2 were designed to determine the interval, after ingestion, when maximum oocyst excretion occurred. Experiments 4 and 5 were designed to learn how long the infected oligochaetes could transmit *G. carpelli* infection to fish. The objective of experiments 3-7 was to learn whether cyprinids other than the common carp could be infected with *G. carpelli* (Table 1). In experiment 8 the effect of repeated oligochaete feeding on the intensity of infection was studied (Table 2). In experiment 9, oocysts intended for infection were collected from the gut of goldfish fingerlings, and these oocysts were mixed into the mud of the dish containing the oligochaetes (Table 3). Until the conclusion of the experiments, the oocysts collected from common carp and goldfish were regarded as the spore stages of a single species, *G. carpelli*.

Infection of fish by oligochaetes. Oligochaetes were fed to the fish at different post-exposure intervals. The times of infection of oligochaetes and fish are indicated in Tables 1-3. Oligochaetes cut into small pieces were used for infecting the fish. Two to three days before the infected oligochaetes were fed to them, individual fasted fish were in a plastic dish containing 0.5 l of water. These fish, sustained on granulated dry fish food, had previously been acclimated to the consumption of coccidia-free oligochaetes. Infected oligochaetes were fed for one to three days; the duration of feeding varied by experiment. The fish were kept in an individual dish throughout the experiment to limit potential sources of exposure. Throughout the experiment, fish were fed the granulated fish food. Water in the dishes was changed daily and kept fresh and replenished with oxygen by aeration. Relatively few fish were assayed owing to the demands required to maintain fish individually in separate dishes. The experiments were carried out at room temperature (21 to 25°C). The fish were killed 10-21 days after infection, when peak oocyst excretion was expected. Feeding of fish food was stopped two days before the fish were killed.

Examination of fish for infection. The gut of the fish was removed in its entirety, cut open lengthwise, and the mucus in which the oocysts were most easily detectable was lifted off the gut wall as far as possible. In a negative case, besides the mucus the lumps of faeces present in the gut and mucosal scrapings taken from different segments of the intestinal wall were also placed on a slide, under a coverslip. The samples were examined under microscope at 200- to 400-fold magnification. In the case of fish of smaller size, the entire gut was examined for the occurrence of oocysts or developmental stages. For histological examination, segments from the intestine were fixed in Bouin's solution and embedded in paraplast. The 5 µm sections were stained with haematoxylin and eosin solutions. Photomicrographs of fresh oocysts in mucus under a coverslip were taken with an Olympus DH-10 digital camera mounted on an Olympus BH2 microscope.

RESULTS

Experiments 1 and 2, in which parasite-free common carp fingerlings were infected with *G. carpelli* oocysts collected from common carp through tubificid vectors, have confirmed that *G. carpelli* infection can be successfully transmitted to the common carp. Oocysts enclosed in yellow bodies could be detected in the mucus, faeces and gut epithelium at room temperature by days 11-20 in these fish (Table 1; Figs 1, 3).

Equivalent experiments demonstrated that parasites derived from the common carp infections did not result in infections of either goldfish or other cyprinid fishes (Table 1).

Table1. Experimental infection of common carp and other cyprinids with *Goussia carpelli* through oligochaete vectors exposed to oocysts for varying periods.

Fish species	No. fish	Oligochaetes exposed to oocysts before infection (days)	Time from exposure to killing (days)	Number of infected fish	Intensity of infection	No. exp.
Common carp	2	4	14	2	+/+++	4
	1	15-17	12	-	-	5
	1	15-17	17	1	+	5
	2	26	14	2	+/++	4
	1	32	9	1	+++	7
	8	32	12	8	+/+++	7
	1	34	11	1	+	1
	2	34	19	2	+++	1
	2	34	20	2	++	1
	4	31-32	15-16	3	+/+++	6
	4	31-32	15-16	2	++	6
	1	46-48	12	-	-	5
	1	46-48	17	1	+	5
	1	68-70	12	-	-	5
	1	68-70	17	1	+	5
	2	72	14	1	+	4
	2	73-74	12	-	-	2
	2	73-74	13	1	+++	2
	2	73-74	14	1	+++	2
	2	73-74	15	1	+++	2
	4	73-74	16	4	++/+++	2
	2	73-74	19	1	+++	2
	2	73-74	20	2	+/++	2
	2	73-74	21	2	+/++	2
Goldfish	2	31-32	15-16	-	-	6
	2	32	9	-	-	7
	2	4-72*	14	-	-	4
	1	15-70*	12	-	-	5
	1	15-70*	17	-	-	5
Common bream	2	32	12	-	-	7
	2	4-72*	14	-	-	4
	2	31-32	15-16	-	-	6
	1	15-70*	12	-	-	5
	1	15-70*	17	-	-	5
Roach	2	32	12	-	-	7
	2	4-72*	14	-	-	4
	2	31-32	15-16	-	-	6
	1	15-70*	12	-	-	5
	1	15-70*	17	-	-	5
Vimba	2	32	12	-	-	7
	2	31-32	15-16	-	-	6
	2	4-72*	14	-	-	4
	1	15-70*	12	-	-	5
	1	15-70*	17	-	-	5
White bream	2	32	12	-	-	7
	2	31-32	15-16	-	-	6
Bleak	2	32	12	-	-	7
	2	31-32	15-16	-	-	6
Barbel	2	32	12	-	-	7
	2	31-32	15-16	-	-	6
	2	4-72*	14	-	-	4
	1	15-70*	12	-	-	5
	1	15-70*	17	-	-	5

+ - a few oocysts in the gut; ++ - easily detectable coccidian infection; +++ - intensive infection; * - feeding by mixed oligochaete groups exposed to oocysts of varying times

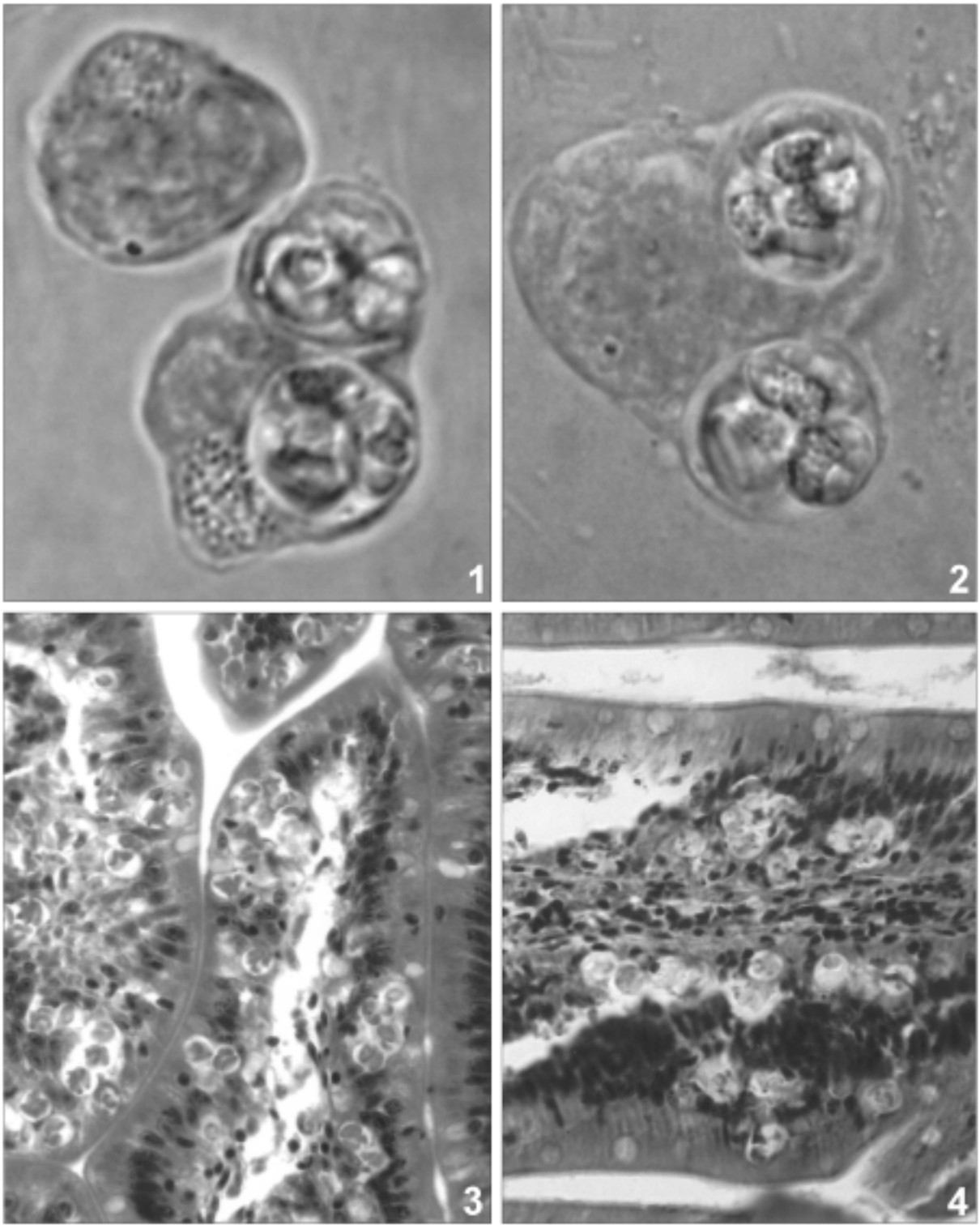

Fig. 1. *Goussia carpelli* oocysts located within a yellow body from the gut of an experimentally infected common carp fingerling. 3000×.
Fig. 2. *Goussia* sp. oocysts located within a yellow body from the gut of an experimentally infected goldfish fingerling. 3000×.
Fig. 3. Sporulated *G. carpelli* oocysts in the gut epithelium of the common carp. Histological section stained with haematoxylin and eosin. 500×.
Fig. 4. Oocysts of a *Goussia* sp. in the gut epithelium of a goldfish. Histological section stained with haematoxylin and eosin. 500×.

Table 2. Common carp and goldfish fingerlings fed for 1-10 days on oligochaetes exposed to *G. carpelli* oocysts.

Fish species	No. fish	Oligochaetes exposed to oocysts before infection (days)	Duration of oligochaete feeding (days)	Time from the first exposure to killing (days)	Number of infected fish Carp/Goldfish	Intensity of infection Carp/Goldfish	No. exp.
Common carp/goldfish	1/1	36	1	15	1/-	++ / -	8
	1/1	36-37	2	15	1/-	++ / -	8
	1/1	36-38	3	15	1/-	++ / -	8
	1/1	36-40	5	15	1/-	++ / -	8
	1/1	36-41	6	15	1/-	++ / -	8
	1/1	36-42	7	15	1/-	+++ / -	8
	1/1	36-43	8	15	1/-	+++ / -	8
	0/1	36-44	9	15	-/-	/ -	8
	0/1	36-45	10	15	-/-	/ -	8

Table 3. Coccidian infection of goldfish and common carp fingerlings after feeding on tubificid oligochaetes exposed to oocysts of a *Goussia* sp. originating from goldfish.

Fish species	No. fish	Oligochaetes exposed to oocysts before infection (days)	Time from exposure to killing (days)	Number of infected fish Goldfish/Carp	Intensity of infection Goldfish/Carp	No. exp.
Goldfish/common carp	3/3	14	9	-/-	-/-	9
	3/3	14	14	3/-	+ to ++/-	9
	2/2	14	20	2/-	+++/-	9
	2/2	14	23	2/-	+++/-	9

The transmission of *G. carpelli* infection from common carp to common carp was successful regardless of whether the oligochaetes had been infected 4, 26, 69 or 72 days before the infection of fish. Oligochaetes infected for longer intervals resulted in less intensive infections in common carp (Table 1).

Oocyst excretion was observed, once, as early as 9 days after infection but regularly from day 13 onwards (Tables 1, 2).

An only minimal increase in the intensity of infection was observed in common carp fed with the oligochaetes for 8 days as compared to those fed oligochaetes for one day (Table 2).

Fish could also be infected through tubificid vectors with oocysts, morphologically appearing identical to *G. carpelli*, collected from goldfish. However, in this case infection developed only in goldfish, while common carp infected in the same way, and serving as controls for the goldfish, remained free of infection (Table 3; Figs 2, 4).

In goldfish, oocyst excretion could also be observed from day 13 post infection (Table 3). At that time the majority of oocysts were still in unsporulated or half-sporulated state in the gut lumen. However, after day 19 an advanced stage of sporulation was observed (Fig. 4).

DISCUSSION

The primary objective of the experiments was to study the host specificity of *G. carpelli* and the possibility to transmit parasites derived from common carp to other cyprinids. To maximize our ability to detect such heterologous infections, we first sought to determine, without robust replication, other transmission characteristics such as the optimum time of infection with oligochaetes and the duration of oocyst formation and oocyst excretion.

As shown by the data of experiments presented in Tables 1-2, *G. carpelli* infection could consistently be

established in infection-free common carp through the feeding of tubificid (*Tubifex, Limnodrilus*) paratenic hosts. Oocyst formation and excretion commenced on days 9-12 post infection and continued at least for further eight days. In all the eight experiments, oocysts could be demonstrated only in common carp in the given period, and other cyprinids remained infection free. It was especially striking that the goldfish did not become infected either, although according to the original description *G. carpelli* is a common parasite of the genus *Cyprinus* and *Carassius* (Léger and Stankovitch 1921, Lukeš *et al*. 1991) could also produce a low-level infection in goldfish during their experiments. In contrast to these results, our experiments suggested that the common carp and the goldfish were infected by two distinct species, and that the common carp could not be infected successfully with oocysts collected from goldfish (Table 3). Otherwise, the intraoligochaete and intrapiscine development of the latter species appeared to be similar to that of *G. carpelli*, and oocyst excretion was recorded from day 13.

The results obtained in this study support the hypothesis that fish coccidia are characterized by relatively strict host specificity (Stankovitch 1921, Léger and Stankovitch 1921, Musselius *et al*. 1965). According to Belova and Krylov (2000), fish coccidia can develop normally only in closely related fish species, but we observe an even greater degree of host specificity in *G. carpelli*, as it cannot cause infection even in those species of *Carassius* most closely related to the common carp, i.e. the goldfish (*Carassius auratus*) and possibly the crucian carp (*C. carassius*) and the gibel carp (*C. auratus gibelio*). The close relationship between these fish species and the common carp is confirmed by morphological characteristics as well as by molecular evidence (Zardoya and Doadrio 1999). Similarly, the common carp cannot be infected with the *Goussia* species collected from goldfish, which, however, can successfully be transmitted experimentally to infection-free goldfish. These facts suggest that the *G. carpelli*-like species parasitising the goldfish might be regarded as a new, hitherto undescribed species. Waiting for the definitive description, this species has been designated as *Goussia* sp.

In these experiments, infection was consistently attempted by the use of oligochaete paratenic hosts. Namely, both in our own experience and according to the findings of Steinhagen and Körting (1990), this is the only method by which *G. carpelli* infection can be reproduced consistently. To date, few authors have attempted to transmit coccidian infections experimentally in fish. Successful infection through direct transmission of oocysts has been reported by Musselius *et al*. (1965), Zmerzlaya (1966), Steinhagen and Körting (1988, 1990) and Steinhagen *et al*. (1989), who conducted their experiments with *G. carpelli* in common carp. Marincek (1973) and Steinhagen (1991) could also successfully infect the same fish species with oocysts of *G. subepithelialis*. As regards to other fish species, Landsberg and Paperna (1985) successfully infected *Tilapia* by direct transmission of *G. cichlidarum* oocysts in tilapia. The paratenic host is necessary for the successful transmission of infection was first suggested by Molnár (1979), who could not induce *G. carpelli* infection in common carp with oocysts mixed in the food but obtained intensive infection by feeding tubificids collected from the natural environment. Similarly, the necessity of mediator organisms has been reported by Solangi and Overstreet (1980) as well as Fournie and Overstreet (1983), who proved that a natural intermediate host, the grass shrimp (*Palaemonetes pugio*), was needed for the development of *Calyptospora funduli*. Kent and Hedrick (1985), who studied *G. carpelli* infection in goldfish (sic?!), successfully induced infection by the use of vectors (tubificids, grass shrimp). However, Steinhagen and Körting (1990) could induce *G. carpelli* infection both directly and indirectly (by the use of vectors), and they regarded tubificids as paratenic hosts only. Nevertheless, the above authors emphasised that infection via vectors was more likely to succeed. Steinhagen (1991) found *G. carpelli* and *G. subepithelialis* sporozoites within parasitophorous vacuoles in the gut epithelial cells of *Tubifex* and established that they maintained their viability for nine weeks. Fournie *et al*. (2000) detected sporozoites of *C. funduli* in the basal epithelial cells of the gut of shrimp, where they became infective in 5 days.

Cross-infection experiments were first reported by Paterson and Desser (1982), who successfully induced *G. iroquoina* infection in the common shiner (*Notropis cornutus*) and fathead minnow (*Pimephales promelas*) with oocysts collected from the common shiner both by direct infection and through *Tubifex* vectors. On the basis of observations made in ponds, Musselius and Laptev (1967) came to the conclusion that if the common carp, silver carp and bighead stocks are reared together the common carp develops only *G. carpelli* infection, whereas the oocysts of *G. carpelli* also can be found in the gut of silver carp and bighead where they occur together with *G. sinensis* and *G. cheni* which

typically infect these fish species. So far, only *G. sinensis* infection has been recorded in silver carp and bighead in Hungary (Molnár 1976), and the oocysts of *G. carpelli* were not detectable from these fish species. The results of the present experiment suggest that it is unlikely that *G. carpelli* infects other fish than the common carp. We suggest that in the experiment of Musselius and Laptev (1967) *G. carpelli* coccidia may have reached the gut of silver carp and bighead with the food owing to conditions applicable only when fish are reared together in aquaria. To rule out this potential source of error, fish in the present experiments were kept in individual dishes, isolated from fish of the same or other species. The only reliable results on the host specificity of *G. carpelli* were obtained by Lukeš *et al*. (1991) who found that this species was strictly host specific. However, in contrast with our results, besides the common carp the above authors could establish a slight infection in the goldfish as well.

Outside the host oocysts from mammals and birds can remain viable and infective for more than one year (Kheysin 1972). However, according to Musselius *et al*. (1965) oocysts remain viable in water only for 20-22 days. According to our own observations (Molnár, unpublished), the period of oocyst viability is actually much shorter, and paratenic hosts play an important role also in the prolonged maintenance of infectivity. In addition to the data reported by Steinhagen (1991), this is supported also by the results of experiments 3 and 4 of this study. In these experiments, besides relatively recently infected oligochaetes, tubificid worms of an oligochaete stock exposed to oocysts four months earlier were also fed to fish. Both stocks established successful infection, though the level of infection with earlier infected oligochaetes was lower than when recently infected tubificid worms were used.

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ACTA
PROTOZOOLOGICA

Trichodinids (Ciliophora: Trichodinidae) from Native and Exotic Australian Freshwater Fishes

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Summary. There are very few data on trichodinids of freshwater fishes in Australia. 2003 fishes were surveyed across Eastern Australia to investigate the diversity of trichodinids present, to determine which species have been introduced with exotic fishes and to determine the extent to which these species have crossed into native fish populations. Twenty-one putative trichodinid species were recovered from the 33 fish species examined. *Trichodina heterodentata*, *T. mutabilis* and *T. reticulata* were the exotic species recovered regularly; a single specimen matched a fourth exotic species, *T. acuta*. All four exotic species are redescribed from Australian material. *Trichodina heterodentata* was recorded from 17 species of fishes, 15 of which were new host records; this species is identified as one of emerging importance in fish parasitology and a list of its known hosts is presented. Two new native species are also described based on silver stained specimens: *T. cribbi* sp. n. from *Hypseleotris galii*, *H. klunzingeri*, and *Hypseleotris* sp. 5; and *T. bassonae* sp. n. from *Selenotoca multifasciata*. *Trichodina cribbi* is characterised by a large circular central inclusion and approximately 28 denticles, which have a blade length slightly greater than the ray length. *Trichodina bassonae* is characterised by a small, round, central inclusion and approximately 25 denticles, which have straight, non tapering rays that are in line with the leading edge of the denticle blade. It is estimated that the Australian trichodinid fauna may include up to 150 as yet undescribed species and represents a major source of unexplored biodiversity.

Key words: Australian, endemic, exotic, richness, *Trichodina bassonae* sp. n., *Trichodina cribbi* sp. n., trichodinids.

INTRODUCTION

Data on trichodinids of Australian freshwater fishes are sparse and fragmentary. No individual species have been described from either native or exotic freshwater fishes in Australia. Lom and Dyková (1992) recorded an unspecified *Trichodina* species from the gills of *Gambusia holbrooki* at Armidale, New South Wales. Langdon (1989) listed *Trichodina* spp. as "parasitic on native and introduced fishes" but provided no data on which *Trichodina* species infected which host species, nor any data on shared *Trichodina* species. Rowland and Ingram (1991) discussed the ectoparasitic diseases of Murray cod (*Maccullochella peeli*), golden perch (*Macquaria ambigua*) and silver perch (*Bidyanus bidyanus*). Although they identified *Chilodonella hexasticha*, *Ichthyophthirius multifilis* and *Ichthyobodo necator* specifically, *Trichodina* spp. (and *Tetrahymena* spp.) were regarded as a single group. These authors recognised that "the identity and

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number of species of *Trichodina* on freshwater fishes in Australia are not known".

The aims of this study were: to identify which trichodinid species occur on Australian populations of exotic freshwater fishes; to investigate the diversity of the Australian trichodinid fauna; and to quantify the degree to which trichodinid species have been exchanged between native and exotic fishes. The null hypothesis that no exchange of trichodinid species between native and exotic fish species has occurred was tested using survey data and comparisons with known trichodinid records on Australia's exotic fishes in their original ranges.

MATERIALS AND METHODS

Collection and dissection protocol

Trichodinid ciliates were collected on an opportunistic basis during a survey for other parasites that encompassed 58 sites throughout Victoria, New South Wales, the Australian Capital Territory and Queensland. In addition, a dedicated survey was conducted at 15 sites in Queensland to obtain accurate data on prevalence, host and microhabitat specificity. Site abbreviations are as follows: **ALR** - Albert River; Queensland (Qld); **ATH** - Atherton, Qld; **BCF** - Crayfish Farm, Beenleigh Qld; **BMP** - Lake Burley-Griffin at Black Mountain Peninsula, Australian Capital Territory (ACT); **BOC** - Boorolong Creek, New South Wales (NSW); **BRB** - Billabong at Bangarang Road, Echuca, Victoria (Vic); **BRF** - Brisbane River at Fernvale, Qld; **CBL** - Condamine River below Leslie Dam, Qld; **CRH** - Condamine River Headwaters, Qld; **CRW** - Condamine River at Warwick, Qld; **EBK** - Lake Burley-Griffin at East Basin Kingston, ACT; **EGC** - England Creek, Qld; **ENC** - Enoggerra Creek, Qld; **ERS** - Edward River at Steven's Weir, Deniliquin, NSW; **GBL** - Gum Bend Lake, Condobolin, NSW; **KB216** - Kalinga Park Creek, Qld; **LGN** - Lake Ginninderra, ACT; **MNC** - Monsildale Creek, Qld; **MOC** - Moody's Creek, north Qld; **NFC** - Narranderra Fisheries Centre, NSW; **NPR** - North Pine River, Qld; **OVR** - Ovens River, Vic; **RGR** - Moggill Creek at Rafting Ground Road, Qld; **SRW** - Shoalhaven River at Warri Bridge, NSW; **WCC** - West Court Creek, north Qld; **WKN** - Walkamin Research Station, north Qld; **WVN** - Lake Wivenhoe, Qld.

Fishes were collected by cast-netting, dip-netting, electrofishing and line fishing, and were separated into species-specific plastic bags on site. In the laboratory, fishes were maintained in aerated 20 l tanks with water from the collection site. Dissections were made within three days to minimise the effect of collection stress on trichodinid prevalence.

Fish were killed by pithing the brain, then identified, measured (caudal fork length) and sexed (if possible), and immediately dissected in freshwater. Each fin and gill was removed and placed in a separate Petri dish in aged water and the skin scraped into another dish of aged water. Each dish was then examined under dissecting microscope for the presence of trichodinids; any present were pipetted onto a clean or albuminised glass slide (depending on staining regimen) and allowed to air dry.

Morphometrics

Stained preparations were made using two silver salt staining protocols: Foissner's modification of Klein's dry silver nitrate technique (Foissner 1991), and Basson's version of Lom's dry silver nitrate technique (Lom 1958). Measurements presented here follow the protocols of Lom (1958). Three denticles were measured from each specimen and the means for denticle measurements are therefore based on 3n denticles. Measurements were made using a system similar to that described by Roff and Hopcroft (1986), with a digitising tablet and a drawing tube attached to a compound microscope. All measurements are given in micrometres. Type material was lodged in the Queensland Museum.

Drawing and photography techniques

Representatives of each putative species from each host species were photographed using a 35 mm camera attached to a DIC compound microscope. These photographs were the basis for the drawings of each trichodinid species. Drawings were made by enlarging the photographs by 200% on a photocopier, overlaying the resulting image with architectural film and tracing the morphological features. The ink drawings were then scanned and the resulting files edited in Adobe Photoshop© 5.0.

RESULTS

A total of 2003 fishes was dissected, representing 33 host species and 58 sites across Queensland, New South Wales, Victoria and the Australian Capital Territory. From these, 21 putative species of trichodinids were recovered, 6 of which are discussed here. Three exotic trichodinid species were recovered: *Trichodina heterodentata*, *T. mutabilis* and *T. reticulata*. A single specimen matching a fourth exotic species, *T. acuta*, was also recovered. These four species of exotic trichodinids are redescribed here to confirm their identity and to provide data on their distribution among hosts in Australia. In addition, two new species of *Trichodina* are described from native fish species: *T. cribbi* sp. n. and *T. bassonae* sp. n. The remaining 15 trichodinid morphotypes are not described here.

Descriptions

Trichodinidae Raabe, 1963
Trichodina Ehrenberg, 1838

***Trichodina heterodentata* Duncan, 1977 (Fig. 1)**

Hosts: [Host (number of infected hosts examined)] Ambassidae: *Ambassis agassizi* (4); Apogonidae: *Glossamia aprion gilii* (1); Cichlidae: *Oreochromis mossambicus* (1); Cyprinidae: *Cyprinus carpio* (2); Eleotridae: *Hypseleotris compressa* (2); *Hypseleotris*

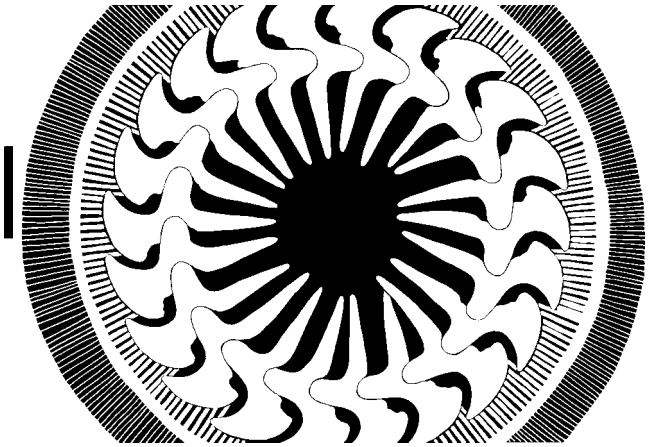

Fig. 1. *Trichodina heterodentata* Duncan, 1977 from the skin of *Ambassis agassizi.* Scale bar 10 µm.

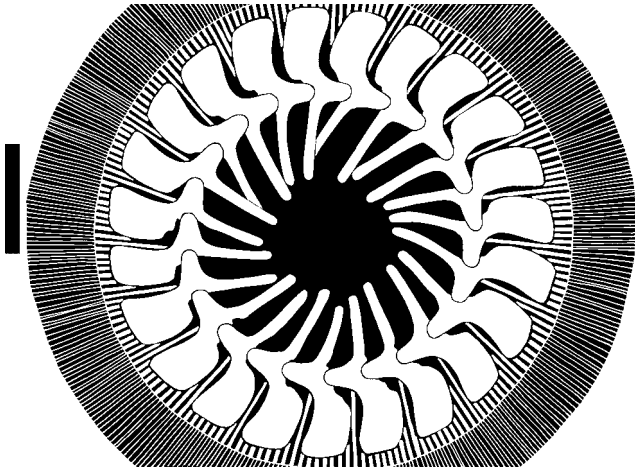

Fig. 2. *Trichodina mutabilis* Kazubski *et* Migala, 1968 from the skin of *Cyprinus carpio.* Scale bar 10 µm.

galii (2); *Hypseleotris klunzingeri* (9); *Philypnodon grandiceps* (1); Galaxiidae: *Galaxias maculatus* (2); *Galaxias olidus* (1); Gerreidae: *Gerres* sp. (1); Percichthyidae: *Macquaria ambigua* (2); Poeciliidae: *Gambusia holbrooki* (5); *Poecilia reticulata* (6); *Xiphophorus helleri* (1); *Xiphophorus maculatus* (6); Pseudomugilidae: *Pseudomugil signifer* (1).

Localities: KB216, WKN, BRB, NPR, NFC, BCF, EBK, WVN, GBL, MNC, BOC, SRW.

Microhabitat: Skin, fins, occasionally gills.

Description: Description based on 18 specimens: [Host species, Autopsy number (number of specimens)] *A. agassizi*, AD334 (3); *C. carpio*, T141 (1); *G. holbrooki*, AD309 (1); *G. aprion gilii*, AD311 (1); *H. compressa*, NPR5 (1); *H. galii*, T137 (1), BCF5 (1), BCF6 (1); *H. klunzingeri*, T130 (1); *O. mossambicus*, NPR12 (1); *P. signifer*, AD252 (1); *X. helleri*, KB20/10/97_8 (1); *X. maculatus*, AD307, AD310, KB20/10/97_1 (1) KB20/10/97_3 (1). Medium to large disc-shaped cell; adhesive disc 56.9 (41.2-89.2) in diameter; denticle ring 31.2 (24.7-37.4) in diameter. Denticles 23 (21-26); denticle span 17.2 (12.5-23.9). Denticle with strongly curved blade ending in a sharp point, blade 5.2 (4-7.4) long, 5.6 (4-8.3) wide. Central part elongate, tip rounded, 3 (2.3-4.6) long, 4.4 (3.1-6.4) wide. Projection of central part not visible. Ray tapering evenly from central part to pointed tip, 7.9 (5.5-11.2) long, 1.9 (1.1-3) wide at widest point. Central disc without inclusions of any kind. Radial pins prominent, 11 (10-13) pins per denticle. Nuclear apparatus not observed.

Taxonomic remarks: *Trichodina heterodentata* is a distinctive species characterised by the robust, strongly sickle-shaped denticle blades with pointed ends, the evenly tapering rays with pointed tips, the absence of any central inclusions and the prominence of the radial pins. It was originally described from cultured cichlids, *Oreochromis mossambicus* from the Philippines, but has subsequently been recorded from a large number of fish species (see Table 1). Morphometric data for the present material fall within the ranges for *T. heterodentata* presented in the original description by Duncan (1977) and subsequent redescriptions by Van As and Basson (1989), Kruger *et al.* (1993) and Basson and Van As (1994). Van As and Basson (1989) and Basson and Van As (1994) noted a degree of morphological variability in isolates of *T. heterodentata*; a similar degree of variability was evident in the present material, especially with respect to the ray length and the degree of curve in the distal blade edge. The distinctiveness of the denticle shape, however, made identification of this species relatively easy.

Trichodina mutabilis Kazubski *et* Migala, 1968 (Fig. 2)

Hosts: Cyprinidae: *Cyprinus carpio* (3); Poeciliidae: *Gambusia holbrooki* (1)

Localities: BCF.

Microhabitat: Fins, gills.

Description: Description based on seven individuals: *C. carpio* BCF12/1/98_1 (4), BCF12/1/98_6 (1);

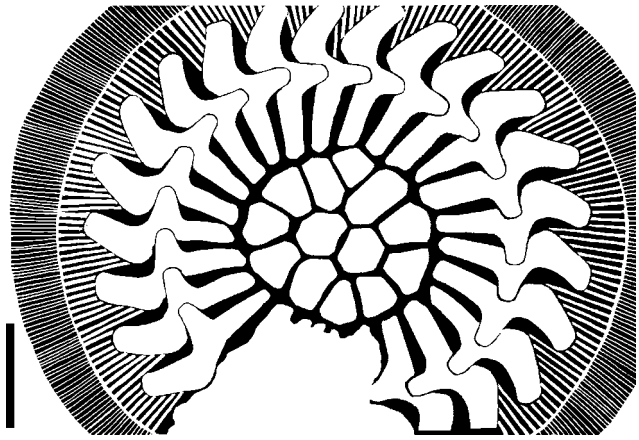

Fig. 3. *Trichodina reticulata* Hirschmann *et* Partsch, 1955 from the skin of *Carassius auratus*. Scale bar 10 µm.

G. holbrooki BCF12/1/98_8 (2). Medium to large disc-shaped cell; adhesive disc 45.9 (40.5-55.7) in diameter, denticle ring 27.6 (24-37.5) in diameter. Denticles 24 (22-26); denticle span 15.4 (11.5-19). Denticle blade oblong, largely parallel to radial of disc, with squared distal end, blade 5.6 (4.6-7) long, 4 (3.4-5.3) wide. Central part narrow, with oblong to rounded overlapping end; 1.9 (1.3-2.4) long, 3.5 (2.7-4.5). Projection of central part not visible. Ray narrow with no taper, tip blunt to square; 7.3 (5.4-9) long, 1.4 (1-2) wide. Central disc without inclusions of any kind. Radial pins 11 (9-13) per denticle. Nuclear apparatus not observed.

Taxonomic remarks: Our material falls within the ranges for this species in the description by Kazubski and Migala (1968) and a subsequent redescription by and Basson and Van As (1994).

Trichodina reticulata Hirschmann *et* Partsch, 1955 (Fig. 3)

Hosts: Cyprinidae: *Carassius auratus* (2), *Cyprinus carpio* (1).

Localities: SRW, GBL, NFC.

Microhabitat: Skin, fins.

Description: Description based on five individuals from *C. auratus* G372. Medium to large disc-shaped cell; adhesive disc 48.6 (40.9-54.2) in diameter, denticle ring 30.4 (26-34.3) in diameter. Denticles 24 (23-25); denticle span 13.8 (11.8-14.9). Denticle blade curved on inside margin but roughly angular on outside margin, with squared distal end, 5.6 (4.8-6.7) long, 4 (3.4-4.6) wide. Central part oblong, with oblong to rounded overlapping end; 2.5 (2.2-2.9) long, 3.7 (3.3-4.3) wide. Projection of central part not visible. Ray with little taper, tip blunt to square; 5.2 (4.2-5.8) long, 1.9 (1.7-2.1) wide at widest point. Central disc with 12 (10-14) icosahedral inclusions (mean diameter 4 (2.5-6.3)); inclusions give impression of a single large inclusion with reticulated markings. Radial pins prominent 10 (9-11) per denticle. Nuclear apparatus not observed.

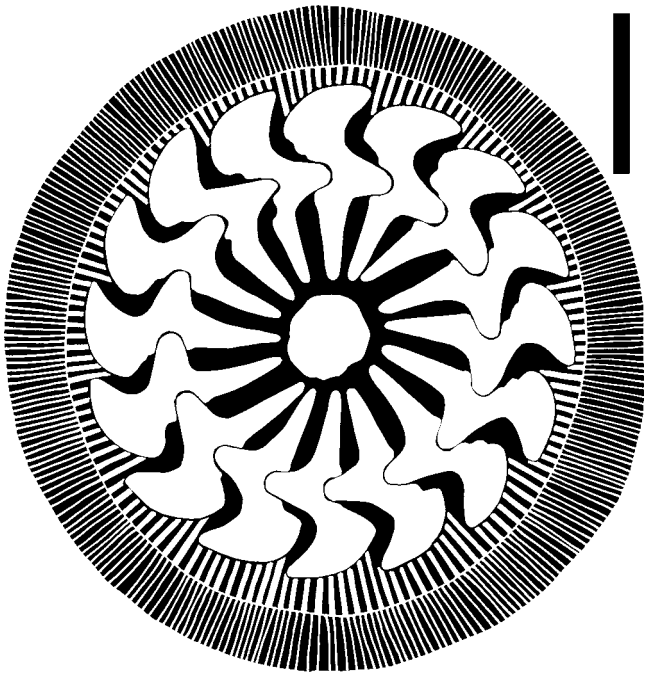

Fig. 4. *Trichodina cf. acuta* Lom, 1961 from the skin of *Poecilia reticulata*. Scale bar 10 µm.

Taxonomic remarks: *Trichodina reticulata* is a highly distinctive species well-known as a pathogen of *Carassius* spp. and other cyprinids in aquaculture and aquaria. The central inclusions are unlike those of almost any other *Trichodina* species, while the angular outer edge to the blade, prominent radial pins and large cell size only serve to confirm the diagnosis. Our material is indistinguishable from the redescription in Basson *et al*. (1983) as well as photographs of this species in Lom (1961), Wellborn (1967) and Van As and Basson (1987). Basson and Van As (1994) observed that this species is primarily a parasite of *Carassius* species and that it has been widely distributed around the world with goldfish, *C. auratus*, and crucian carp, *C. carassius* and is now present in the former USSR, Eastern Europe, Iran, North Korea, Japan, China, Indonesia, Israel and the USA. Neither *C. auratus* nor *Cyprinus carpio* is a new host record for this parasite (see Lom and Dyková 1992).

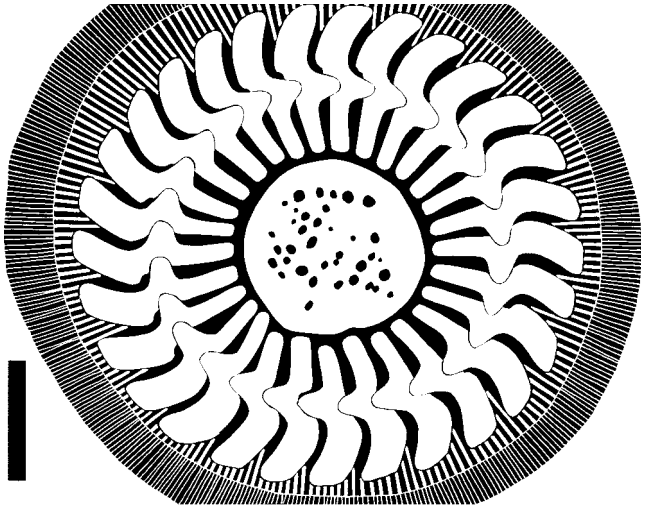

Fig. 5. *Trichodina cribbi* sp. n., from the skin of *Hypseleotris klunzingeri*. Scale bar 10 µm.

Trichodina cf. acuta Lom, 1961 (Fig. 4)

Hosts: Poeciliidae: *Poecilia reticulata* (1)
Localities: WKN.
Microhabitat: Skin

Description: Description based on the single specimen from T144. Medium disc-shaped cell; adhesive disc 33.8 in diameter; denticle ring 20.7 in diameter. Denticles 16; denticle span 12.4 (12.1-12.8). Denticle with curved blade ending in a sharp point, blade 4 (3.9-4.1) long, 4.5 (4.1-4.6) wide. Central part slightly elongate, tip rounded, 2.4 (2.3-2.6) long, 3.8 (3.6-4) wide. Projection of central part not visible. Ray tapering evenly from central part to pointed tip, 5.3 (4.8-5.7) long, 1.8 wide at widest point. Central disc with single, roughly circular inclusion 5.1 in diameter. 10 (9-11) radial pins per denticle. Nuclear apparatus not observed.

Taxonomic remarks: *Trichodina cf. acuta* is a problematic specimen. It is certainly very similar to figures of *T. acuta* from a number of sources (Lom 1961; Kulemina 1968; Basson *et al*. 1983; Van As and Basson 1989; Basson and Van As 1993, 1994), more so in fact than *T. compacta*, which was only discriminated from *T. acuta* by Van As and Basson (1989). One significant difference concerns the number of denticles. Our specimen has 16 denticles, which is two fewer than the smallest number seen in any of the figures mentioned above and three fewer than the mean in the original description of Lom (1961). The absolute range given in Lom (1961), however, is from 15-23, a range which incorporates our specimen. The central disc of our specimen is also rather small at 5.1, slightly more than half the diameter of the disc measured in Van As and Basson (1989). It may be worth noting, though, that the specimens in that paper were the largest and had the highest number of denticles of any description we are aware of (20-23). Apparently the species shows considerable morphological variability. The characteristics of this specimen are sufficient to assign tentatively this specimen to *T. acuta*, but the examination of more specimens is essential to confirm the diagnosis.

Trichodina cribbi sp. n. (Fig. 5)

Type host: Eleotridae: *Hypseleotris klunzingeri* (3)
Other hosts: Eleotridae: *Hypseleotris galii* (1), *Hypseleotris* sp. 5 (1)
Type locality: Black Mountain Peninsula, Lake Burley Griffin, Canberra, Australia
Localities: BCF, EC, NFC, ERS, CRW, GBL.
Microhabitat: Gills
Etymology: This species is named after Australian parasitologist Thomas H. Cribb.

Description: Description based on 12 specimenss from *H. klunzingeri*: T121 (1), T126 (5), T136 (6). G464586-G464590 Medium to large disc-shaped cell; adhesive disc 51.7 (45.6-59.2) in diameter, denticle ring 28.2 (23.7-31.9) in diameter. Denticles 29 (25-31); denticle span 13.7 (11.6-15.5). Denticle blade curved on inside margin but roughly angular on outside margin, with bluntly-squared distal end, 6.3 (5.1-7.7) long, 3.3 (2.4-4.3) wide. Central part oblong, with slightly-tapered, rounded overlapping end; 2.0 (1.4-2.7) long, 2.9 (2.3-3.5) wide. Projection of central part not visible. Ray with little taper, sometimes curved anteriorly, tip blunt to rounded; 4.6 (3.5-6) long, 1.2 (0.9-1.9) wide at widest point. Central disc with 1 large, roughly circular inclusion (mean diameter 14.3 (10.6-17.4)); sometimes with darker argentophilic spots towards its centre. Radial pins indistinct, 10 (9-11) per denticle. Nuclear apparatus and adoral spiral not observed.

Taxonomic remarks: This species is somewhat similar to the population of *T. jadranica* Raabe, 1958 as described by Lom and Dyková (1992), but differs in having blunter denticle blades, thicker denticle rays and in having twice the diameter of the adhesive disc. In morphology, the denticles are very similar to *T. porocephalusi* Asmat, 2001, a species also described from eleotrid fishes. There are several important differences, however. The mean disc width of our material (51.7) is greater than the maximum reported for *T. porocephalusi* (50.5). We also recorded a mean

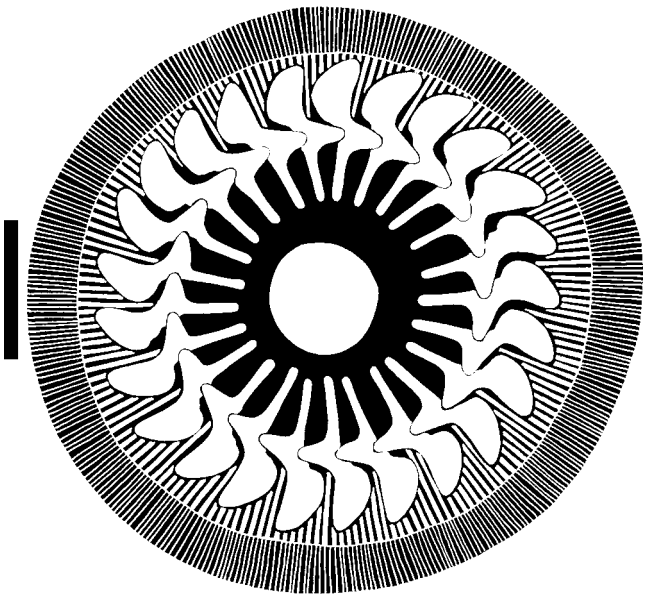

Fig. 6. *Trichodina bassonae* sp. n., from the fins of *Selenotoca multifasciata*. Scale bar 10 µm.

denticle number (29) five greater than that of Asmat's material (Asmat 2001). Finally, our mean number of pins per denticle (10) is greater than the maximum number in *T. porocephalusi* (9). These differences serve to unequivocally separate the species, though the similarity of morphology and hosts suggests a close relationship.

***Trichodina bassonae* sp. n. (Fig. 6)**

Type host: Scatophagidae: *Selenotoca multifasciata* (1)

Type locality: Albert River, South-East Queensland, Australia.

Microhabitat: Fins, particularly the anal fin.

Etymology: This species is named after South African parasitologist Linda Basson, whose contribution to the taxonomy and systematics of trichodinids has been extensive.

Description: Description based on 12 specimens from T143. G464591-G464593. Small to medium-sized disc-shaped cell; adhesive disc 33.3 (30.2-37.9) in diameter, denticle ring 22.9 (19.7-26.4) in diameter. Denticles 24 (23-26); denticle span 9.8 (8.7-10.8). Denticle blade straight on leading edge, roughly rounded on trailing margin, with bluntly-pointed distal end, 3.9 (3.4-4.4) long, 2.9 (2.5-3.6) wide. Central part oblong, with slightly-tapered, rounded overlapping end; 1.4 (1.0-1.8) long, 2.8 (2.2-3.5) wide. Projection of central part not visible. Denticle ray with no taper, tip blunt to rounded; 3.5 (3.0-4.4) long, 1.0 (0.5-1.4) wide at widest point. Central disc with 1 small to medium, roughly circular inclusion of mean diameter 9.0 (5.9-11.3). Radial pins 10 (9-11) per denticle. Nuclear apparatus and adoral spiral not observed.

Taxonomic remarks: This material is characterised by a unique configuration of having a denticle blade with a straight leading edge and a ray which is not tapered. This conformation, in combination with the circular central inclusion, is not similar to any published trichodinid species.

DISCUSSION

Exotic trichodinids

Of the 21 putative trichodinid species recovered, four have probably been introduced with exotic fishes. *Trichodina mutabilis* has almost certainly been introduced along with carp, *Cyprinus carpio*. Since its original description by Kazubski and Migala (1968), it has become a well-known and characterised parasite of carp as well as a number of other cyprinid species (Basson *et al*. 1983). In addition to carp, it also infected the unrelated exotic mosquitofish, *Gambusia holbrooki*, at the sole site from which it was recovered in this study, a crayfish farm near Beenleigh, Queensland. *Trichodina mutabilis* may not be widespread in Australia, however, despite the extensive distribution of carp in inland drainages. It was not recovered from any carp or mosquitofish (combined sample size N > 400) collected in inland areas and, as such, appears to be restricted to the Albert River carp population (the ultimate source of the crayfish farm fish). We have collected additional parasitological evidence that suggests that this population has a distinct and separate origin to that of the inland, Boolarra strain of carp; carp at the crayfish farm harbour a dactylogyrid monogenean species (*Dactylogyrus cf arcuatus*) and a bomolochid copepod which are not found on inland carp, which only harbour *Dactylogyrus anchoratus* (Dove and Ernst 1998).

Trichodina reticulata appears to have been introduced with its type host, the ornamental goldfish *Carassius auratus*. In the survey, it was restricted to the two cyprinid fish species: carp, *Cyprinus carpio*, and goldfish, *Carassius auratus*. Based on these data it may be premature, however, to conclude that this species is restricted to these two host species. Kazubski (1982a, 1988), for example, has documented infections of this parasite on tadpoles of the frog *Rana temporaria*

Table 1. Hosts of *Trichodina heterodentata* Duncan, 1977.

Family	Host species	Locality	Reference
Anura: Ranidae	*Kassina senegalensis*	experimental	Kruger *et al.* 1993
Anura: Ranidae	*Rana fuscigula*	experimental	Kruger *et al.* 1993
Anura: Xenopidae	*Xenopus laevis*	South Africa	Kruger *et al*. 1993
Ambassidae	*Ambassis agassizi*	Australia	Present study
Apogonidae	*Glossamia aprion gilii*	Australia	Present study
Balitoridae	*Crossostoma lacustre*	Taiwan	Basson and Van As 1994
Characidae	*Hydrocynus forskali*	Egypt	Al-Rasheid *et al*. 2000
Characidae	*Micralestes actuidens*	South Africa	Van As and Basson 1989
Cichlidae	*Chetia flaviventris*	South Africa	Van As and Basson 1989
Cichlidae	*Oreochromis aureus*	Israel	Van As and Basson 1989
Cichlidae	*Oreochromis aureus* × *niloticus*	Israel	Van As and Basson 1989
Cichlidae	*Oreochromis mossambicus*	Philippines	Duncan 1977
		South Africa	Van As and Basson 1989
		Venezuela	Van As and Basson 1989
		Taiwan	Van As and Basson 1989
		Australia	Present study
Cichlidae	*Pseudocrenilabrus philander*	South Africa	Van As and Basson 1989
Cichlidae	*Sarotherodon galilaeus*	Israel	Van As and Basson 1989
Cichlidae	*Tilapia rendalli swierstrae*	South Africa	Van As and Basson 1989
Cichlidae	*Tilapia spearmanii*	South Africa	Van As and Basson 1989
Cichlidae	*Tilapia zillii*	Philippines	Duncan 1977
		Israel	Van As and Basson 1989
Cobitidae	*Misgurnus anguillicaudatus*	Taiwan	Basson and Van As 1994
Cyprinidae	*Acanthobrama* sp.	Israel	Van As and Basson 1989
Cyprinidae	*Aristichthys nobilis*	Taiwan	Albaladejo and Arthur 1989
Cyprinidae	*Barbus eutaenia*	South Africa	Van As and Basson 1989
Cyprinidae	*Barbus marequensis*	South Africa	Van As and Basson 1989
Cyprinidae	*Barbus paludinosus*	South Africa	Van As and Basson 1989
Cyprinidae	*Barbus trimaculatus*	South Africa	Van As and Basson 1989
Cyprinidae	*Candida barbata*	Taiwan	Basson and Van As 1994
Cyprinidae	*Carassius auratus*	Israel	Van As and Basson 1989
Cyprinidae	*Ctenopharyngodon idella*	Israel	Van As and Basson 1989
		Taiwan	Albaladejo and Arthur 1989
Cyprinidae	*Cyprinus carpio*	South Africa	Van As and Basson 1989
		Israel	Van As and Basson 1989
		Indonesia	Albaladejo and Arthur 1989
		Australia	Present study
Cyprinidae	*Hypopthalmichthys molitrix*	Taiwan	Basson and Van As 1994
Cyprinidae	*Labeo cylindricus*	South Africa	Van As and Basson 1989
Cyprinidae	*Neobola brevianalis*	South Africa	Van As and Basson 1989
Cyprinidae	*Sarcocheilichthys nigripinnis*	Taiwan	Basson and Van As 1994
Eleotridae	*Hypseleotris compressa*	Australia	Present study
Eleotridae	*Hypseleotris galii*	Australia	Present study
Eleotridae	*Hypseleotris klunzingeri*	Australia	Present study
Eleotridae	*Philypnodon grandiceps*	Australia	Present study
Galaxiidae	*Galaxias maculatus*	Australia	Present study
Galaxiidae	*Galaxias olidus*	Australia	Present study
Gerreidae	*Gerres* sp.	Australia	Present study
Gobiidae	*Glossogobius giurus*	South Africa	Van As and Basson 1989
Gobiidae	*Rhinogobius brunneus*	Taiwan	Basson and Van As 1994
Mormyridae	*Marcusenius macrolepidotus*	South Africa	Van As and Basson 1989
Mormyridae	*Petrocephalus catostoma*	South Africa	Van As and Basson 1992
Percichthyidae	*Macquaria ambigua*	Australia	Present study
Poeciliidae	*Aplocheilichthys johnstonii*	South Africa	Van As and Basson 1989
Poeciliidae	*Gambusia affinis*	Taiwan	Basson and Van As 1994
Poeciliidae	*Gambusia holbrooki*	Australia	Present study
Poeciliidae	*Poecilia reticulata*	Australia	Present study
Poeciliidae	*Xiphophorus helleri*	Australia	Present study
Poeciliidae	*Xiphophorus maculatus*	Australia	Present study
Pseudomugilidae	*Pseudomugil signifer*	Australia	Present study
Synodontidae	*Synodontis zambezensis*	South Africa	Van As and Basson 1989

in Poland. The frog species which are commonly sympatric with carp and goldfish in Australia include *Limnodynastes tasmaniensis, L. fletcheri, L. interioris, Litoria peroni, L. leseuri* and *Crinia parinsignifera* (pers. obs.) and a number of other species. Tadpoles were not examined during the survey, but it is conceivable that *T. reticulata* may be using these hosts also. Further, the two sites from which infected fishes were collected, the Shoalhaven River and Gum Bend Lake, are separated by more than 500 km and the Great Dividing Range, which suggests that the parasite is widespread or, at least, occurs at multiple foci. The importance of *T. reticulata* may have been underestimated in this study.

The presence of *Trichodina acuta* in Australia, although equivocal because it is based on a single specimen, is a pattern consistent with published records of successful trichodinid invasions of alien systems. Kulemina (1968) recorded *T. acuta* from redfin, *Perca fluviatilis*, in Poland, whereas Basson *et al.* (1983) recorded the species from fry of *Tilapia* sp. and Basson Van As (1993) recorded it from cage-cultured rainbow trout, *Oncorhynchus mykiss*, in Qua-Qua, South Africa. Australia has populations of all three of these host species, and therefore any one of them is the potential source for this parasite in Australia. The Barron River drainage, however, harbours only one, *Tilapia* sp. (in this case *T. mariae*) as well as the closely-related cichlid *Oreochromis mossambicus*, seemingly identifying the source host species. Interestingly, however, *T. acuta* was not recovered from any of the *T. mariae* or *O. mossambicus* examined in this study. Until more specimens can be examined, *T. acuta* will remain an enigma in Australia.

Undoubtedly the most significant exotic trichodinid recovered in terms of host range and zoogeography was *Trichodina heterodentata*. The origin of Australian populations of *T. heterodentata* is unclear. The simplest explanation is that because it was originally described from cultured cichlids, *Oreochromis mossambicus*, in the Philippines by Duncan (1977), it has been imported to Australia with that species, which is now widespread along coastal drainages of Queensland (Mather and Arthington 1991). However, some specimens were collected from sites more than 1000 km away and on the other side of the Great Dividing Range from the nearest wild *O. mossambicus* population. This suggests either that the pattern of spread of this species in Australia is somewhat more complex or that *T. heterodentata* is, in fact, a species native to Australia, giving it a Gondwanan distribution. Assuming it is introduced, then a likely culprit in the dissemination of *T. heterodentata* in Australian waterways is the mosquitofish, *Gambusia holbrooki*. This species has spread to become perhaps the most widely-distributed freshwater fish in Australia (Merrick and Schmida 1984) and, unlike any other fish in the study, has a range which incorporates the entire known range of *T. heterodentata*. Mosquitofish may well have acted as a vector for spreading this parasite to the hosts and localities observed in the present study. The possibility that *T. heterodentata* is a native Australian species cannot, however, be excluded.

Native trichodinids

The native fishes of Australia harbour a species-rich fauna of native trichodinids, with at least 17 species of undescribed trichodinids detected from 33 species of native fishes examined from all three survey sources. From the sampling regimes employed, it was apparent that these species vary greatly in zoogeography, microhabitat-specificity and host-specificity and should not be regarded as a single homogenous assemblage. Undoubtedly, a number of cryptic species lie within the 17 native "species" discriminated in this study, also.

The richness of trichodinids recovered during the study can be used to estimate the biodiversity of Australian trichodinids, using the technique of Cribb *et al.* (1994). Native fishes for which more than 9 specimens were examined had a mean trichodinid richness of 2.4 species. Cribb *et al.* (1994) considered that 9 specimens was sufficient to conservatively estimate the parasite richness on a host. Given the host range of *T. heterodentata*, this can be assumed to represent 1.4 native trichodinids, plus *T. heterodentata*. Given the low sampling effort for most species (the most heavily-sampled species, *Hypseleotris klunzingeri*, harboured 5 trichodinid species, the most of any fish species) it seems reasonable to round this figure up to 2 trichodinid species per fish species. The average number of host species per native trichodinid species was also 2.4. Using the following formula, the species-richness of the Australian trichodinid fauna can be calculated.

$$R = \frac{h \times ph}{hp}$$

where R is the richness of the fauna, h is the number of host species, ph is the number of parasite species per host species and hp is the number of host species per parasite species. In this case:

$$R = \frac{180 \times 2}{2.4}$$

$$R = 150$$

In other words, it may be expected that the Australian native freshwater fish fauna will harbour about 150 species of trichodinids. This number approaches the total number of trichodinid species described from all host families to date!

Flux of trichodinids

We have shown that there have been host-switching events whereby parasites have crossed from native fishes to exotic and *vice versa*. In this study there were three records of host-switches involving trichodinids; *Trichodina* sp. J was recorded from *Gambusia holbrooki* at the crayfish farm near Beenleigh, Queensland; *Trichodina* sp. P was recorded from *Cyprinus carpio* from the same site; and *Trichodina heterodentata* was recorded from 11 native species and 6 exotic species at 13 different sites in Eastern Australia. The first record is somewhat intriguing in that *Trichodina* sp. J was not recorded from any other host at the crayfish farm near Beenleigh, however it's only other focus of infection, the Albert River, is the water source for the farm and the two water bodies may be considered to be freely interacting (Ben Brookman, pers. comm.). The second record is not surprising and supports the hypothesis proposed in the section above, that *Trichodina* sp. P is one of the species most likely to undergo host-switching, in this case to the exotic fish, *C. carpio*. It is the third case of host-switching, however, that provides the most informative data on parasite flux between native and exotic hosts.

Trichodina heterodentata was the most widespread and least host-specific trichodinid recovered. The fact that it is an exotic parasite makes this fact all the more remarkable. We contend that *T. heterodentata* is a parasite of emerging importance in studies of parasites of freshwater fishes, especially those which include species introductions. The fact that it was described as late as 1977 (Duncan 1977) demonstrates the lack of attention paid to the taxonomy of this group in the earlier 20th century. However, enough data have now been collected to indicate that *T. heterodentata* may be as important as any of the other, more prominent parasite species often associated with fish introductions: *Argulus* spp. (Branchiura), *Myxobolus cerebralis* (Myxozoa), *Bothriocephalus acheilognathi* (Cestoda), *Camallanus cotti* (Nematoda) and *Lernaea* spp. (Copepoda).

Van As and Basson (1989) discussed the biology of *T. heterodentata* in some detail. They contended that, although described from the Philippines, the parasite is almost certainly native to Eastern South Africa. They based their contention on the fact that they collected *T. heterodentata* from sites in the Limpopo River system which have never been exposed to exotic fish introductions, and that the host from which Duncan (1977) described the species, *Oreochromis mossambicus*, is native to that area. This contradicts the theory of Kazubski (1982b) that their material was not representative of *T. heterodentata* sensu Duncan (1977). Van As and Basson (1989) also recorded a number of new host records for *T. heterodentata*. A revised host list for this species is given in Table 1. The presence of this species on at least 50 host species including fishes from at least 16 diverse families and the tadpoles of three frog species indicates that it is a truly adaptable species.

The results of this study highlight the importance of taxonomy for studies of exotic animal phenomena. It is convenient to suggest that parasites are introduced with exotic fishes and that they are therefore directly responsible for declines in native fishes. That may be so in some cases, but it is far from a safe assumption. This study has clearly demonstrated that Australia has a unique and species-rich native trichodinid fauna, perhaps as many as 150 spp. and that species have crossed from native to exotic hosts and *vice versa*. These complex patterns of species richness and flux require a more rigorous approach to taxonomy than that which has been employed in the past.

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ACTA
PROTOZOOLOGICA

Description of a New Species with a Remarkable Cyst Structure in the Genus *Naegleria*: *Naegleria angularis* sp. n.

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Summary. A vahlkampfiid amoeba was isolated from a water sample collected in Peru. Ribosomal DNA sequence analysis showed that the isolate belongs to the genus *Naegleria*, that it represents a novel species and that its closest relative is *N. pussardi*. Although there is very little sequence difference between the new isolate and *N. pussardi*, and both species grow at 40°C, their cyst morphology is very different. The cysts of the new species are angular to star-like in appearance and so do not resemble the cysts of any described *Naegleria* species. The strain did not form flagellates in distilled water but did transform in Prescott's and James's solution. The name *N. angularis* sp. n. is proposed for this isolate with angular cysts.

Key words: 5.8S rDNA, cyst morphology, *Naegleria angularis* sp. n.

INTRODUCTION

Over the last decade, ribosomal DNA (rDNA) sequences of many strains of the family Vahlkampfiidae have been compared and the evolutionary relationships which have been inferred from these analyses are not congruent with the phenotype-based classification of vahlkampfiid species (De Jonckheere and Brown 1995, De Jonckheere 1997, De Jonckheere *et al.* 1997, Brown and De Jonckheere 1999). The least controversial consequence was the reassignment of most species of the phenotypically-diverse and poorly-defined genus *Vahlkampfia* to other genera (Brown and De Jonckheere 1999). Several *Vahlkampfia* species were reassigned to the genus *Tetramitus*, but these, and other vahlkampfiid species which cluster with the type species *T. rostratus* in rDNA phylogenetic trees, do not conform to the morphological definition of the genus (De Jonckheere and Brown 2003). Consequently the genus *Tetramitus* now includes phenotypically-diverse species which may (or may not) have a flagellate stage with (or without) a rostrum and with two (or four) flagella, and whose cysts may (or may not) have pores. Despite the observed phenotypic variation, all *Tetramitus* species have very similar internal transcribed spacer (ITS) and 5.8S rDNA sequences.

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The genus *Naegleria* is also now defined by molecular criteria (De Jonckheere 2002) but, in contrast with *Tetramitus*, somewhat less morphological variation exists within the genus, whilst ITS and 5.8S rDNA sequences of *Naegleria* species are more divergent. The morphological variation in *Naegleria* is mostly in the presence or absence of the flagellate stage (De Jonckheere *et al.* 2001), and whether or not the flagellate stage can divide. For example, *N. minor* was originally described as *Willaertia minor* on the basis that the flagellates can divide, as is the case in *W. magna* (Dobson *et al.* 1993). However, small subunit (SSU) rDNA sequence analysis demonstrated that *W. minor* is, in fact, a *Naegleria* sp. Similarly, *N. robinsoni* has a 5.8S rDNA sequence which clusters within the genus *Naegleria*, although also in this species the flagellates do divide (De Jonckheere and Brown 1999). Within the genus some slight variation is seen in the cyst morphology. Page (1975) attempted to differentiate *N. gruberi* strains on the basis of the cyst wall morphology, which he characterised as smooth, rough or angular. In fact, the use of molecular methods established later that the *N. gruberi* strains he investigated represented several distinct species. These species are *N. pringsheimi*, *N. pagei* (De Jonckheere 2002), *N. schusteri* and *N. americana* (De Jonckheere 2004). Subsequently, Pussard and Pons (1979) tried to differentiate *Naegleria* isolates on the basis of the number of pores in the cyst wall and the shape of the plugs in these pores. In addition to those species which Page investigated, Pussard and Pons included in their study the *N. gruberi* strains which were later differentiated by molecular techniques as *N. gallica*, *N. americana* (De Jonckheere 2004) and *N. gruberi sensu stricto* (De Jonckheere 2002), and two even turned out to belong to another genus, *Willaertia* (De Jonckheere *et al.* 1984). However, despite the fact that Page (1975) and Pussard and Pons (1979) observed significant differences in cyst morphology amongst *Naegleria* isolates which we now know are different species, they failed to obtain morphological criteria that could be used in species identification, because cyst morphology varies according to culture age and conditions.

Here we present a new *Naegleria* sp. which has a pronounced and consistent difference in cyst morphology from its closest relative, *N. pussardi*. These two species are very closely related: there is only a small difference in their ITS sequences (4 bp in the ITS1 and 3 bp in the ITS2) and their 5.8S rDNA sequences are identical.

MATERIALS AND METHODS

Strain T692 was isolated from a sample kindly provided by Humberto Guerra. The freshwater sample containing sediment was collected in Iquitos, Peru, on June 24, 2003, and held at ambient temperature until February, 2004, before it was processed for the isolation of amoebae. Sediment in the water sample was placed on non-nutrient (NN) agar streaked with *Escherichia coli* and the plates were incubated at 37°C. The vahlkampfiid strain which was isolated was later tested for growth at 40°C and 42°C. Light microscopical observations were made using Nikon Eclipse TS100 and Olympus BH2 microscopes.

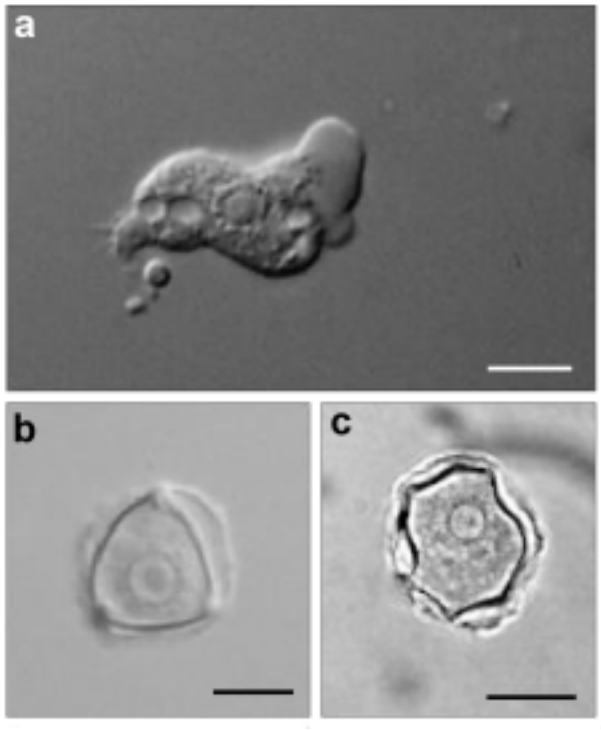
DNA was isolated from pelleted trophozoites of strain T692 using the UNSET method (Hugo *et al.* 1992). The ITS1, 5.8S and ITS2 rDNA were PCR-amplified using the ITS forward primer and the ITS reverse primer, corresponding to the 3' end of the SSU rDNA and the 5' end of the large subunit (LSU) rDNA, respectively (De Jonckheere 2004). Two pairs of ITS primers were employed, one pair designed for amplifying from *Naegleria* spp. specifically (De Jonckheere 1998) and a second less specific pair for amplifying from vahlkampfiid species (De Jonckheere and Brown 2003). Employing the latter primer pair the PCR product was sequenced (both strands) without cloning using a Beckman CEQ2000 sequencer using the CEQ Dye Terminator Cycle Sequencing kit (Beckman Coulter Inc., Fullerton, CA, USA).

The 5.8S rDNA sequences were aligned using ClustalX (Thompson *et al.* 1997) and the alignment was manually adjusted in SepPub9 (D. G. Gilbert, http://iubio.bio.indiana.edu). Because of long indels in the ITS1 and ITS2 sequences, these sequences were not included in the alignment. Phylogenetic trees were constructed from these alignments of 177 nucleotides using the DNAPARS (parsimony), DNADIST (distance matrix), NEIGHBOR (Neighbor joining and UPGMA) and Drawgram programs of the PHYLIP (version 3.572c) package (Felsenstein 1989).

The ITS1, 5.8S rDNA and ITS2 sequence of strain T692 has been deposited in EMBL with the accession number AJ785756.

RESULTS

Trophozoites of strain T692 (Fig. 1a) move very rapidly across the substratum. During steady, unidirectional, locomotion the trophozoites are limax, with length 28.8-40.0 µm (mean 33.5 µm) and breadth 4.8-8.0 µm (mean 6.9 µm); L/B ratio 3.8-5.8 (mean 4.9). Locomotory cells are not markedly eruptive, but the hyaloplasm does spill (from the front) to alternate sides, creating a slight left, then right, pattern of progression. The frontal hyaline zone is relatively-large; usually deeper than broad, often accounting for one third of the cell length. Trophozoites make frequent changes in direction and are very eruptive when changing direction and when stationary. All trophozoites observed had a perinuclear layer of small granules. The nucleus is vesicular and round, or oval if squashed. The nucleolus has a diameter approx.



Figs 1a-c. a - trophozoites of strain T692 showing the typical eruptive pseudopodes during locomotion; **b** - angular cyst of strain T692 with three pores clearly visible; **c** - more irregularly shaped cyst. Bars 10 µm.

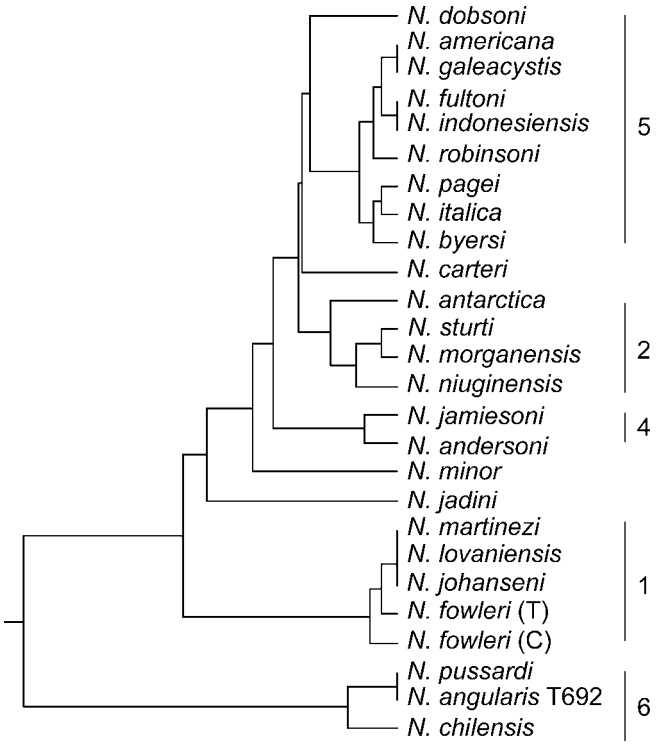


Fig. 2. Phylogenetic tree inferred from comparisons of the 5.8S rDNA sequences using the Kimura two-parameter correction and the UPGMA method. Numbers at the right indicate the clusters as defined previously (De Jonckheere 2004). Only cluster 3 (*N. carteri* and *N. minor*) does not form here.

one third to a half the diameter of the nucleus. All trophozoites observed were uninucleate, with one, two or three contractile vacuoles per cell. The posterior of the cell is rounded. Uroidal filaments were observed at the rear of a small proportion of cells.

Cysts have two walls and are unevenly-round or irregular in shape (Fig. 1c). The two walls always meet at a point at excystment pores. The cyst shape is often angular, usually with three or four points each ending at a pore. In the latter cases the cysts have a star-like appearance (Fig. 1b). The triangular and quadrangular cysts each account for approximately one third of cysts in a culture. Exact proportions cannot be provided as they vary from culture to culture. The proximity of the outer and inner cyst walls varies greatly, even around the perimeter of a single cyst. The diameter of the outer wall varies from 12.9-24.6 µm (mean 18.6 µm). All were uninucleate with a perinuclear layer of small granules.

A temporary flagellate stage was not observed when the trophozoites were suspended in distilled water. However, after suspension in Prescott's and James's solution (Page 1988) flagellates did appear. Strain T692 grows at 40°C but not at 42°C.

A strong PCR product was obtained when using the general vahlkampfiid ITS primers while only a faint band was obtained with the more specific *Naegleria* ITS primers. Therefore, the PCR product using the vahlkampfiid primers was sequenced using the same primers. The sequence lengths of the ITS1, 5.8S and ITS2 of strain T692 are 40, 176 and 91 bp, respectively. While the 5.8S rDNA sequence is identical to that of *N. pussardi* (De Jonckheere 2004), the strain differs from the latter species in 4 bp in the ITS sequence (Table 1) and 3 bp in the ITS2 sequence (Table 2). Because the 5.8S rDNA sequence of strain T692 is the same as that of *N. pussardi* both cluster in phylogenetic analyses of the aligned 5.8S rDNA with other *Naegleria* (Fig. 2), whichever method used.

DISCUSSION

Prior to the DNA sequence analysis, our initial phenotypic observations made us uncertain about which vahlkampfiid genus strain T692 belongs to. The amoe-

Table 1. ITS1 sequence.

	10 20 30 40
T692:	5′TTGGTAAAAGATTTGGGTAAAACCAAATTTT**T**TT**G**CC**TA**C3′
N.pussardi:	5′TTGGTAAAAGATTTGGGTAAAACCAAATTTT**A**TT**A**CC**CC**C3′

Table 2. ITS2 sequence.

	10 20 30 40
T692:	5′GCTGCTTTCGAGTTTGCGCCCTATTCAGAGGAAGCGTGTCAAAA**AAG**T
N.pussardi:	5′GCTGCTTTCGAGTTTGCGCCCTATTCAGAGGAAGCGTGTCAAAA-**TT**T
	50 60 70 80 90
	ATTTTTGATACGAATTTTAATCCGAAAGGATTTCAATTATTAC3′
	ATTTTTGATACGAATTTTAATCCGAAAGGATTTCAATTATTAC3′

bae have a layer of refractile granules around the nucleus which seems to be typical for the genera *Naegleria* and *Willaertia* (Page 1988), but the morphology of the cysts, together with the absence of transformation in distilled water, are not characteristic of these genera. Even so, the 5.8S rDNA sequence conclusively placed the strain within the genus *Naegleria*, although a better PCR product was obtained with the more general vahlkampfiid primers than with the more specific *Naegleria* primers. This unexpected result prompted the re-isolation, PCR-amplification and sequencing of DNA from strain T692 to check that the first result was not the consequence of mislabelling. However, the same result was obtained and was confirmed by determining the sequence three times in both directions.

Phylogenetic analyses of the 5.8S rDNA sequences with those of other *Naegleria* species revealed that strain T692 is most closely related to *N. pussardi* (Fig. 2). These two species, which have a 5.8S rDNA of 176 bp, constitute a separate branch 6 (De Jonckheere 2004) with *N. chilensis*, which has a 5.8S rDNA of 177 bp. The latter is the longest 5.8S rDNA length detected in *Naegleria*, as deduced from an alignment of *Naegleria* 5.8S rDNA sequences which is more accurate than the alignment published originally (De Jonckheere 2002). All other *Naegleria* spp. have a 5.8S rDNA length of 175 bp, except *N. andersoni*, *N. jamiesoni* (the two species in branch 4), and *N. carteri* (one of the two species in branch 3), which have a length of 174 bp.

Cysts of *N. pussardi* are typical in appearance for the *Naegleria* genus: round in shape with a double wall, and with plugged pores (Pernin and De Jonckheere 1996). The cysts of the new isolate T692 also have a double wall with pores, but the cyst shape is usually angular, often with three or four points ending in a pore. Although Page (1975) described angular cysts in *Naegleria* spp. he did not depict or describe cysts that had the star-like appearance which we observed in strain T692. When cysts have the star-like appearance they bear a superficial resemblance to those of *Acanthamoeba* strains, especially *A. triangularis*. Therefore the cyst also resembles the cyst of *Vahlkampfia angularis* (Robinson 1980). The species *V. angularis* is not a valid name, because it has never been published in a regular journal but a description of it is in preparation using strains that were isolated more recently (Robinson *et al.*, in preparation). The absence of the flagellate stage made Robinson assign his isolates to the genus *Vahlkampfia*, despite the presence of pores in its cysts. Unpublished molecular results (JFDJ) indicate that the new strains of *V. angularis* do not belong to the genus *Naegleria* nor to the genus *Vahlkampfia*, but to the genus *Tetramitus*. Hence the strains of B. Robinson will be described as *T. angularis*. In contrast to *T. angularis*, which was reported to be unable to grow above 30°C, strain T692 does grow very quickly at 37°C. It even grows at 40°C, but not at 42°C. In its temperature tolerance it resembles *N. pussardi*, its phylogenetically closest relative.

Because of the morphological similarity of the cysts with those of *V. angularis* we decided to also use the species name *angularis* for strain T692 which, however, belongs to the genus *Naegleria*. The special morphology of the cysts of this new species further increases the degree of phenotypic diversity observed within the genus *Naegleria*.

Description of *Naegleria angularis* sp. n.

The trophozoites have the typical appearance of *Naegleria* and they can be induced to transform into flagellates. The cysts have an angular shape, with the points ending in a pore. Because of the angular shape a high proportion of the cysts have a star-like appearance, not seen in any other described *Naegleria* sp. The strain grows at 40°C, but not at 42°C.

Because of the morphological similarity of the cysts with those of *T. angularis* molecular identification is required, especially since flagellates might be difficult to obtain in *N. angularis*. The species can be identified from the ITS1 and ITS2 sequences, which differ by 4 bp and 3 bp, respectively, from those of *N. pussardi*. The 5.8S rDNA sequence is identical to that of *N. pussardi*.

The type strain was isolated from a water/sediment sample collected in Peru.

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Gregarina tibengae sp. n. (Apicomplexa: Eugregarinida) Described from *Zophobas atratus* Fabricius, 1775 (Coleoptera: Tenebrionidae)

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Summary. *Gregarina tibengae* sp. n. (Apicomplexa: Eugregarinida) is described from larvae and adults of *Zophobas atratus* (Coleoptera: Tenebrionidae). Trophozoites are solitary, attached to intestinal epithelium or located between epithelium and peritrophic membrane. Epimerites are spherical, ellipsoid, ovoid, or conical; protomerites are very broadly ovoid to depressed ovoid; deutomerites are obovoid to broadly obovoid. Association is precocious, caudofrontal and biassociative. Assocations are located between intestinal epithelium and peritrophic membrane; early associations are axial-symmetric, late associations bilateral-symmetric. Primite protomerites are very broadly to depressed ovoid in early associations, broadly obovoid to very broadly obovoid in late associations. Primite deutomerites are obovoid to broadly obovoid, panduriform, with truncated posterior end. Satellite protomerites are very broadly ovoid to depressed ovoid; satellite deutomerites are obovoid to broadly obovoid, or cylindrical, panduriform, with truncated and emarginated posterior end. Total length of association 96 to 611 µm. Gametocysts are spherical, form 7-12 sporoducts. Oocysts are ellipsoid and measure 5.8 by 4.3 µm. They dehisce in chains.

Key words: Apicomplexa, cultured insects, Eugregarinida, *Gregarina tibengae* sp. n., Tenebrionidae, *Zophobas atratus.*

Abbreviations: AVEC-DIC - Allen video enhanced differential interference contrast, DIC - differential interference contrast, LD - deutomerite length, LE - epimerite length, LG - gametocyst length, LO - ooycst length, LP - protomerite length, ND - nucleus diameter, NL - nucleus length, NoD - nucleolus diameter, Pri - primate, Sat - satellite, TL - total length, TLA - total length of association, WD - deutomerite width, WE - epimerite width, WEO - equatorial oocyst width, WG - gametocyst width, WLO - width of oocyst ligaments, WP - protomerite width, ZSRO - Zoological Collection of the University of Rostock, Germany.

INTRODUCTION

Théodoridès and Jolivet (1990) described *Gregarina zophobasi*, a eugregarine parasite from the darkling beetle *Zophobas opacus* Sahlberg, 1823 in Brazil. In 1977 a related coleopteran, *Z. atratus* Fabricius, 1775 (syn. *Z. morio* Kraatz, 1880), was introduced from its habitat in Central and South America to Germany by Pepe Alcaraz (Friederich and Volland 1992). It is now commercially cultured as a forage insect for insectivorous animals. The present study reports a new eugregarine species, *G. tibengae* sp. n., from the intestine of *Z. atratus* larvae and adults.

MATERIALS AND METHODS

Zophobas atratus larvae were purchased from commercial sources (Tropenwelt, Hamburg; Faunatopics, Marbach/Neckar; Germany) and subcultured by the author. Friederich and Volland (1992) give information on the cultivation of *Zophobas*. For this study the larvae

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were kept at 23-27°C in the dark. Rolled oats and pieces of apple and carrot were provided as food items.

Zophobas atratus larvae were eviscerated and intestines were dissected in Ringer's solution for insects (Seifert 1995) under a dissecting microscope. The gregarines were separated from the luminal content and transferred on cover slips using a pipette. They were fixed (2% glutaraldehyde in 0.05% Na-cacodylate buffer, pH 7.4) and hanging drop preparations were made. Measurements were taken using a micrometer; drawings were made with the aid of a drawing tube.

For permanent preparation wet smears were fixed in AFA (Seifert 1995) for 10 min, stained with Masson's haematoxyline according to Romais (1989) and counterstained in a 2% aqueous eosin solution, dehydrated in ascending ethanol concentrations, 100% n-propanol, Rotihistol (Carl Roth GmbH & Co, Karlsruhe, Germany) and mounted in neutral balsam. Gametocysts were collected from the intestine and the feces, transferred in Ringer's solution on cover slips and held in moist chambers for maturation and dehiscence. The obtained oocysts were fixed in 2% picric acid in Ringer's solution.

Photomicrographs of live gregarines in Ringer's solution were taken using a Zeiss Axiovert (bright-field, phase contrast) and a Reichert-Jung Polyvar (DIC). AVEC-DIC photomicrographs of oocysts were made using a Nikon Diavert with Hamamatsu Photonic Microscopy System C1966.

For morphometric measurements only gregarines from larvae of the last and last but one instar were used. Measurements were taken at their widest points, except for deutomerite lengths that were calculated as differences of total lengths and protomerite lengths. Measurements and indices in micrometers (µm) are given as range values followed by means, standard deviations, and sample sizes in parentheses. For the most part they are those used by Clopton (1998). Terminology of shapes follows that suggested by the Systematics Association Committee for Descriptive Biological Terminology (1962).

RESULTS

Taxonomic position: Eugregarinida Léger, 1900 *sensu* Levine *et al*. (1980); Septatina Lankester, 1885 *sensu* Levine *et al*. (1980); Gregarinicae Chakaravarty, 1960; Gregarinidae Labbé, 1889 *sensu* Levine (1988), with characters of the genus *Gregarina* Dufour, 1828 *sensu* Clopton (2002): epimerite conical, button-shaped, globular, or cylindrical; association precocious; oocysts doliform, navicular, or spherical; in intestine of insects.

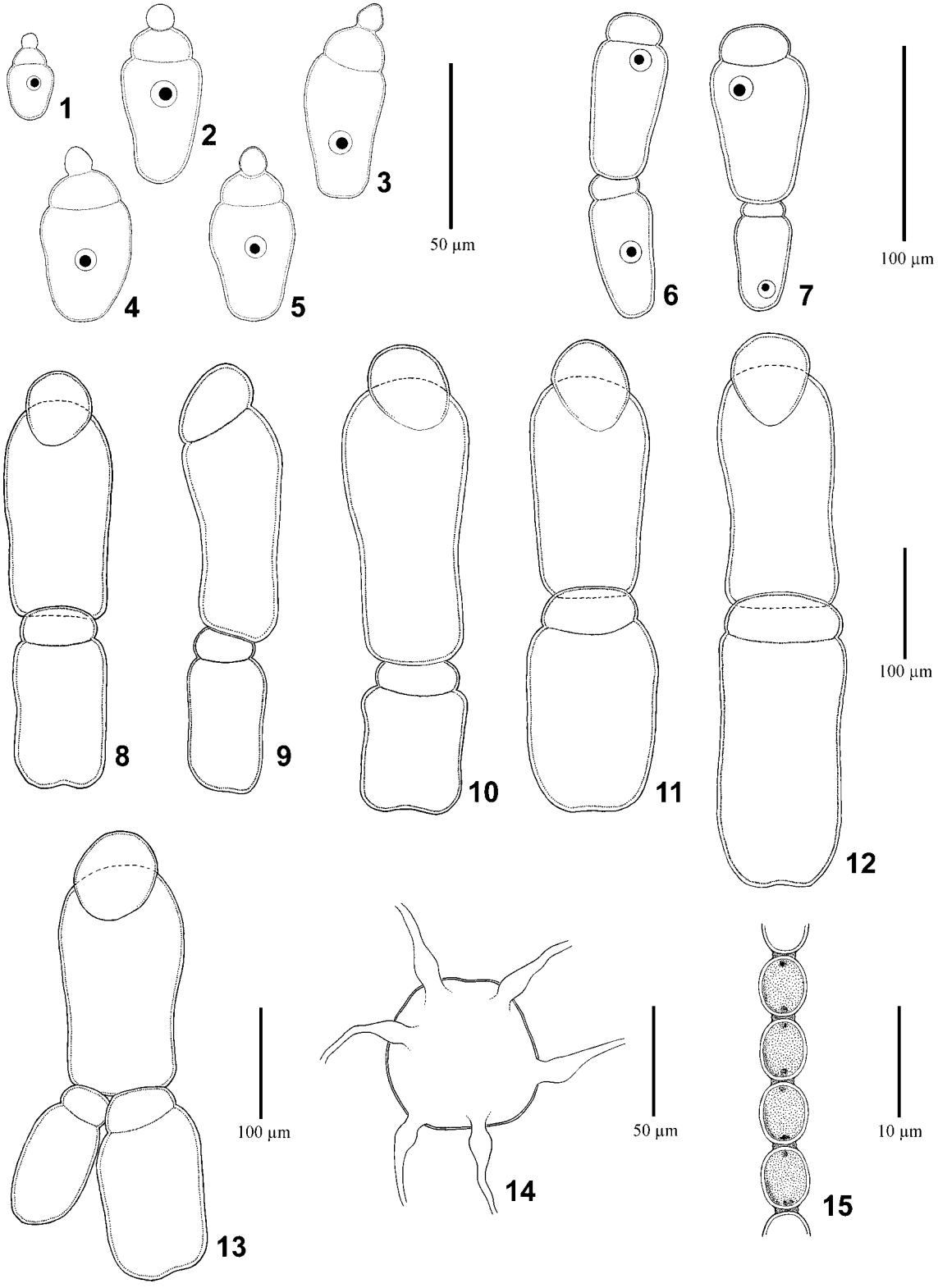
Gregarina tibengae sp. n.

Trophozoite (Figs 1-5, 17): Cephalont (Figs 1-5; measurements and indices given in Table 1) attached to host intestinal epithelium by epimerite, in posterior half of mesenteron. Solitary, never in association. Earliest cephalonts circular to broadly ovoid (Fig. 1), minor constriction at protomerite-deutomerite septum. Older cephalonts more oblong. Epimerite simple, spherical (Fig. 2), ellipsoid to broadly ovoid (Fig. 5), or cone-shaped (Figs 3, 4); without diamerite; surface smooth, without folds or impressions. Protomerite very broadly to depressed ovoid, tapered anteriorly to junction with epimerite; moderate posterior constriction at protomerite-deutomerite septum. Deutomerite shape obovoid to broadly obovoid, with shallow sinuate margin at half to second third of length on each side, rounded to truncated posteriorly, maximum width at anterior third; septum concave. Nucleus spherical; typically with single spherical nucleolus, eccentrical in location, young cephalonts occasionally with 2-3 nucleoli. Sporont (Fig. 17) solitary; free, located between host intestinal epithelium and peritrophic membrane; morphology similar to late cephalonts but epimerite absent; epimerite residue absent.

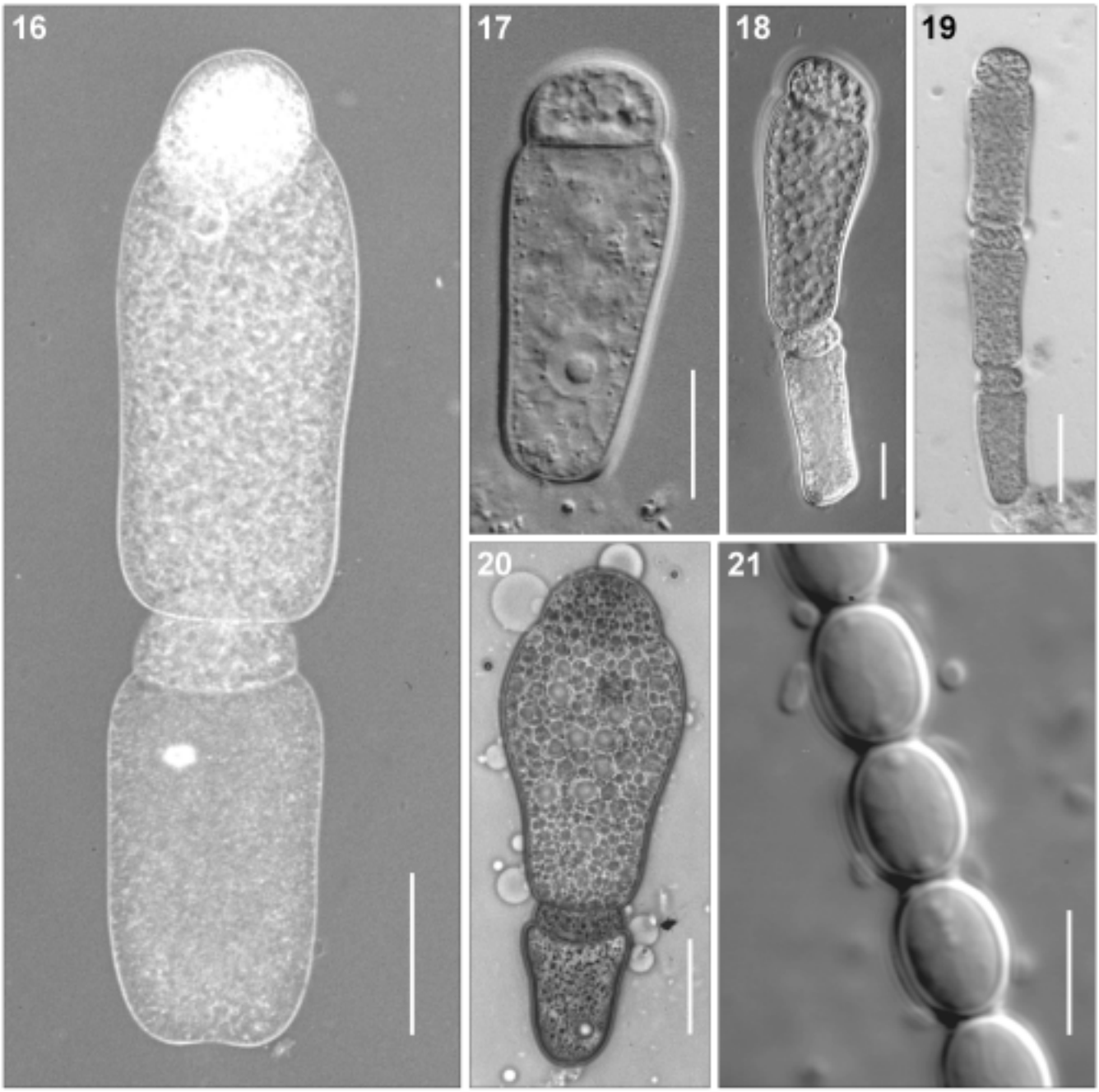
Association (Figs 6-13, 16, 18-20): Measurements and indices given in Table 1). Syzygy early (precocious), caudofrontal; multiple associations of one primite with two satellites occasionally observed (Fig. 13), linear multiple association seen only once (Fig. 19); located between host intestinal epithelium and peritrophic membrane, in second third of mesenteron of host. Early associations axial-symmetric (Figs 6, 7), morphology of gamonts similar to that of sporonts, some associations with noticeable small satellites (Fig. 20). Late associations bilaterally symmetric, dorsoventrally flattened, the side bearing the contact areas between primite protomerite and primite deutomerite as well as between primite deutomerite and satellite protomerite is (hereby) defined as "ventral" (on the left in Fig. 9).

Primite: Protomerite very broadly to depressed ovoid in early associations; or broadly to very broadly obovoid in late associations, situated on ventral side; with moderate constriction at protomerite-deutomerite septum. Deutomerite obovoid to broadly obovoid; anterior end rounded; septum concave; with shallow to moderate sinuate margin at half to second third of length on each side (panduriform); maximum width typically in anterior third, in some late associations anterior and posterior third with similar width, maximum width rarely in posterior third, close to the posterior end (never more than 10 µm wider than anterior third); truncated posteriorly. Nucleus spherical; abaxial in location; in all parts of deutomerite, mostly in anterior half; concealed by granula in late associations; with one spherical nucleolus, eccentrically located.

Satellite: Protomerite very broadly to depressed ovoid; contact area with primite deutomerite ventral; rounded anteriorly; with minor constriction at protomer-



Figs 1-15. *Gregarina tibengae* sp. n. **1-5** - cephalonts; **6-7** - early associations; **8, 10-12** - late associations, ventral view; **9** - late association, lateral view; **13** - multiple association; **14** - dehisced gametocyst with sporoducts; **15** - oocysts.



Figs 16-21. *Gregarina tibengae* sp. n. **16** - late association (bright-field, inverted); **17** - sporont (DIC); **18** - early association (DIC); **19** - linear multiple association (DIC, inverted); **20** - early association (phase-contrast, inverted); **21** - oocysts (AVEC-DIC). Scale bars 5 µm (21); 20 µm (17, 18); 50 µm (19, 20); 100 µm (16).

ite-deutomerite septum. Deutomerite of early associations obconoid to obovoid; with shallow sinuate margin in middle to second third of length; rounded or truncated posteriorly. Deutomerite of late associations obovoid to broadly obovoid or cylindrical, often with square outline (Fig. 10); septum concave; with shallow to moderate sinuate margin in middle of length; truncated and emarginated posterior end, sinus elongated anteriorly

Table 1. Measurements (in µm) and indices of trophozoites and associations of *G. tibengae* sp. n.

Measurements	Trophozoites	Associations	
	Cephalonts	Primites	Satellites
LE	2-11 (**6** ± 1.7; 78)		
WE	5-10 (**7** ± 1.2; 78)		
WE/LE	0.60-3.00 (**1.27** ± 0.41; 78)		
LP	3-22 (**12** ± 4.1; 78)	15-99 (**54** ± 23.8; 202)	6-61 (**30** ± 13.0; 202)
WP	11-35 (**20** ± 5.2; 78)	25-91 (**57** ± 18.4; 202)	13-135 (**62** ± 29.5; 202)
WP/LP	1.31-3.67 (**1.83** ± 0.44; 78)	0.77-1.93 (**1.15** ± 0.29; 202)	1.31-3.29 (**2.07** ± 0.33; 202)
LD	12-89 (**39** ± 15.1; 78)	51-263 (**141** ± 52.0; 202)	24-258 (**125** ± 58.0; 202)
WD	13-55 (**27** ± 7.9; 78)	31-138 (**82** ± 29.9; 202)	15-157 (**74** ± 35.2; 202)
WD/LD	0.45-1.33 (**0.71** ± 0.14; 78)	0.41-0.79 (**0.59** ± 0.08; 202)	0.39-0.91 (**0.59** ± 0.09; 202)
TL	20-119 (**57** ± 20.1; 78)	66-343 (**195** ± 74.4; 202)	30-309 (**154** ± 70.4; 202)
LE/TL	0.06-0.21 (**0.11** ± 0.03; 78)		
LP/TL	0.14-0.29 (**0.21** ± 0.03; 78)	0.18-0.38 (**0.27** ± 0.04; 202)	0.12-0.30 (**0.19** ± 0.03; 202)
LD/TL	0.57-0.78 (**0.69** ± 0.04; 78)	0.63-0.82 (**0.73** ± 0.04; 202)	0.70-0.95 (**0.81** ± 0.03; 202)
LP/LD	0.20-0.50 (**0.30** ± 0.05; 78)	0.22-0.60 (**0.37** ± 0.08; 202)	0.14-0.42 (**0.24** ± 0.04; 202)
WP/WD	0.64-0.95 (**0.77** ± 0.06; 78)	0.59-0.91 (**0.71** ± 0.07; 202)	0.65-1.00 (**0.84** ± 0.07; 202)
ND	4-11 (**6** ± 1.6; 54)	8-32 (**17** ± 6.1; 102)	4-28 (**13** ± 5.0; 81)
NoD	2-4 (**3** ± 0.8; 52)	3-12 (**7** ± 2.2; 102)	2-11 (**6** ± 2.2; 81)
TLA		96-611 (**345** ± 137.5; 202)	
Pri TL/TLA		0.46-0.77 (**0.57** ± 0.05; 202)	
Sat TL/TLA		0.30-0.55 (**0.44** ± 0.05; 202)	
Pri WD/Sat WD		0.75-2.57 (**1.20** ± 0.31; 202)	
Pri WD/Sat WP		0.90-2.95 (**1.44** ± 0.34; 202)	

boldface type - mean

into shallow longitudinal groove. Nucleus spherical; abaxial in location, in all parts of deutomerite, mostly in posterior half; concealed by granula in late associations; with one spherical nucleolus; eccentrically located. In developing associations satellites smaller than primites (Figs 8-10); in mature associations satellites reach or exceed primite length and width. Index Pri TL/TLA decreases in large associations; satellite size increases in

Table 2. Characters of late associations of *G. zophobasi* and *G. tibengae* sp. n. Measurements in µm.

Character	*G. zophobasi*	*G. tibengae* sp. n.
Primite		
Maximum TL	> 400	343
WD	**50**	31-138 (**82**)
Protomerite	anterior dilatation, ampullaceous or "champignon" aspect, situated on anterior pole	very broadly obovoid, without anterior dilatation, situated on ventral side
Satellite		
Maximum TL	> 300	309
WD	**70**	15-157 (**74**)
Protomerite	quadrangular, fine transversal epicytical striation	depressed ovoid, striation absent
Deutomerite	attenuated posterior end	emarginated posterior end
Protomerite-deutomerite septum	triangular	concave
Nucleus	ellipsoid, > 30 µm in lenght, always in posterior part of deutomerite	spherical, up to 32 µm in diameter, in all parts of deutomerite, mostly in anterior part

boldface type - mean; >: exceeding

Table 3. Measurements (in µm) and characters of species similar to *G. tibengae* sp. n. 1 - *G. amoji*, 2 - *G. ceropriae*, 3 - *G. decourti*, 4 - *G. dragescoi*, 5 - *G. gonocephali*, 6 - *G. inclinata*, 7 - *G. joliveti*, 8 - *G. maculata* var. *banulensis*, 9 - *G. mesomorphi*, 10 - *G. niphandrodes*, 11 - *G. steini*, 12 - *G. tibengae*

Character	1	2	3	4	5	6	7	8	9	10	11	12
Associations												
TLA	-	-	-	> 1000	> 175	355-470	-	-	-	-	-	96-611 (**345**)
Pri TL	-	?-200	[> 300]	430-550	78-91	160-203	165-300	115-165	250-300	143-377 (**233**)	108-187 (**147**)	66-343 (**195**)
Pri WD	-	45	-	-	36-38	63-85	> 300	-	108-149	102-235 (**146**)	13-33 (**20**)	31-138 (**82**)
Pri LP/TL	-	-	-	-	0.17-0.22	0.17-0.30	-	-	**0.17**	0.17-0.40 (**0.26**)	0.10-0.18 (**0.13**)	0.18-0.38 (**0.27**)
Sat TL	-	?-300	[> 350]	445-540	78-91	181-260	150-400	115-145	250-300	102-347 (**218**)	84-141 (**113**)	30-309 (**154**)
Sat WD	-	45	-	-	33-40	52-85	-	-	108-149	61-214 (**114**)	11-22 (**15**)	15-157 (**74**)
Sat LP/TL	-	-	-	-	0.12-0.18	0.11-0.17	-	-	**0.13**	0.15-0.33 (**0.19**)	0.10-0.17 (**0.13**)	0.12-0.30 (**0.19**)
Bilateral symmetry	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
Curved	no	yes	yes	no	yes	yes	no	yes	yes	yes	no	yes
Margine of deutomerites	sinuate	sinuate	convex	sinuate	sinuate or convex	sinuate	convex	sinuate	convex	convex	sinuate	sinuate
Panduriform deutomerites in late associations	yes	no	no	no	no	no	no	yes	no	no	yes	yes
Emaginated posterior end of satellite deutomerite	no	no	no	no	no	no	no	no	no	no	no	yes
Nuclei	spherical	ellipsoid	spherical	ellipsoid	spherical	spherical	ellipsoid	ellipsoid	spherical	spherical	spherical	spherical
Pri NL; Sat NL	-	30; 30	-	-	8-16; 8-16	**20; 20**	40-50; 40-50	-	**34; 30**	**36; 36**	**18; 18**	**32; 28**
Gametocysts												
Shape	ellipsoid	ellipsoid	-	-	-	spherical or ellipsoid	spherical	-	spherical	spherical	ellipsoid	spherical
Diameter	70 x 54	140-200	-	-	-	**200**	150	-	200	225	85 × 60 - 160 × 100[2]	146-191 (**167**)
Oocysts:												
LO × WO	4.4 × 2.3	4 × 2	-	-	-	6 × 4	6 × 3	-	5.6 × 4.2	8.8 × 5.5	4.9 × 4.9[1], 5 × 3[2]	5.8 × 4.3

Boldface - means; > - exceeding; [ ] - not given by the authors, assumed from illustration; [1] from Clopton *et al*. (1991); [2] from Geus (1969)

large associations; indices Pri WD/Sat WD and Pri WD/Sat WP constantly decreasing during growth of association.

Cytology: Cytoplasm brownish; primite cytoplasm always filled with numerous large vesicles; vesicles absent in satellite cytoplasm (Fig. 18); paraglycogene glanula present in both cells.

Gametocysts (Fig. 14): Roughly spherical; with hyaline gametocyst coat, coat thickness (in Ringer's solution) 2-6 µm; located between host intestinal epithelium and peritrophic membrane, egested with feces. Gametocysts isolated from intestine: LG (including coat) 139-176 (**161** ± 9.1, 21), WG 129-173 (**153** ± 11.1, 21), WG/LG 0.87-1.0 (**0.95** ± 0.04, 21). Gametocysts collected from feces: LG 146-191 (**167** ± 15.0, 15), WG 173-187 (**159** ± 15.2, 15), WG/LG 0.90-1.0 (**0.96** ± 0.03, 15). Gametocysts collected from fresh feces mature and dehisce within 50-80 h. Oocysts extruded in chains through sporoducts. Sporoducts with wide basis, proximal bulbus and distal tube; length 25-50 µm; usually 7-12 per gametocyst, sometimes more primordial but not evaginated.

Oocysts (Figs 15, 21): Ellipsoid, connected in chains by "ligaments"; uniform in size and shape; LO 5.7-6.2 (**5.8** ± 0.13, 64), WEO 4.0-4.5 (**4.3** ± 0.11, 64), WLO 1.7-2.3 (**2.1** ± 0.1, 64), WEO/LO 0.68-0.77 (**0.74** ± 0.02, 64), WLO/LO 0.27-0.39 (**0.36** ± 0.02, 64), WLO/WEO 0.38-0.54 (**0.48** ± 0.03, 64); cross-section roughly circular; two, sometimes numerous, polar oocyst residua present.

Taxonomic summary

Type specimens: Eight syntype slides (19 March 2002-17 October 2002) were deposited in the ZSRO (# ZSRO Pro 0001a - Pro 0001h; label: *Gregarina tibengae* (Apic., Eugregarinida) ex. *Zophobas atratus* Fabricius, 1775 (Col., Tenebrionidae), [syntype], leg. & det. Martin Jahnke, Germany: Rostock, cultured host).

Type host: *Zophobas atratus* Fabricius, 1775 (Tenebrionidae: Tenebrioninae: Tenebrionini); larvae and adults

Symbiotype: One adult host specimen was deposited in the ZSRO (# ZSRO Pro 0001i; label: Germany, Rostock, 2003-V-12, leg. & det. Martin Jahnke, Tenebrionidae, *Zophobas atratus* Fabricius, 1775, cultured, [symbiotype], type host of *Gregarina tibengae*). Two additional voucher specimens were labeled analogous and have got collection numbers ZSRO Pro 0001j - ZSRO Pro 0001k.

Infection site: Mesenteron.

Prevalence: High; 83% (n=30).

Type locality: Germany, Rostock

Other localities: Germany: Hamburg, Marbach (Neckar), Tübingen; cultured.

Etymology: The specific epithet refers to the Swabian word "Tibenga" for Tübingen were the author found this gregarine for the first time.

DISCUSSION

The present gregarine is the second eugregarine species described from *Zophobas*. A comparision of the characters of *Gregarina zophobasi* Théodoridès *et* Jolivet, 1990 and *G. tibengae* is given in Table 2. In both species the gamonts of late associations are alike in size. However, *G. zophobasi* can be distinguished from *G. tibengae* based on its elongated gamonts, the "champignon" view of primite protomerites, the attenuated posterior end of deutomerite, the triangular shape of the protomerite-deutomerite septum, and the ellipsoid nucleus which is always situated in the posterior part of deutomerite. Of the 56 species of *Gregarina* known from Tenebrionid beetle hosts, the following 11 species exhibit a similar primite protomerite: *G. amoji* Datta, Ghose *et* Haldar, 1991, *G. ceropriae* Théodoridès *et* Desportes, 1967, *G. decourti* Théodoridès, Desportes *et* Jolivet, 1975, G. *dragescoi* Théodoridès *et* Desportes, 1966, *G. gonocephali*, Obata, 1953, *G. inclinata* Hoshide, 1979, *G. joliveti* Théodoridès, 1958, *G. maculata* Léger, 1904 var. *banyulensis* Théodoridès, 1955, *G. mesomorphi* Devdhar *et* Deshpande, 1971, *G. niphandrodes* Clopton, Percival *et* Janovy, 1991, and *G. steini* Berndt, 1902. A comparision of these species with *G. tibengae* is given in Table 3. The associations of all species are bilateral-symmetric and most are slightly curved, similar to those of *G. tibengae*. *Gregarina decourti*, *G. joliveti*, *G. mesomorphi*, and *G. niphandrodes* can be destinguished from *G. tibengae* based on their convex margins of deutomerites. In *G. gonocephali* only early associations have deutomerites with sinuate margins wereas those of late associations are convex. *Gregarina amoji*, *G. ceropriae*, *G. dragescoi*, *G. inclinata*, *G. maculata* var. *banyulensis*, and *G. steini* exhibit sinuate margins of deutomerites but only *G. amoji*, *G. maculata* var. *banyulensis*, and *G. steini* possess panduriform deutomerites similar to those of *G. tibengae*. *Gregarina amoji* lack the emarginated posterior end

of the satellites, moreover, *G. amoji* has ellipsoid gametocysts. *Gregarina ceropriae*, *G. maculata* var. *banyulensis*, and *G. steini* can be distinguished from *G. tibengae* based on their attenuate deutomerites. *Gregarina inclinata* possess characteristic unciform associations different from *G. tibengae*. In comparision with *G. tibengae* the associations of *G.dragescoi* are plainly larger (maximum TLA 611 µm versus >1000 µm). The combination of diagnostic characters of the described gregarine, especially associations possessing bilateral symmetry, panduriform margin of deutomerites, and emarginated posterior end of satellite deutomerite, seem to differentiate it from known *Gregarina* species of Tenebrionidae.

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Filamoeba sinensis sp. n., a second species of the genus *Filamoeba* Page, 1967, isolated from gills of *Carassius gibelio* (Bloch, 1782)

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Summary. *Filamoeba sinensis*, a new species of amphizoic amoeba isolated from the gills of *Carassius gibelio* (Bloch, 1782) sampled in Hubei Province, China, is described. Both morphology and SSU rRNA gene sequences were employed to compare this species with *Filamoeba nolandi* Page, 1967, the type and only species of the genus. Trophozoites differ from *F. nolandi* in having robust multipolarly branched pseudopodia. A less pronounced hyaloplasmic zone produces a lower number of filiform subpseudopodia. The greatest dimensions of extended trophic forms reach 40-50 µm. Cysts, with a relatively thick and homogeneous wall, are smooth and free of pores. The diameter of rounded cysts ranges from 14 to 20 µm. Cristae of mitochondria are tubular. They are arranged in parallel or irregular mutual positions, their anastomoses or branches were not observed. Phylogenetic analysis revealed a close relationship to *F. nolandi*.

Key words: amphizoic amoebae, *Filamoeba sinensis* sp. n., morphology, SSU rDNA, phylogeny.

INTRODUCTION

Despite the widely recognised diversity of organisms currently assigned to Amoebozoa Lühe, 1913 *sensu* Cavalier-Smith (2003), diagnostic criteria are not well defined within all groups of amoebae. Morphological features that could possibly differentiate the taxa have not been adequately described in "neglected" groups of amoebae. It is most probably due to the exceptional variability of these protists, methods of observation available in amoeba research as time progressed and, possibly, also due to the rare frequency of many findings. As a consequence of diagnostic difficulties, many species have not been reported again since their descriptions and some were never accommodated to suprageneric taxons. Molecular characterisation of amoebae based mostly on SSU rRNA gene sequences has become gradually an integral part of the description of new species. For synthesis of both morphological and molecular approaches in amoeba studies, molecular characterisations of type strains are of a great value, since they facilitate comparisons and serve as a basis for studies of intra-amoebozoan phylogeny.

A freshwater amoeba described as *Filamoeba nolandi* Page, 1967 was isolated from shallow water on

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the sandy shore of Little Deer Lake in Itasca County, Minnesota. Prior to its description in 1964, Page deposited the strain in the former Culture Collection of Algae and Protozoa (CCAP), presently the UK National Culture Collection (UKNCC). Page (1967) did not settle the systematic position of this organism in the original description. In his publications "An Illustrated Key to Freshwater and Soil Amoebae" and "A New Key to Freshwater and Soil Amoebae" Page (1976, 1988) placed the genus *Filamoeba* in the family Echinamoebidae Page, 1975. Phylogenetic analyses published thus far (Amaral Zettler *et al*. 2000, Bolivar *et al*. 2001, Peglar *et al*. 2003) included the SSU rRNA gene sequence of American Type Culture Collection (ATCC 50430) strain of *F. nolandi* (NC-AS-23-1) and not of that deposited in UKNCC as a type strain of the species. Contrary to the type strain, the ATCC strain denoted as *F. nolandi* (NC-AS-23-1) is of marine origin. It was isolated from a sediment core 23 nautical miles off coast of Wilmington, NC, in 1993.

This paper presents a new amoeba species, the second one of the genus *Filamoeba* Page, 1967, which was isolated from a freshwater fish host.

MATERIALS AND METHODS

The strain denoted as CH26, was isolated from gill tissue of *Carassius gibelio* (Bloch, 1782) sampled in Hubei Province, China, in April 2002. It was recovered from decomposing tissue stored for 14 days in eppendorf tubes and washed repeatedly with sterile tap water in order to separate spores of *Myxobolus* sp. detected in gill tissue of the opposite gill arch. A homogeneous population of trophozoites (passage No. 8), cultured on non-nutrient agar was cryopreserved in September 2002. A clone was derived in November of the same year from passage No. 13.

Amoeba trophozoites were observed in hanging drop preparations using an Olympus BX51 microscope equipped with the Nomarski DIC system. For ultrastructural studies, trophozoites and cysts were fixed on agar plates with 2.5% cacodylate buffered glutaraldehyde, then pelleted *via* centrifugation, postfixed with cacodylate buffered 1% osmium tetroxide, dehydrated with series of acetones and embedded in Spurr's resin. The same methods of culturing, observation and harvesting were applied to the strain of *Filamoeba nolandi*. It was ordered for purposes of comparison from UKNCC where it is listed in the type culture collection as CCAP 1526/1 strain.

Our original clone used in this study was cryopreserved and is stored in the culture collection of the Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice.

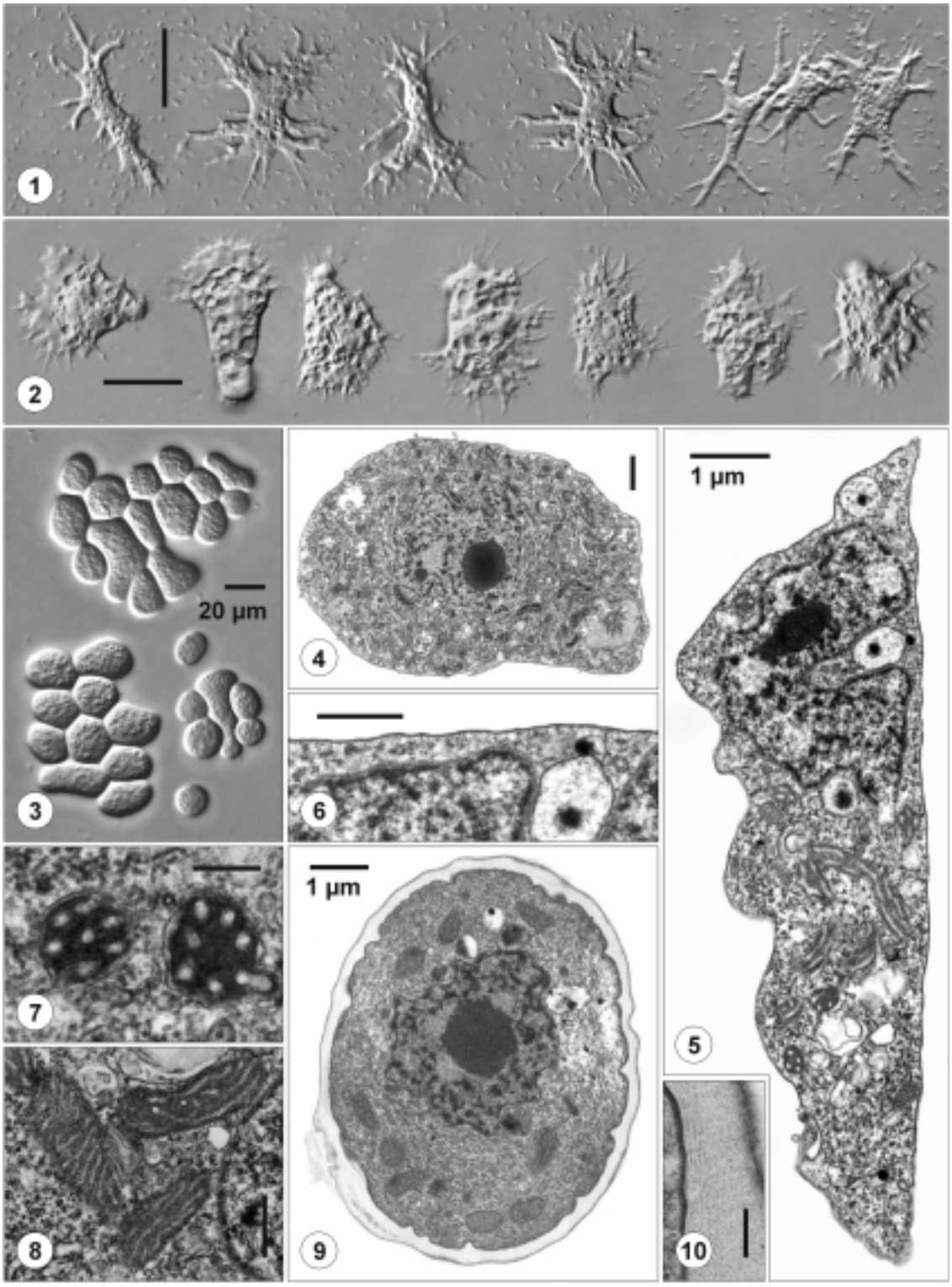
DNA isolation, amplification and sequencing. Morphological characterisation of clones derived from both strains (CH26/I and CCAP 1526/1) was completed with phylogenetic analysis of small-subunit ribosomal RNA gene sequences. DNA was extracted from the cell pellets using the DNeasy™ Tissue kit, according to the protocol of the manufacturer (Qiagen). The SSU rRNA genes of both strains were amplified by polymerase chain reaction (PCR) using universal eukaryotic primers ERIB 1 (5´- ACCTGGTTGAT CCTGCCAG - 3´) and ERIB 10 (5´ - CTTCCGCAGGTTCACCTACGG - 3´) (Barta *et al*. 1997). Each 25 µl reaction included 50 ng of genomic DNA, 10 pmol of each oligonucleotide, 250 ìM of each dNTP, 2.5 µl 10 × PCR Buffer (Top-Bio, Czech Republic), and 1 unit of Taq-Purple polymerase (Top-Bio, Czech Republic). The mixture was preheated 5 min at 95°C. Thermocycling conditions were as follows: 5 cycles consisting of 94°C for 1 min, 44°C for 1.5 min and 72°C for 2 min, 25 cycles consisting of 94°C for 1 min, 48°C for 1.5 min and 72°C for 2 min, then a 10 min incubation at 72°C. The PCR products were isolated from agarose and cloned into the pCR® 2.1 TOPO cloning vector (Invitrogen). Vectors with inserts were sequenced in both directions using CEQ DTCS Dye Kit (Beckman Coulter) on the CEQ TM 2000 automatic sequencer (Beckman Coulter).

Alignments and phylogenetic analyses. The alignments were done using the Clustal_X program (Thompson *et al*. 1997) with various alignment parameters. Ambiguously aligned regions were excluded. The SSU rRNA gene sequences from the newly sequenced *Filamoeba* strains were aligned against sequences of the ATCC strain of *Filamoeba nolandi* and 37 eukaryotic taxa available through GenBank, including representatives of Amoebozoa sensu Bolivar *et al*. (2001). *Hexamita inflata* was selected as an outgroup. The final alignment consisted of 1615 characters (1022 characters were parsimony informative).

The phylogenetic relationships between taxa were determined using maximum parsimony (MP) and maximum likelihood (ML) methods carried out in the program package PAUP*, version 4.0b8 (Swofford 2001). The MP analysis was performed using a heuristic search with random addition of taxa. Gaps were treated as missing data. Transition/transversion/ (Ts:Tv) ratio was 1:2. For ML, the likelihood ratio test (LRT) implemented in the Modeltest v. 3.06 (Posada and Crandall 1998) was used to determine the best model of evolution. ML analyses were done with the TrN+G model of evolution selected by LRT. Clade support was assessed by bootstraping (MP, 1000 replicates; ML, 500 replicates).

RESULTS

Description of *Filamoeba* strain CH26. The strain under study was characterised by an irregular, multipolarly branched, uninucleate trophozoites (Fig. 1). Robust pseudopodia were pointed, non-anastomosing subpseudopodia were filiform and up to 12 µm long. The greatest dimensions of locomotive and extended forms were 40-50 µm. A rounded or lobe-shaped nucleus was mostly difficult to distinguish in Nomarski DIC. The cytoplasm usually contained about 10 contractile vacuoles. Floating forms were rounded. Cysts (Fig. 3) were smooth, rounded, ovoid, irregularly ovoid or reniform with homogeneous and rather thick wall that lacked pores and closely applied to the amoeba plasmalemma.



Figs 1-10. Morphology of strains of *Filamoeba* spp. **1** - trophozoites, representatives of *Filamoeba sinensis* sp. n., strain CH26. **2** - trophozoites, representatives of *Filamoeba nolandi* Page, 1967, type strain from UKNCC (CCAP1526/1). **3** - cysts of *F. sinensis* formed from trophozoites in groups, note their irregular shape. **4, 5** - ultrastructure of *F. sinensis* trophozoites in two different levels of sectioning show a lobular nucleus, rounded nucleolus, several vacuoles in the cytoplasm and mitochondria sectioned in various planes. **6** - the surface of trophozoite of *F. sinensis*. **7** - mitochondria of *F. sinensis* in cross section. **8** - roughly longitudinal sections through mitochondria showing the shape and arrangement of their cristae. **9** - electron micrograph of mature cyst of *F. sinensis*. **10** - detail of cyst wall structure of *F. sinensis*. Scale bars: 20 µm (1-3); 1 µm (4, 5, 9); 500 nm (6, 8); 200 nm (7, 10).

The diameter of rounded cysts was 14-20 µm, the greatest dimension of oval cysts was 37 µm. The outline of cysts was evidently modified by close contact with neighbouring ones, i.e. by the space available for each cysts, which was observed also in old cultures.

At the ultrastructural level (Figs 4-6), trophozoites were truly "naked". The organic layer coating the surface membrane was very thin, almost invisible (Fig. 6). The rounded as well as lobe-shaped nuclei contained rounded nucleoli (Figs 4, 5). Numerous mitochondria, mostly of elongated shape were randomly distributed within the cytoplasm of trophozoites. They had tubular cristae (Fig. 7) situated irregularly or parallel to each other (Fig. 8). Neither cross-, nor longitudinal sections through mitochondria revealed anastomoses or branches of cristae. The cyst wall was homogeneous (Figs 9, 10). The nucleus of cysts sectioned at various levels was mostly rounded, with the diameter about 3 µm.

Based on comparison with the true type strain of *F. nolandi* (CCAP 1526/1) (Fig. 2), which we had cultured under identical conditions, the strain CH26 differed morphologically having robust multipolarly branched pseudopodia, less pronounced hyaloplasmic zone and a lower number of contractile vacuoles. Details of ultrastructure were not given in the original description of *F. nolandi*. The arrangement of tubular cristae in mitochondria of *F. sinensis* showed no signs of ramification.

SSU rRNA data and phylogenetic analysis of *Filamoeba* strains. The complete SSU rRNA gene sequences obtained from strains CH26 and CCAP 1526/1 have been deposited in the EMBL/GenBank database under accession numbers AY714369 and AY714368 respectively. They matched with the sequence of *F. nolandi* (AF293896) available in GenBank, but still could not be identified with it. The sequence of SSU rRNA gene of our strain CH26 is 1839 bp. The GC content is 50.73%. A sequence of the same length but differing in the GC content (49.59%) was obtained for the *F. nolandi* strain CCAP 1526/1. Similarities based on comparisons of complete sequences of SSU rRNA gene were as follows: 96.3% for strain CH26 and *F. nolandi* type strain CCAP 1526/1; 97.1% when CH26 and *F. nolandi* ATCC strain NS-AS-23-1was compared, 97.7% for *F. nolandi* ATCC strain NS-AS-23-1 and *F. nolandi* CCAP strain 1526/1.

As revealed by phylogenetic (ML) analysis (Fig. 11), two *Filamoeba* strains sequenced in this study plus ATCC strains of *F. nolandi* form monophyletic group (supported by 100% bootstrap) that is independent of Gymnamoebia *sensu stricto* according Bolivar *et al*. (2001) and of LH, V and PV lineages sensu Peglar *et al*. (2003). *Gephyramoeba* sp. clusters with *Filamoeba* strains with a bootstrap support lower than 50%. Within the group of *Filamoeba* strains, the true type strain of *F. nolandi* (CCAP 1526/1) is closely related to the ATCC strain of *F. nolandi* of marine origin, while our CH26 strain branches out of these two strains. The MP analysis resulted in three trees with the same length (8701 steps). The phylogenetic position of *Filamoeba* strains as well as relation to *Gephyramoeba* sp. was the same as obtained in ML.

Tentative assignment of strain CH26 to the genus *Filamoeba* Page, 1967, araising from comparison of morphological data available on freshwater naked amoebae, was supported by results of phylogenetic analyses. The SSU rRNA gene sequence we have obtained for the type strain of *F. nolandi* Page, 1976 complemented molecular data on *Filamoeba* spp. represented to date by the one sequence of the ATCC strain of *F. nolandi*, which contrary to the type species is of marine origin.

Details distinguishing the strain CH26 from the type strain of *F. nolandi* Page, 1967 together with results of phylogenetic analyses based on SSU rRNA gene sequences allowed to establish the new species, *F. sinensis*, the second one within the genus.

***Filamoeba sinensis* sp. n.**

Origin of type material: Gills of the Prussian carp, *Carassius gibelio* (Bloch, 1782) (Cypriniformes: Cyprinidae).

Type locality: Fish farm in Hubei Province, China.

Type material: Photosyntypes (light micrographs), nos. 13870-13891, transmission electron micrographs, nos. 17151-17165 and 17251-17275, and cryopreserved culture (clone CH26/I), deposited in the Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice.

Etymology: The species name is given according to the geographic origin of fish host. It was derived from Latin adjective "Chinese".

Diagnostic summary. Trophozoites uninucleate with narrow peripheral hyaloplasmic zone, robust multipolarly branched pseudopodia and numerous contractile vacuoles; greatest dimensions not exceeding 50 µm; mitochondria with tubular cristae arranged in parallel or irregular mutual positions. Cysts rounded (14-20 µm in

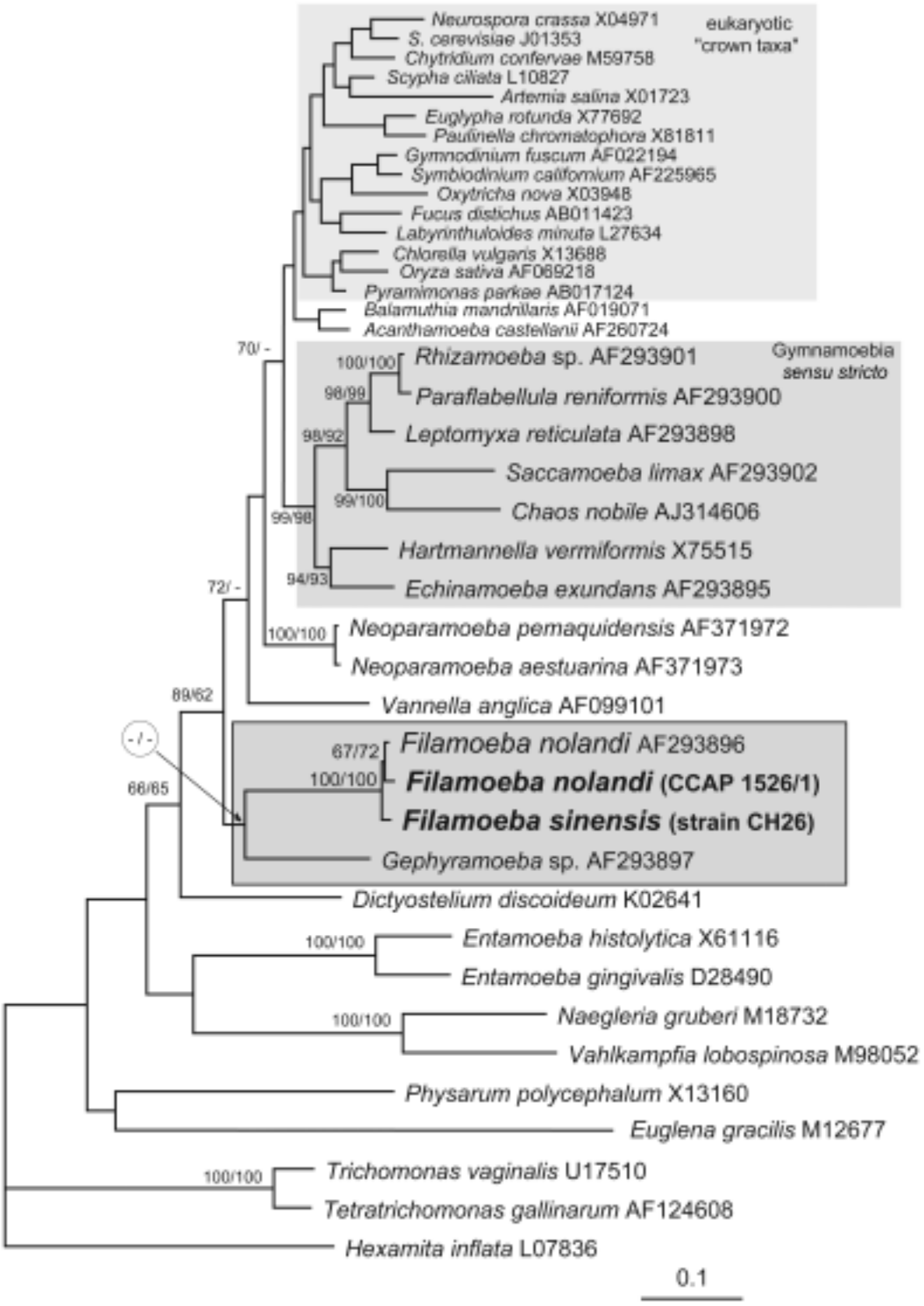


Fig. 11. Phylogenetic position of *Filamoeba* strains inferred from ML analysis (-ln = 25926.0376, α shape parameter = 0.4927). Bootstrap values (ML and MP Ts/Tv = 1:2) are shown for the nodes gaining more than 50% support (except for eukaryotic "crown" taxa). The distance scale (substitution/site) is given under the tree. GenBank accession numbers are indicated.

diameter) or ovoid with homogeneous wall free of pores. *F. sinensis* differs from type species of the genus in morphology of trophozoites and SSU rRNA gene sequence.

DISCUSSION

Tentative generic diagnosis of amoeba under study, morphology of which was unique among amoebae we have isolated from fish, was possible thanks to thorough original description of *F. nolandi* (Page 1967), supplemented with several line drawings of good quality and documentation in monographs by Page (1988, 1991). Description of the type species of the genus *Filamoeba* Page, 1967 was based on light microscopy and some unpublished observations in TEM (Page 1967). For almost 40 years, *F. nolandi* was the unique species representing the genus. The number of reported isolates was surprisingly low and most of them were not documented (Page 1976, Coutinho Salazar 1982, Ariza Astolfi *et al*. 1988). This seems to be the reason why in phylogenetic studies dealing with relationships among amoebae, one only SSU rDNA sequence of *Filamoeba* sp. (ATTC strain) was used.

Amaral Zettler *et al*. (2000), who discovered that distance and likelihood methods united *Filamoeba nolandi* and *Gephyramoeba* sp., also discovered consistent sister relationship of *Acanthamoeba - Balamuthia* with *Gephyramoeba - Filamoeba*. The bootstrap support for common ancestry of both couples was marginal in their analyses. On the basis of an "unexpected relationship" between *Gephyramoeba* and *Filamoeba*, and on the basis of morphological data, Amaral Zettler *et al*. (2000) suggested reconsideration of the current systematic placement of both genera. The strong union of *Gephyramoeba* and *Filamoeba* characterised by Amaral Zettler *et al*. (2000), based on distance and likelihood methods, weakened in studies by Bolivar *et al*. (2001) and Peglar *et al*. (2003). Also in our study, this union is supported by low bootstrap only. In accordance with Peglar *et al*. (2003), representatives of four lineages of gymnamoebae included in our analysis are not related to the cluster of *Filamoeba* strains.

In the overview of morphological features important in amoeboid taxa subjected to phylogenetic analysis, tubular and branching cristae of mitochondria were mentioned by Amaral Zettler *et al*. (2000) in *F. nolandi* and *Gephyramoeba* sp. To the best of our knowledge, this feature was not documented in any ultrastructural study. The branching pattern of mitochondrial cristae of *F. sinensis*, if any, can hardly be identified with typical branching pattern as known, e.g. in *Acanthamoeba* spp.

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Short communication

Effect of Hormones on the Concentration of a Digoxin-like Material in *Tetrahymena*

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Summary. The unicellular *Tetrahymena pyriformis* contains digoxin-like materials (digoxin, digitoxin, digoxigenin). The effect of two amino-acid-type hormones (histamine and serotonin) and a peptide hormone (insulin) was studied on the level of the hormone-like steroid, digoxin, in the concentrations of 10^{-6} and 10^{-9} by using immunocytochemistry and flow cytometric and confocal microscopic analysis. Each hormone elavated significantly the digoxin concentration at the end of the 30 min treatment. As *Tetrahymena* was maintained in a tryptone-yeast extract medium during the treatment, it seems to be likely that steroid synthesis was enhanced. The most effective was histamine, the effect of which was significantly more expressed than that of the others and the less effective (however significant), was serotonin. The results call attention to hormonal interactions inside the unicellular organism and on the possibility of mutual intercellular influences, considering the low effective hormone concentration which was needed for the striking effect.

Key words: digoxin, evolution, histamine, hormonal interactions, insulin, serotonin, *Tetrahymena*.

INTRODUCTION

Many members and characteristics of the hormonal system of higher ranked animals can be demonstrated in the unicellular *Tetrahymena*. It contains receptor-like structures in the plasma membrane and cytosol (Csaba1980, 1985; Christopher and Sundermann 1995; Christensen *et al*. 2003), hormones similar to mammalian ones (LeRoith *et al*. 1980, 1983; Lenard 1992; Kőhidai and Csaba 1995; Kőhidai *et al*. 2000, 2002a, b) and signal transduction pathways (Kuno *et al*. 1979; Muto *et al*. 1983; Kovács and Csaba 1990, 1997) using the same components which are working in mammals. Hormones, received by the receptors can influence a lot of physiological processes of the unicellular animal, e.g. cell division, phagocytosis, chemotaxis etc. (Wheatley *et al*. 1993, Csaba 2000), nevertheless our knowledge on the interactions of these hormones inside the *Tetrahymena* is very scarce. Recently we studied the effect of three amino acid- or peptide-type hormones (which are present in *Tetrahymena*) on the concentration of epidermal growth factor (EGF), a peptide hormone, also present in *Tetrahymena* (Csaba *et al*. 2004). As this

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was demonstrated, in the present experiment the effect of the same hormones on digoxin, a hormone-like steroid molecule, is studied.

MATERIALS AND METHODS

Populations of *Tetrahymena pyriformis* GL were grown axenically in a 0.1% yeast extract containing 1% tryptone medium at 28°C. In the experiments cells in the mid-exponential growth phase (48h cultures; ~ 5×10^5 cells/ml) were used. The cells were washed with fresh culture medium and harvested by centrifugation at ~ 400 g for 3 min at room temperature, and were adjusted to ~ 5×10^5 cells/ml with fresh culture medium. Five samples were prepared for each measurement. The samples contained *Tetrahymena* in fresh medium and the hormones (except control) in 10^{-6} and 10^{-9} M concentrations for 30 min. The hormones were the following: histamine dihydrochloride (Sigma, St. Louis, USA); serotonin HCl (Sigma) or insulin (Semilente MC, Novo, Copenhagen, Denmark). After treatment the cells were studied by flow cytometry and confocal microscopy.

Flow cytometric analysis

Samples of cells were fixed with 4% paraformaldehyde solution (dissolved in pH 7.2 PBS) for 5 min, and then washed twice in wash buffer (0.1% BSA; 20 mM Tris-HCl; 0.9% NaCl; 0.05% Nonidet NP-40; pH 8.2). To block nonspecific binding of antibodies the cells were treated with blocking buffer (1% BSA in PBS) for 30 min at room temperature. Aliquots from cell suspensions (50 ml) were transferred into tubes, and 50 ml primary antibody (monoclonal anti-digoxin developed in mouse ascites; [Sigma, D-8156, Clone Di-22; dilution 1:200 in antibody buffer (1% BSA in wash buffer)] was added for 30 min at room temperature. Negative controls were carried out with 50 ml PBS containing 10 mg/ml BSA instead of primary antibody.

After washing four times with wash buffer to remove excess primary antibody the cells were incubated with secondary antibody (FITC-labeled monoclonal anti-mouse IgG developed in goat; Sigma, St. Louis USA; dilution 1: 50 with antibody buffer) for 30 min at room temperature.

For controlling the specificity, autofluorescence of the cells and aspecificity of the secondary antibodies were detected. The measurement was done in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, USA), using 25,000 cells for each measurement. In the cell populations the digoxin content (concentration) had been compared. For the measurement and analysis CellQuest Pro program was used. The numerical comparison of detected values was done by the comparison of percentual changes of geometric mean channel values to the appropriate control groups by using Origin program and Student t-test.

Confocal microscopic analysis

After the flow cytometric analysis the cells were subjected to confocal microscopic analysis in a BioRad MRC 1024 confocal laser scanning microscope, equipped with krypton-argon mixed gas-laser as a light source, at an excitation wavelength of 480 nm line. Three independent series of experiments were carried out with identical results.

RESULTS AND DISCUSSION

Tetrahymena is able to synthesize steroid molecules. One of them is the tetrahymanol for the synthesis of which squalene tetrahymanol synthase enzyme is responsible (Saar *et al*. 1991). The unicellular eukaryotes also contain 20a-hydroxysteroid dehydrogenase (Inazu *et al*. 1994)). In addition to the synthesized steroid hormones (as testosterone and estradiol: Csaba *et al*. 1998) digoxin, a cardioactive glycoside can be found, which also has a steroid structure. In earlier experiments (Kovács *et al*. 1998) the presence of digoxin, digitoxin and digoxigenin was demonstrated in *Tetrahymena*. The monoclonal antibody used in the present experiments was produced against digoxin however, it had a high affinity also to digoxigenin.

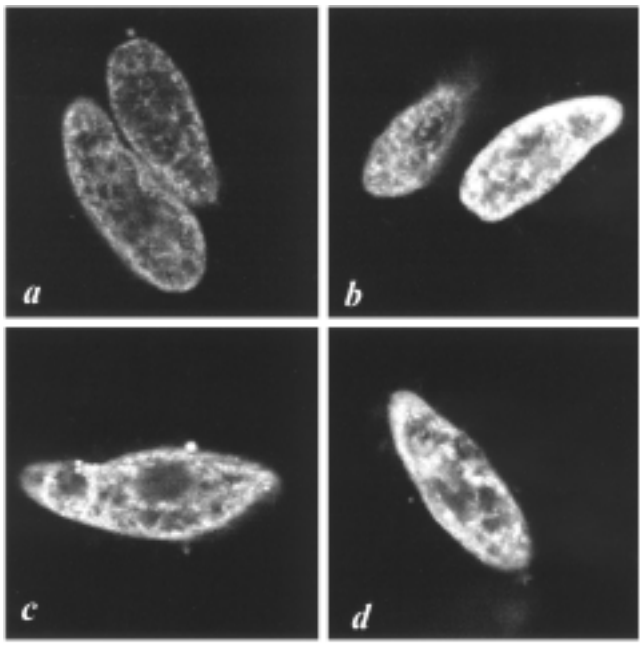
The flow-cytometric results unanimously show that the hormones used for treatment significantly elevated the concentration of the digoxin-like materials in *Tetrahymena* (Table 1). The confocal microscopic pictures support the flow cytometric quantitative results. However, there is no difference between the control and hormone treated cells in the localization of the digoxin-like material (Fig. 1).

As simple tryptone-yeast extract medium was administered for maintaining *Tetrahymena* it is not likely that complete digoxin was taken up from it. This means that the (amino-acid and peptide) hormones which are also physiologically present in *Tetrahymena*, enhanced the steroid synthesis. The most effective hormone was histamine which was similarly effective in the EGF-experiments done earlier (Csaba *et al*. 2004). However, in these earlier experiments serotonin was as effective as histamine and now serotonin was the less active, and in the present case the difference between the effects of histamine and the other two hormones is significant. This emphasizes the important role of histamine at this low level of phylogeny, which is supported by the presence of histamine and histamine synthesizing enzyme (HDC) genes already in bacteria (Ruby *et al*. 2002, Darvas and Falus 2004), and in *Tetrahymena* (Hegyesi *et al*. 1999). The fact, that there was no significant difference between the two concentrations used shows the strong influence of histamine and the other two hormones in a very low (nanomolar) concentration. Considering this,

Table 1. Effect of hormones on the content of a digoxin-like material in *Tetrahymena*.

Treatnent	Concentration	Geo-mean	Signif. to control	Signif. to insulin	Signif. to serotonin
Untreated control		108.65 ± 14.62			
Histamine	10^{-6}	163.0 ± 13.18	p<0.001	p<0.05	p<0.01
	10^{-9}	150.96 ± 7.07	p<0.001	p<0.05	p<0.01
Serotonin	10^{-6}	128.28 ± 10.7	p<0.05	n.s.	
	10^{-9}	131.2 ± 6.6	p<0.05	n.s.	
Insulin	10^{-6}	141.63 ± 13.82	p<0.01		n.s.
	10^{-9}	136.118 ± 7.46	p<0.01		n.s.

There are no significant differences between the results of treatments (with the same hormone) in 10^{-6} and 10^{-9} M concentrations.



Figs 1a-d. Confocal microscopic pictures (median sections) of *Tetrahymena*. **a** - control; **b** -histamine treated; **c** - serotonin treated; **d** - insulin treated. Hormone concentrations 10^{-9} M. There is no striking differences in the localization of the digoxin-like molecule however, the quantitative differences can be observed. Magnification ×1400.

not only the influence of these hormones on the inside hormone metabolism can be imagined, but the effect to other members of the population by the secretion of hormone-like materials.

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ACTA
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Letter to the Editor

Ghazi S. M. ASMAT

Department of Zoology, University of Chittagong, Chittagong, Bangladesh

Dear Editor,

I have gone through an article of August issue, 2004, entitled "First Record of *Trichodinella epizootica* (Raabe, 1950) Šramek-Hušek, 1953, with description of *Trichodina notopteridae* sp. n. (Ciliophora: Peritrichida) from Freshwater Fishes of India" by Amlan Kumar Mitra and Durga P. Haldar. *Acta Protozool*. **43:** 269-274 (2004)**.**

For your kind notice I am presenting some misdeeds about the presentation and information of the article as follows.

a) The authors have given the latest references (e.g., Mitra and Haldar 2003) but intentionally omitted the following references (including the articles published in *Acta Protozool.*). They have cited the papers in which the name of Haldar is present, why? Astonishingly, the reviewers also overlooked the matter, intentionally or unintentionally I don't know, they might have satisfactory answer.

1) Ghazi S. M. Asmat (2000) *Trichodina cuchiae* sp. n. (Ciliophora: Trichodinidae) from Gangetic mudeel, *Monopterus cuchia* (Hamilton-Buchanan, 1822) (Synbranchiformes: Synbranchidae) in India. *The Chittagong Univ. J. Sc*. **24 (1):** 55-61
2) Ghazi S. M. Asmat (2000) First record of *Trichodina acuta* Lom, 1961 (Ciliophora: Trichodinidae) from India. *The Chittagong Univ. J. Sc.* **24 (1):** 63-70
3) Ghazi S. M. Asmat (2001a) *Trichodina cancilae* sp. n. (Mobilina: Trichodinidae) from the gills of a freshwater gar, *Xenentodon cancila* (Hamilton) (Belonidae). *Acta Protozool.* **40:** 141-146
4) Ghazi S. M. Asmat (2001b) *Trichodina canningensis* sp. n. (Ciliophora: Trichodinidae) from an Indian estuarine fish, *Mystus gulio* (Hamilton) (Bagridae). *Acta Protozool.* **40:** 147-151
5) Ghazi S. M. Asmat (2001c) *Trichodina porocephalusi* sp. n. (Ciliophora: Trichodinidae) from an Indian flathead sleeper, *Ophiocara porocephalus* (Valenciennes) (Eleotrididae). *Acta Protozool*. **40:** 297-301
6) Ghazi S. M. Asmat (2001d) Occurrence and morphology of *Trichodinella epizootica* (Raabe, 1950) Šramek-Hušek, 1953 in India. *The Chittagong Univ. J. Sc.* **25 (2):** 37-44
7) Ghazi S. M. Asmat (2002a) Trichodinid ciliates (Ciliophora: Trichodinidae) from Indian fishes with description of two new species. *Bangladesh J. Zool.* **30 (2):** 87-100
8) Ghazi S. M. Asmat (2002b) Occurrence and morphology of some *Tripartiella* sp. (Ciliophora: Trichodinidae) from freshwater and estuarine fishes in India. *Univ. j. zool. Rajshahi Univ*. **21:** 49-55
9) Ghazi S. M. Asmat (2002c) Two new species of trichodinid ciliates (Ciliophora: Trichodinidae) from Indian fishes. *Univ. j. zool. Rajshahi Univ*. **21:** 31-34

b) The Indian *Trichodinella epizootica* is already reported in a Bangladeshi journal vide ref. 6. How authors could publish it again knowing the matter, it is highly disgraceful, I think. You have to do something relating this article and information.

c) According to Article 29.2 (International Code of Zoological Nomenclature, Fourth Edition, 1999, p. 32), we know the suffix - *idae* is used for a family name. But the new species name described in the said paper is "*... notopteridae*". Would it be valid?

d) The description of the new species is not also valid, I think. I described the same kind of individuals as *Trichodina nigra*. If they disagree, they may compare their specimens with *T. nigra* of my paper (Asmat 2002 a). There is no reason to compare these with *T. luciopercae*. The Indians never ever identified *T. nigra* properly. I wonder that the reviewers of the article also forgot the features of *T. nigra*?

It would be highly appreciated if you could kindly go through this matter seriously and let me know the decision of the editorial board of *Acta Protozoologica* regarding this article.

Thanking you.

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Letter to the Editor

Amlan Kumar MITRA and Durga P. HALDAR

Protozoology Laboratory, Department of Zoology, University of Kalyani, Kalyani, West Bengal, India

Sir,

In the August, 2004 issue, we published a paper incorporating our first record of *Trichodinella epizootica* (Raabe, 1950) Šramek-Hušek, 1953 from a freshwater fish in India. Recently, it was pointed out to us that *Trichodinella epizootica* has already been reported from an Indian fish by Asmat (2001) from the host fish *Puntius serana* in Kalyani, West Bengal, India. As such, our claim of first record of this species in India is not tenable. However, the species obtained by us was recorded from *Puntius gelius* from a different locality. The matter was perhaps overlooked by the experts who reviewed the paper, because the description of the first report of *T. epizootica* was published in a University journal in Bangladesh which is neither available here nor in the Internet. In view of the fact that the particular species was obtained by several authors outside India, our "second" record of the species from this subcontinent only has a great significance so far as the distribution and host ranges of trichodinid ciliophorans are concerned.

Yours etc.

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Books: Swofford D. L. (1998) PAUP* Phylogenetic Analysis Using Parsimony (*and Other Methods). Ver. 4.0b3. Sinauer Associates, Sunderland, MA

Articles from books: Neto E. D., Steindel M., Passos L. K. F. (1993) The use of RAPD's for the study of the genetic diversity of *Schistosoma mansoni* and *Trypanosoma cruzi*. In: DNA Fingerprinting: State of Science, (Eds. S. D. J. Pena, R. Chakraborty, J. T. Epplen, A. J. Jeffreys). Birkhäuser-Verlag, Basel, 339-345

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