ACTA

PROTOZOOLOGICA

ACTA
PROTOZOOLOGICA

Paramecium Morphometric Analysis and Taxonomy

Sergei I. FOKIN and Sergei M. CHIVILEV

Biological Institute of St. Petersburg State University, St. Petersburg, Russia

Summary. Morphometric analysis of 13 cell attributes in 24 stocks of 6 freshwater *Paramecium* morphospecies (including three sibling species of the *P. aurelia* complex- *P. biaurelia*, *P. tetraurelia* and *P. pentaurelia*) was carried out. The full morphometric data for the main freshwater *Paramecium*: *P. caudatum*, *P. multimicronucleatum*, *P. jenningsi*, *P. bursaria*, *P. putrinum* were shown for the first time. The data were compared with the authors morphometric material for the brackishwater species of the genus (25 stocks of *P. calkinsi*, *P. nephridiatum*, *P. woodruffi* and *P. duboscqui*) and *P. polycaryum* with the aim of revising the taxonomy of the genus (the existence of the "putrinum", "woodruffi" and "aurelia" subgroups and their composition). Morphological characteristics were transformed to numerical coding and relationships among these paramecia are represented as dendrorgams and two-dimensional figures using cluster and multidimensional scaling analysis. According to this analysis, the "aurelia" subgroup is a real cluster of species as well as the brackishwater paramecia (the "woodruffi" subgroup without *P. polycaryum*). *P. polycaryum* is not grouped with either the "woodruffi" or the "aurelia" subgroups. *P. bursaria* and *P. putrinum* do not have close morphological similarity as was previously believed. They are not clustered with each other or with other paramecia. Thus, neither type of previous taxonomy (2 subgroups of Woodruff and 3 subgroups in *Paramecium* taxonomy of Jankowski) is supported by morphometric analysis.

Key words: morphometry, *Paramecium*, subgroups, taxonomy.

Abbreviations: BC - buccal cavity, BO - buccal overture, C - cytoproct, CV - contractile vacuole, MA - macronucleus, MDS - multidimensional scaling, MI - micronucleus, PCV - pore of contractile vacuole, UPGMA - unweighted pair-group method of arithmetic averages.

INTRODUCTION

Since 1752 when Hill published his History of Animals, the organisms named *Paramecium* by him have been prominently mentioned in the scientific literature (Mueller 1786, Woodruff 1945, Wichterman 1953, Vivier 1974). Identification of species has however,

often been neglected by those who have discussed *Paramecium* in the literature. As was mentioned by Wenrich (1928) "indeed, very few of the descriptions of species previous to the time of Maupas (1888) were accurate enough to make recognition possible for later workers". In spite of Maupas' attempt to stress the importance of morphometric data for accurate species determination, such information has been limited up to now. The number of *Paramecium* species varied between the earlier reviews: 8 (Ehrenberg 1838), 2 (Dujardin 1841), 8 (Claparède and Lachmann 1858), 5 (Kent 1881-1882) and, apparently, only a few species from these lists

---

can be considered to be true paramecia. At the beginning of this century only 4 valid species of *Paramecium* were determined: *P. aurelia, P. caudatum, P. bursaria* and *P. putrinum (trichium)*. Today the *Paramecium* genus includes at least 16 valid morphospecies (Wichterman 1986, Shi *et al.* 1997, Fokin and Chivilev 1999, Fokin *et al.* 1999a), but the morphometric data for many of them are still absent.

Generally, two species groups are distinguished by authors within the genus *Paramecium:* "aurelia" and "bursaria" (Woodruff 1921). Jankowski (1969, 1972) recognized three such groups: "putrinum", "woodruffi" and "aurelia". They are regarded by this author as taxonomic subgenera which differ in many morphological characters (body size and shape, position of the cytostome, deepen in the buccal cortex, number of kineties and so on). However, the morphometric data for these subgenera representatives were not shown by Jankowski or anybody else.

The goals of this study were the morphometric characterization of the common freshwater *Paramecium* species and the revision of the genus taxonomy (the existence of the "putrinum", "woodruffi" and "aurelia" subgroups and their composition) using comparison between morphometric data for 11 fresh and brackishwater *Paramecium* species.

MATERIALS AND METHODS

A number of clonal cultures of *P. putrinum, P. bursaria, P. biaurelia, P. tetraurelia, P. pentaurelia, P. jenningsi, P. caudatum* and *P. multimicronucleatum* were used in this study. They were isolated from the natural populations in various places, mainly in the former Soviet Union (Table 1). Data about the origin, morphometry and biological peculiarities of *P. calkinsi, P. woodruffi, P. nephridiatum, P. duboscqui* and *P. polycaryum* were reported in a previous publication (Fokin and Chivilev 1999).

The morphology of the ciliates was first studied in a living condition. The cells were immobilized by a device for controlling the cover slide (Skovorodkin 1990) and were observed by phase contrast or DIC optic (Axioskop, Zeiss, Germany).

Cultures were fixed (Bouin's fixative) and stained by the Feulgen reaction for the measurement of the nucleus apparatus. Silver-staining technique by the Chatton and Lwoff procedure (Corliss 1953) was used for the impregnation of cortical structures. For this purpose the samples were fixed 24 h after feeding. 20-25 properly oriented and well-impregnated specimens were selected from each stock and measured. The 13 attributes which were used are listed in Table 2. The points of the direct measurements are represented in Fig. 1. The measurements of the attributes were carried out using the same microscopes equipped with a micrometer (Carl Zeiss, Germany).

For each stock and for each attribute, the mean, standard deviation, coefficient of variation, minimum value and maximum value were calculated. Graphical representation of the similarity matrix was performed with multidimensional scaling analysis (MDS) (Kruskal and Wish 1978). Cluster analysis by the unweighted pair-group method with arithmetic averages (UPGMA) (Sneath and Sokal 1973) using the program package CLASS developed by the Research Institute for Nature Conservation of the Arctic and the North (RINCAN, Russia) and STATISTICA for Windows (StatSoft, Inc.) was made as well. Classification and ordination were made on the basis of Braverman (1965) recalculations of the Euclidean distance without transformation of primary data. The permutation test of "ANOSIM" procedure from the program package PRIMER (Plymouth Marine Laboratory, Great Britain) was used for testing differences between groups of species.

As a result of our recent study on brackishwater paramecia (Fokin and Chivilev 1999) we have learned that the length of the cell is a very "heavy" characteristic and a number of the actual measurements have a high correlation with it. The ratios, in fact, are more informative than the actual morphological measurements, because they give us the proportions (plan of construction of the ciliates). This "cytotopography" in our opinion is much more important for species comparison than the actual size of the cells. Therefore, we have used for the clustering and MDS analysis only a subset of ratios (Table 2). The UPGMA analysis of all 49 *Paramecium* stocks has shown a very complicated picture of similarity (Fig. 14). Then we have used for the UPGMA only the mean values for the species (Figs. 15, 16).

RESULTS

Morphometrical analysis permitted the determination of the main parameters of the cell for the 8 investigated *Paramecium* species (Tables 3-5, Figs. 2-13)

Paramecium putrinum (Tables 1, 3; Figs. 2, 3)

The average size of the cell in 3 investigated stocks was 91.4 x 34.2 μm. The number of cilia rows (kineties) varied from 44 to 54 (49 on average). The BO was located strongly to the anterior end from the cell's equator (34% of the cell's length). The distance from the anterior end of the cell to the single PCV of the anterior CV was approximately 24% of the cell's length. The distance from the single PCV of the posterior CV to the posterior end of the cell was 18% of the cell's length. The size of the BC, on average, was 22% of the body length. The endoral membrane was composed of 8-15 units (11 on average).

The ovoid or slightly ellipsoidal MA was usually situated near the cell equator. Its size varied from 11 x 20 to 18 x 29 μm (12.9 x 23.5 μm on average).

The single spheroid or spindleform MI had a size ranging from 2.7 x 3 to 8 x 11 μm (5.3 x 6.6 μm on average). It belongs to the compact type "c" following our classification (Fokin 1997). Usually, the MI was located

Table 1. Origin of the cultures

Species	Stock	Number	Origin	Isolator
P. putrinum (trichium)	Mon-1	pu1	St. Petersburg district, Russia	Fokin
	NA-5	pu2	St. Petersburg district, Russia	Fokin
	So 2	pu3	Nagano, Japan	Hiwatashi [a]
P. bursaria	Br80-6	bu1	Belgorod district, Russia	Fokin
	Br80-10 [c]	bu2	Belgorod district, Russia	Fokin
	B-51	bu3	Irkutsk district, Russia	Fokin
	D12-7	bu4	Vladivostok district, Russia	Skoblo
P. biaurelia	T10-1	ba1	Irkutsk district, Russia	Fokin
	MB3-2	ba2	Muenster, Germany	Fokin
	GSK2-1	ba3	Stuttgart, Germany	Fokin
P. tetraurelia	51	ta	Spencer, USA	Preer [a]
P. pentaurelia	87	pa1	Philadelphia, USA	Preer [a]
	Br9-3	pa2	Belgorod district, Russia	Fokin
P. jenningsi	Ia	je1	Bangalore, India	Padmarathi [b]
	Ma	je2	Madagascar	Nibois [b]
	Mb	je3	Madagascar	Nibois [b]
P. caudatum	M-571	ca1	St. Petersburg district, Russia	Skoblo
	M571-11	ca2	St. Petersburg district, Russia	Skoblo
	R21-2	ca3	St. Petersburg district, Russia	Fokin
	DP6-1	ca4	St. Petersburg district, Russia	Fokin
	R20-16	ca5	St. Petersburg district, Russia	Fokin
P. multimicronucleatum	Gl-5	mu1	Tbilisi district, Georgia	Borchsenius
	Or-7	mu2	St. Petersburg district, Russia	Fokin
	As1-4	mu3	Munchen, Germany	Fokin

[a] stocks were received from Dr. Fujishima, Yamaguchi, Japan
[b] stocks were received from Dr. Przyboś, Krakow, Poland
[c] experimental made amicronuclear stock

Table. 2. Description of attributes employed in the analysis of *Paramecium*

Number	Abbreviation	Description
1	L	Maximum rectilinear length
2	W	Maximum rectilinear width
3	B	Maximum rectilinear buccal cavity
4	AE	Distance from the anterior edge to the equator of the buccal opening
5	OC	Distance from posterior edge of the buccal opening to the anterior edge of cytoproct
6	AP	Distance between the anterior PCV and the edge of the cell
7	PP	Distance between the posterior PCV and the edge of the cell
8	WL	W/L %
9	AEL	AE/L%
10	OCL	OC/L%
11	APL	AP/L%
12	PPL	PP/L%
13	BL	B/L%

Abbreviations: are the same as on Fig. 1; PCV - pore of the contractile vacuole
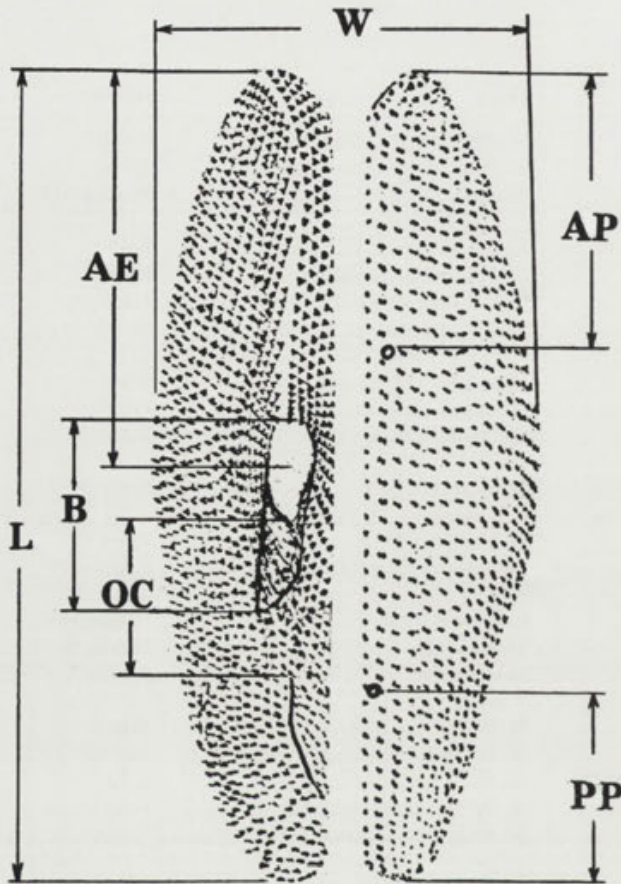
Fig. 1. The points of the direct measurements of the cell features. AE - distance from the anterior edge to the equator of the buccal opening, AP - distance between anterior PCV and the edge of the cell, B - maximal buccal cavity length, L - maximum rectilinear length, OC - distance from posterior edge of the buccal opening to the anterior edge of cytoproct, PP - distance between the posterior PCV and the edge of the cell, W - maximum rectilinear width

close to the MA, usually on the top of it or close to the equatorial line of the nucleus.

Paramecium bursaria (Tables 1, 3; Figs. 6, 7)

The average size of the cell in 4 investigated stocks was 112.7 x 40.0 μm. The number of kineties varied from 62 to 85 (71 on average). The BO was located visibly to the anterior end from the cell's equator (42% of the cell's length). The distance from the anterior end of the cell to the adjacent PCV (2-4 in number) of the anterior CV was approximately 30% of the cell's length. The distance from the posterior end of the cell to the adjacent PCV (2-5 in number) of the posterior CV was 27% of the cell's length. The size of the BC, on average, was 23% of the body length. The endoral membrane was composed of 7-14 units (10 on average).

The ellipsoidal MA was usually located near the cell's equator. Its size varied from 12 x 25 to 25 x 38 μm (15.4 x 29.8 μm on average).

The single, relatively large, compact MI (type "b") had a size between 2.0 x 4.2 and 10 x 15 μm (5.2 x 10.2 μm on average). Usually, it was located very close to one of the ends of the MA.

Paramecium biaurelia (Tables 1, 4; Figs. 10, 11)

The average size of the cell in 3 investigated stocks was 133 x 31.5 μm. The number of kineties varied from 57 to 67 (61 on average). The BO was located behind the cell equator line (54% of the ciliate length). The distance from the anterior end of the cell to the single PCV of the anterior CV was 35% of the cell's length. The distance from the single PCV of the posterior CV to the posterior end of the ciliate was 19% of the cell's length. The size of the BC, on average, was 14% of the body length. The endoral membrane was composed of 13-20 units (16.5 on average).

The ellipsoidal MA was usually situated near the cell's equator. Its size varied from 12 x 24 to 18 x 32 μm (14.0 x 27.5 μm on average).

Two spheroid "vesicular" type MI had a size between 2.5 x 2.7 and 3.7 x 3.9 μm (3.2 x 3.3 μm on average). They were usually located close to the MA, but sometimes were found in different parts of the cytoplasm.

Paramecium tertaurelia (Tables 1, 4; Figs. 10, 11)

The average size of the cell in the single investigated stock was 127.7 x 28.2 μm. The number of kineties was 60-68 (64 on average). The BO was located anteriorly from the ciliate equator (42% of the cell length). The distance from the anterior end to the single PCV of the anterior CV was 32% of the cell length. The distance from the single posterior PCV to the posterior end of the ciliate was 19% of the body length. The size of the BC was 20% of the cell's length. The endoral membrane was composed of 15-21 units (17.5 on average).

The ellipsoidal MA was usually situated near the cell's equator. Its size was from 11 x 21 to 17 x 35 μm (15.5 x 30 μm on average).

Two spherical "vesicular" MI had a size between 2.8 x 2.9 and 3.9 x 3.9 μm (3.3 x 3.3 μm on average). They were located close to the MA.

Paramecium pentaurelia (Tables 1, 4; Figs. 10, 11)

The average size of the ciliate in two investigated stocks was 144.9 x 32.3 μm. The number of kineties was 60-72 (66 on average). The BO was located near the equatorial
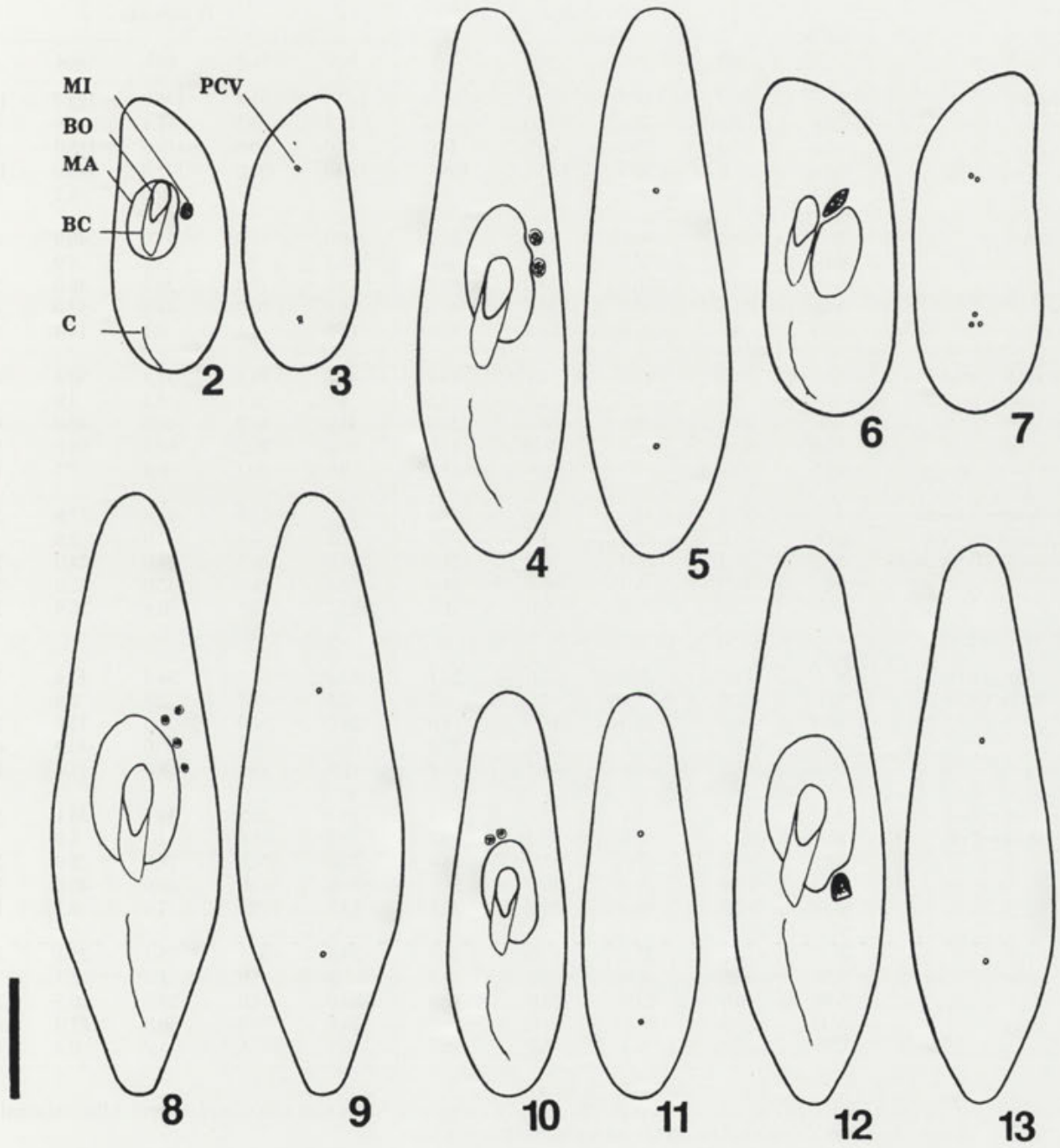
Figs. **2-13**. Ventral and dorsal views of 6 *Paramecium* freshwater morphospecies. **2**, **3** - *P. putrinum*, **4**, **5** - *P. jenningsi*, **6**, **7** - *P. bursaria*, **8**, **9** - *P. multimicronucleatum*, **10**, **11** - *P. aurelia* complex, **12**, **13** - *P. caudatum*. BC - buccal cavity, BO - buccal overture, C - cytoproct, MA - macronucleus, MI - micronuclei, PCV - pore of contractile vacuole. All the drawings are made after paramecia cells morphometry. Scale bar: 40 μm

Table 3. Morphometric characterization of *Paramecium putrinum* and *P. bursaria*

Character	Statistics	P. putrinum				P. bursaria				
		pu1	pu2	pu3	M	bu1	bu2	bu3	bu4	M
Body length	x̄	96.4	84.2	93.8	91.4	107.1	91.4	126.9	125.2	112.7
	SD	8.8	4.8	8.0	9.0	12.3	5.2	11.4	9.9	12.9
	Min	80.0	75.0	80.0	78.0	85.0	80.0	110.0	115.0	98.0
	Max	110.0	90.0	110.0	103.0	120.0	100.0	145.0	140.0	126.0
	CoV	9.1	5.7	8.5	9.8	11.5	5.7	9.0	8.2	11.4
Body width	x̄	36.6	34.6	31.1	34.2	40.0	37.0	41.9	40.9	40.0
	SD	3.2	4.0	4.2	4.3	4.7	3.1	3.8	5.9	5.0
	Min	28.0	26.0	24.0	26.0	32.0	32.0	36.0	36.0	34.0
	Max	44.0	40.0	40.0	41.0	48.0	40.0	48.0	52.0	47.0
	CoV	8.7	11.5	13.5	12.6	11.7	8.4	9.1	14.4	12.5
Distance from anterior end to middle of buccal overture	x̄	30.7	31.2	32.1	31.4	49.8	40.2	51.3	50.4	48.0
	SD	3.9	2.4	2.8	4.1	4.5	4.1	4.3	3.9	4.9
	Min	24.0	28.0	24.0	25.0	44.0	36.0	44.0	48.0	43.0
	Max	40.0	36.0	36.0	37.0	56.0	48.0	60.0	68.0	58.0
	CoV	12.7	7.7	8.7	13.0	9.0	10.2	8.4	7.7	10.2
Distance from proximal edge of buccal overture to anterior end of cytoproct	x̄	35.1	32.3	33.4	33.6	27.0	22.3	27.4	27.8	26.1
	SD	3.8	2.9	3.0	4.0	2.5	3.4	2.9	2.5	3.9
	Min	28.0	28.0	28.0	28.0	24.0	16.0	24.0	24.0	22.0
	Max	40.0	36.0	40.0	38.0	32.0	28.0	32.0	32.0	31.0
	CoV	10.8	9.0	9.0	11.9	15.2	9.3	10.6	8.9	14.9
Distance from anterior end to anterior CVP	x̄	21.8	22.6	22.0	22.1	33.8	27.9	34.4	37.4	33.4
	SD	2.4	3.8	3.2	4.2	2.5	3.7	2.9	2.8	4.2
	Min	16.0	12.0	16.0	15.0	28.0	24.0	28.0	32.0	28.0
	Max	24.0	28.0	28.0	27.0	40.0	40.0	40.0	40.0	40.0
	CoV	11.0	16.8	14.5	19.0	11.5	13.3	8.4	7.4	12.6
Distance from posterior end to posterior CVP	x̄	18.5	14.4	15.9	16.3	25.1	28.5	34.1	32.1	30.0
	SD	3.1	2.8	3.2	3.4	3.2	3.8	2.7	3.0	4.2
	Min	14.0	12.0	12.0	13.0	20.0	24.0	28.0	28.0	25.0
	Max	22.0	20.0	20.0	21.0	30.0	32.0	40.0	40.0	36.0
	CoV	16.7	19.4	20.1	20.8	12.7	13.3	7.9	9.3	14.0
Buccal length	x̄	18.7	22.3	19.5	20.2	28.5	26.5	25.5	25.1	26.4
	SD	3.5	1.4	2.6	3.8	1.9	2.0	1.5	2.3	2.7
	Min	16.0	20.0	16.0	17.0	20.0	24.0	20.0	20.5	21.0
	Max	24.0	24.0	24.0	24.0	24.0	28.0	26.0	27.0	26.0
	CoV	18.7	6.3	13.3	18.8	6.7	7.5	5.9	9.2	10.2

CoV - coefficient of variation, CVP - contractile vacuole pore, M - mean of the species, Max - maximal size of the trait, Min - minimal size of the trait, SD - standard deviation, x̄ - mean of the stock. All measurements in μm

line (49% of the body length on average). The distance from the anterior end to the single PCV of the anterior CV was 34% of the cell length. The distance from the single posterior PCV to the posterior end of the ciliate was 23% of the body length. The size of the BC was 13% of the body length. The endoral membrane was composed of 15-20 units (17.3 on average).

The ellipsoidal Ma was usually situated near the cell's equator. Its size was from 12 x 20 to 20 x 39 μm (16.2 x 32.4 μm on average).

Fig. 14. Non-metric MDS ordination of 49 stocks of 13 *Paramecium* species (on the base of ratio of linear characteristics to the length) (stress = 0.060). ba - *P. biaurelia,* bu - *P. bursaria,* ca - *P. caudatum,* cl - *P. calkinsi,* du - *P. duboscqui,* je - *P. jenningsi,* mu - *P. multimicronucleatum,* nr - *P. nephridiatum,* pa - *P. pentaurelia,* po - *P. polycaryum,* pu - *P. putrinum,* ta - *P. tetraurelia,* wo - *P. woodruffi*
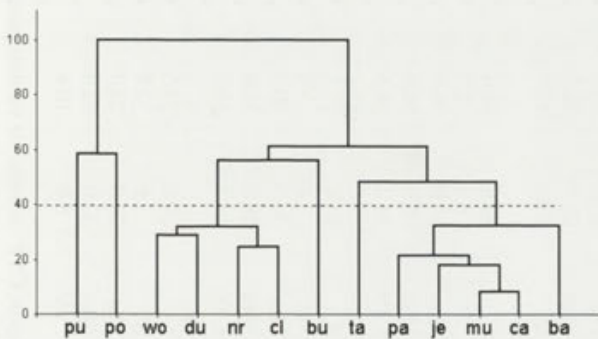
Fig. 15. Dendrogram for hierarchical clustering of 13 *Paramecium* species (UPGMA linking of Euclidean distance similarity on the base of ratio of linear characteristics to the length). Y axis - the standardized scale of the linkage distance (ratio of Euclidean distance to the maximum Euclidean distance). X axis - *Paramecium* species. Abbreviations are the same as on Fig. 14
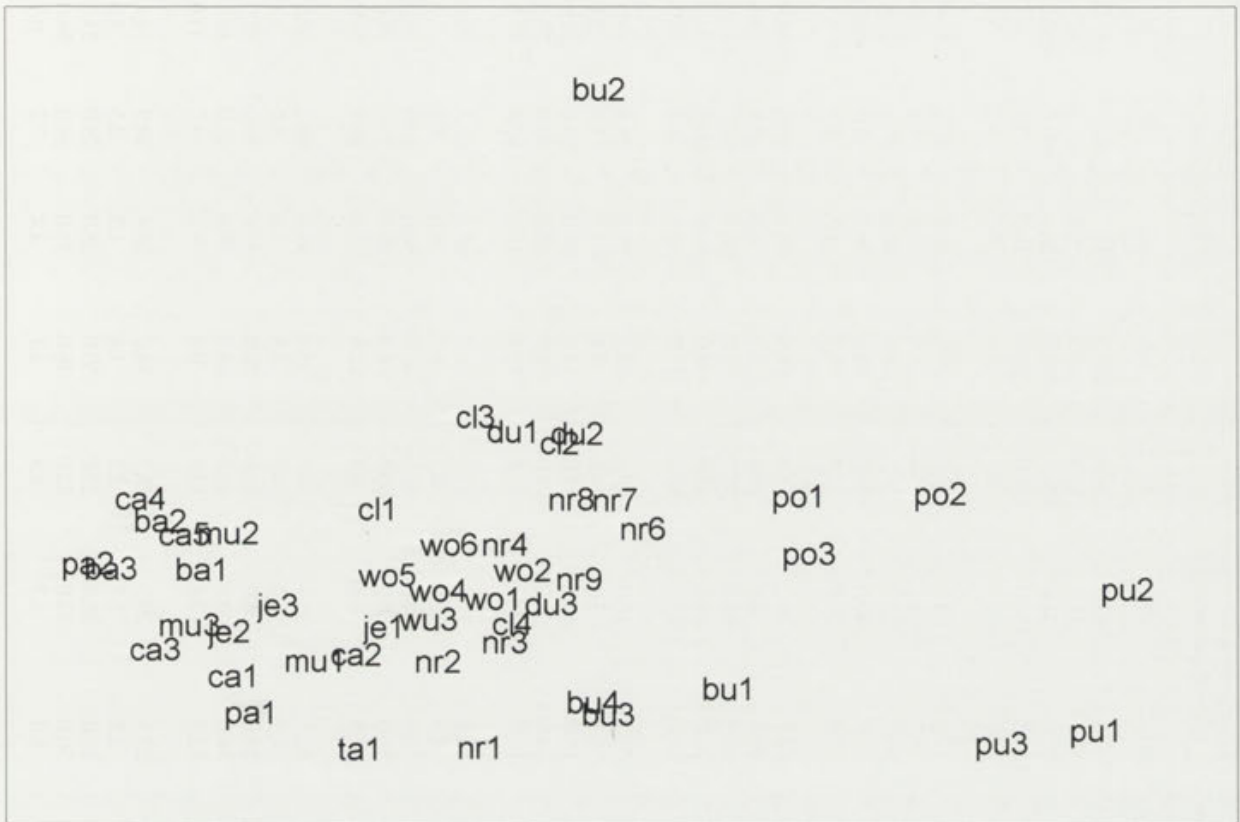

Fig. 16. Non-metric MDS ordination of 13 *Paramecium* species (on the base of ratio of linear characteristics to the length) (stress = 0.060). Abbreviations are the same as on Fig. 14

Table 4. Morphometric characterization of *Paramecium aurelia* complex and *Paramecium jenningsi*

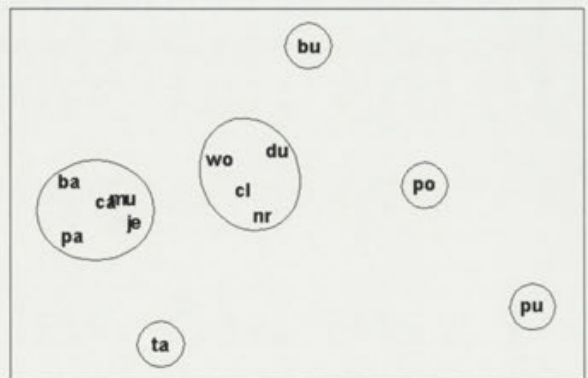| Character	Statistics	P. biaurelia				P. tetraurelia	P. pentaurelia			P. jenningsi			
		ba1	ba2	ba3	M	ta	pa1	pa2	M	jn1	jn2	jn3	M
Body length	x̄	135.0	125.2	137.4	133.0	127.7	147.5	142.1	144.9	185.0	188.1	182.0	185.0
	SD	6.3	10.4	7.4	11.3	9.2	11.5	11.6	12.4	13.5	9.8	12.3	12.3
	Min	128.0	112.0	128.0	112.0	110.0	120.0	124.0	120.0	160.0	168.0	160.0	160.0
	Max	148.0	152.0	156.0	156.0	140.0	155.0	160.0	160.0	210.0	210.0	210.0	210.0
	CoV	4.7	8.3	5.4	8.5	7.2	7.8	8.2	8.5	7.3	5.2	6.7	6.6
Body width	x̄	30.4	31.8	30.2	31.5	28.2	32.5	32.0	32.3	50.6	47.0	47.5	48.4
	SD	3.7	6.7	3.0	4.5	3.4	5.2	4.1	5.4	4.9	3.8	4.2	5.0
	Min	24.0	24.0	24.0	24.0	24.0	20.0	24.0	24.0	40.0	40.0	40.0	40.0
	Max	36.0	40.0	40.0	40.0	36.0	40.0	40.0	40.0	60.0	55.0	55.0	60.0
	CoV	10.3	12.2	21.0	9.6	14.3	12.0	16.0	12.8	16.7	9.7	8.1	8.8
Distance from anterior end to middle of buccal overture	x̄	71.1	67.7	76.0	71.8	53.7	64.7	78.2	71.4	88.0	93.0	89.8	90.3
	SD	3.5	4.8	3.8	5.2	6.8	10.5	4.8	10.8	9.7	6.3	7.5	10.0
	Min	65.0	48.0	48.0	48.0	44.0	55.0	65.0	55.0	72.0	80.0	80.0	72.0
	Max	80.0	76.0	80.0	80.0	64.0	70.0	84.0	84.0	100.0	100.0	100.0	100.0
	CoV	4.9	7.1	5.0	7.2	12.7	16.2	6.1	15.0	11.0	6.8	8.3	11.0
Distance from proximal edge of buccal overture to anterior end of cytoproct	x̄	19.6	17.7	16.6	18.0	25.1	20.1	17.0	18.6	31.0	27.5	28.4	29.0
	SD	3.4	2.3	1.9	3.7	2.8	3.1	3.8	4.0	4.8	3.7	3.9	5.1
	Min	12.0	12.0	12.0	12.0	20.0	15.0	12.0	12.0	24.0	24.0	24.0	24.0
	Max	24.0	20.0	20.0	24.0	36.0	24.0	20.0	24.0	38.0	35.0	36.0	38.0
	CoV	17.3	13.0	11.4	20.5	11.1	15.4	22.3	21.5	15.5	13.4	13.7	17.6
Distance from anterior end to anterior CVP	x̄	44.9	48.4	48.1	47.1	41.0	48.8	51.1	49.9	53.9	65.5	62.5	60.6
	SD	6.4	7.2	4.3	7.5	3.8	4.3	4.5	4.7	6.7	5.0	6.5	7.0
	Min	40.0	40.0	40.0	40.0	36.0	40.0	44.0	40.0	44.0	56.0	50.0	44.0
	Max	60.0	56.0	52.0	60.0	48.0	56.0	60.0	60.0	64.0	76.0	70.0	76.0
	CoV	14.2	14.8	8.9	15.8	9.0	8.8	8.8	9.4	12.4	7.6	10.4	11.9
Distance from posterior end to posterior CVP	x̄	23.8	25.5	25.8	25.0	29.2	32.5	35.4	34.0	35.8	41.4	40.2	39.0
	SD	3.4	3.9	3.7	4.2	3.6	4.2	4.4	4.7	3.5	3.4	3.9	4.3
	Min	20.0	20.0	20.0	20.0	24.0	24.0	28.0	24.0	28.0	36.0	30.0	28.0
	Max	28.0	32.0	32.0	32.0	32.0	36.0	40.0	40.0	44.0	48.0	48.0	48.0
	CoV	14.3	15.3	14.3	16.8	12.3	12.9	12.4	13.8	9.8	8.2	9.7	11.0
Buccal length	x̄	29.6	27.0	27.4	28.0	27.6	27.3	29.7	28.5	34.2	35.4	34.9	34.8
	SD	2.6	2.8	2.6	3.5	1.4	2.2	1.7	2.5	1.4	1.3	2.5	3.0
	Min	24.0	24.0	24.0	24.0	23.0	22.0	26.0	22.0	30.0	28.0	28.0	24.0
	Max	32.0	32.0	32.0	32.0	30.0	30.0	32.0	32.0	36.0	32.0	38.0	38.0
	CoV	8.8	10.4	9.5	10.7	5.1	8.0	5.7	8.8	4.1	4.4	7.2	9.1

CoV - coefficient of variation, CVP - contractile vacuole pore, M - mean of the species, Max - maximal size of the trait, Min - minimal size of the trait, SD - standard deviation, x̄ - mean of the stock. All measurements in μm

Table 5. Morphometric characterization of *Paramecium caudatum* and *P. multimicronucleatum*

Character	Statistics	P. caudatum						P. multimicronuclatum			
		ca1	ca2	ca3	ca4	ca5	M	mu1	mu2	mu3	M
Body length	$\bar{x}$	210.0	196.0	189.8	174.7	169.7	188.0	195.7	186.9	227.8	203.5
	SD	12.9	10.4	14.9	9.8	14.8	22.1	15.3	9.2	10.0	19.4
	Min	170.0	180.0	150.0	150.0	148.0	160.0	170.0	170.0	200.0	180.0
	Max	230.0	210.0	210.0	200.0	180.0	206.0	220.0	212.0	240.0	224.0
	CoV	6.1	5.8	7.8	5.6	8.7	11.7	7.8	4.9	4.4	9.5
Body width	$\bar{x}$	47.2	50.6	49.0	49.2	48.0	48.8	52.6	52.3	59.6	54.8
	SD	5.7	6.5	5.5	5.0	6.0	7.4	4.6	4.3	6.5	5.0
	Min	40.0	40.0	36.0	36.0	36.0	38.0	44.0	40.0	44.0	43.0
	Max	60.0	60.0	54.0	56.0	56.0	57.0	60.0	60.0	72.0	64.0
	CoV	12.1	12.8	11.2	10.2	12.5	15.1	8.7	8.2	10.9	9.1
Distance from anterior end to middle of buccal overture	$\bar{x}$	100.4	92.2	96.6	91.0	89.4	94.0	89.4	100.7	113.6	101.2
	SD	4.3	5.2	8.3	5.9	4.7	9.6	6.1	3.8	4.8	7.4
	Min	90.0	80.0	90.0	80.0	80.0	84.0	80.0	92.0	100.0	91.0
	Max	112.0	100.0	120.0	104.0	96.0	106.0	100.0	112.0	132.0	115.0
	CoV	4.3	5.6	8.6	6.5	5.2	10.2	6.8	3.8	4.2	7.3
Distance from proximal edge of buccal overture to anterior end of cytoproct	$\bar{x}$	25.5	23.9	27.9	20.7	19.2	23.4	26.2	25.0	25.6	25.6
	SD	2.1	2.2	3.2	4.5	4.4	4.9	4.2	4.7	3.4	5.4
	Min	20.0	20.0	20.0	12.0	10.0	16.0	20.0	20.0	20.0	20.0
	Max	28.0	28.0	32.0	28.0	24.0	28.0	36.0	32.0	32.0	33.0
	CoV	8.2	9.2	11.5	21.7	22.9	20.1	16.0	18.8	13.3	21.1
Distance from anterior end to anterior CVP	$\bar{x}$	63.1	55.8	64.9	68.8	61.5	62.8	57.8	66.3	77.1	67.1
	SD	2.8	4.3	6.2	9.1	6.2	9.4	8.9	5.3	4.4	9.6
	Min	56.0	52.0	58.0	48.0	52.0	53.0	40.0	56.0	64.0	53.0
	Max	72.0	60.0	72.0	88.0	80.0	74.0	80.0	76.0	80.0	79.0
	CoV	4.4	7.7	9.5	13.2	10.2	15.0	15.4	8.0	5.7	14.3
Distance from posterior end to posterior CVP	$\bar{x}$	46.1	44.5	55.6	44.5	40.5	46.2	46.8	41.0	54.6	47.5
	SD	3.7	3.3	9.0	5.7	5.1	9.5	5.4	3.1	3.4	4.0
	Min	40.0	36.0	32.0	36.0	28.0	34.0	36.0	36.0	48.0	40.0
	Max	52.0	48.0	60.0	48.0	40.0	50.0	56.0	44.0	60.0	53.0
	CoV	8.0	7.4	16.2	12.8	12.6	20.5	11.5	7.6	6.2	8.4
Buccal length	$\bar{x}$	39.5	38.0	34.0	38.8	35.7	37.2	37.2	37.8	36.4	37.1
	SD	1.6	2.3	2.3	1.9	4.5	4.8	1.2	3.7	2.7	3.9
	Min	36.0	32.0	28.0	36.0	32.0	33.0	28.0	32.0	32.0	31.0
	Max	42.0	40.0	36.0	40.0	40.0	40.0	40.0	44.0	40.0	41.0
	CoV	4.0	6.0	6.8	4.9	12.6	12.9	3.2	9.8	7.4	10.5

CoV - coefficient of variation, CVP - contractile vacuole pore, M - mean of the species, Max - maximal size of the trait, Min - minimal size of the trait, SD - standard deviation, $\bar{x}$ - mean of the stock. All measurements in μm

Two spheroid "vesicular" MI had a size between 2.2 x 2.2 and 3.9 x 3.9 μm (2.9 x 2.9 on average). They were located close to the MA.

Paramecium jenningsi (Tables 1, 4; Figs. 4, 5)

The average size of the cell in 3 investigated stocks was 185.0 x 48.4 μm. The number of kineties varied from 78 to 88 (82 on average). The BO was located near the equatorial line (49% of the body length on average). The distance from the anterior end to the single PCV of the anterior CV was 33% of the cell length. The distance from the single posterior PCV to the posterior end of the ciliate was 21% of the body length. The size of the BC was 19% of the body length. The endoral membrane was composed of 18-22 units (19.6 on average).

The ellipsoidal MA was usually situated around the cell's equator. Its size was from 22 x 38 to 33 x 60 μm (29.2 x 49.5 μm on average).

Two spheroid "chromosomal" MI had a size between 3.2 x 3.9 and 5.9 x 6.3 μm (4.5 x 5.1 μm on average). They were located close to the MA, sometimes in a depression of the latter.

Paramecium caudatum (Tables 1, 5; Figs. 12, 13)

The average size of the cell in 5 investigated stocks was 188.0x48.8 μm. The number of kineties varied from 70 to 90 (80 on average). The BO was located near the equatorial line (50% of the body length on average). The distance from the anterior end to the single PCV of the anterior CV was 33% of the cell length. The distance from the single posterior PCV to the posterior end of the ciliate was 24% of the body length.

The size of the BC was 20% of the body length. The endoral membrane was composed of 22-31 units (25.6 on average).

The ellipsoidal MA was usually situated near the cell's equator. Its size was from 35x47 to 46x78 μm (41.8 x 58.2 μm on average).

The single, ovoid or spheroid, large compact MI (type "a") had a size between 3.5x5.0 and 5.0x13.0 μm (6.4 x 8.7 μm on average). The MI was located close to the MA, often in a shallow cavity of the nucleus.

Paramecium multimicronucleatum (Tables 1, 5; Figs. 8, 9)

The average size of the cell in 3 investigated stocks was 203.5x54.8 μm. The number of kineties varied from 84 to 102 (90 on average). The BO was located near the equatorial line (50% of the body length on average). The distance from the anterior end to the single PCV of the anterior CV was 33% of the cell length. The distance from the single posterior PCV to the posterior end of the ciliate was 23% of the body length. The size of the BC was 18% of the body length. The endoral membrane was composed of 18-30 units (23 on average).

The ellipsoidal MA was usually situated near the cell's equator. Its size was from 34x46 to 48x75 μm (43.4x 61.5 μm on average).

Several (2-5) spheroid MI ("vesicular" type) had a size between 1.6 x 1.7 and 1.9 x 1.9 μm (1.8 x 1.8 μm on average). The MI were usually located close to the MA.

Classification and ordination the morphometric data for all 49 stocks used (data are not presented) and MDS analysis of the material (Fig. 14) showed quite complicated pictures. Many points (stocks) within "aurelia" and "woodruffi" subgroups were overlapping each other because of their morphometric similarity. Then we have used for the UPGMA and the MDS analysis only the mean values for the species (Figs. 15, 16).

However, stocks of some other species (*P. purtinum, P. polycaryum* and, partly *P. bursaria*) made up a distinctive subgroups. Location of the stock bu2 (*P. bursaria*), which was situated unusually far from other of the species, apparently reflects it's amicronuclearity. Many features of amicronuclear cells are often different from the "normal" (binuclear) paramecia (Golikova 1978, Fokin 1983).

Classification and ordination the morphometric data for all freshwater *Paramecium* species (the mean values) were made and compared with the data for brackishwater species (Figs. 15, 16) which were previously obtained by the authors (Fokin and Chivilev 1999). The UPGMA procedure indicated that all of the *Paramecium* species studied are distinguishable from each other (Fig. 15). The ratio-based dendrogram shows 3 separate, easily distinguishable groups (Fig. 15). On the dendrogram, *P. bursaria* is located closer to the brackishwater *Paramecium* cluster. All of the "aurelia" subgroup species (except *P. tetraurelia*) formed another branch. *P. polycaryum* and *P. putrinum* make up the third cluster. However, as is shown on the MDS plot (Fig.16), only two groups of the species are real clusters. Testing for differences between these groups ("ba+ca+je+mu+pa" and "cl+du+nr+wo") showed that all paramecia are more similar within the groups than between them (significance level 0.8%). Meanwhile, *P. bursaria, P. putrinum* and *P. polycaryum* did not manifest close similarity to each other and to the clusters. *P. tetraurela* was also situated quite far from the other species of the "aurelia "subgroup (Fig. 16).

DISCUSSION

A lot of information has been available since the beginning of the century for *Paramecium species* (Woodruff 1945; Wichterman 1953, 1986). Nevertheless, comprehensive morphometric analysis was done for only a few of the species of the *P. aurelia* complex (Gates *et al.* 1974, Powelson *et al.* 1975, Gates and Berger 1976). Some morphometric data were shown for *P. caudatum* (Jennings 1911, Fokin 1988), *P. calkinsi* (Zawoiski 1951), *P. bursaria* (Golikova 1978) and *P. polycaryum* (Takayanagi 1960) but, usually, it was done for some special purpose and without any comparison with other

Paramecium species. However, recently we carried out a morphometric analysis for brackishwater paramecia and *P. polycaryum* (Fokin and Chivilev 1999, Fokin *et al.* 1999b).

It is very important for an accurate comparison to have data which were obtained by the same method and by the same observers. Now it is possible to verify the division of the *Paramecium* genus into subgroups using our results of the morphometry of 11 *Paramecium* morphospecies.

The quantity of morphometric data in the literature are very different for the investigated species. In a mono-graphic article on *P. putrinum* Jankowski (1972) gave only the morphometry of the MI for 14 stocks from 4 syngens of the ciliate (4.2 x 6.6 μm on average). The range of the MI size, which according to Jankowski (1972) connected with it's heteroploidy was from 3.1x 3.9 μm to 5.6 x 8.5 μm. He did not show other morphometric character-istics as tables, but mentioned the range and the average cell size for three morphotypes of *P. putrinum*: *P. p. vernalis* (69-97 x 33-41 μm, mean 82 x 38 μm); *P. p. cheni* (81-119 x 32-42 μm, mean 96 x 37 μm); *P. p. apomictum* (64-83 x 27-32 μm, mean 72-30 μm). Concerning the morphology of the MA only the following was mentioned: "the MA is elongated, ovoid or curved, relatively large (12-16 x 25-30 μm)". These data are confirmed by our results (Fokin 1997, present work). Very recently the cell surface of *P. trichium (putrinum)* was observed using three different methods (Takahashi *et al.* 1998). The main traits of morphology of the non-dividing cells which were observed in this study fit well with our morphometric results.

Some morphometric data for *P. bursaria* were pre-sented by Golikova (1978) who made a measurements on 147 clonal cultures: cell size- from 73 x 27 μm to 114 x 53 μm; the MA size- from 11 x 20 μm to 19 x 40 μm; the MI size- from 1.6 x 6.5 μm to 8.4 x 19.5 μm. Because she used many more stocks than we did, and only cells fixed by Nissenbaum's fixative were used, our results are a bit different.

According to the literature *P. putrinum* and *P. bursaria* are considered to be closely related species (Jankowski 1969, 1972). Following the author, these species have a number of similar morphological characteristics. How-ever, the results of our morphometric analysis using a subset of cell attribute ratio show that these species are quite different from the morphological point of view. In fact, only the ratio width/length is quite similar for them (0.37 - *P. putrinum*, 0.35 - *P. bursaria*). Other character-istics (ratios) - position of buccal overture, location of the PCV, distance between proximal edge of the BO and the

beginning of the C - are different. As a result, these species are located far from each other both on the dendrogram and MDS plot (Figs. 15, 16).

Other distinctive characteristics, which are connected with the species morphology, are the morphology of the CV and the MI structure. The CV of *P. bursaria* are located close to the cell's surface and have 5-7 radial canals with 2-5 pores in each of them (Fokin 1986). The CV of *P. putrinum* are located well below the surface and have very long excretory tubes with single outlet pore in each of them. Radial canals in this type of CV are absent (Wichterman 1953, Jankowski 1972). The MI looks similar in both species, but it has different ultrastructure (Fokin 1997). Thus, we conclude that *P. bursaria* and *P. putrinum* according to their morphology are not so similar as was previously believed (Jankowski 1969, 1972). Moreover, the "putrinum" subgroup does not exist according to our data (Figs. 15, 16).

Using the morphometric data for *P. polycaryum* ob-tained previously (Fokin and Chivilev 1999) for the analysis of relationships between *Paramecium* species we have found an error in the taxonomical position of this species within the genus. It should be excluded from the "woodruffi" subgroup (brackishwater paramecia; "woodruffi" group or subgenus *Cypreostoma* according to Jankowski 1969, 1972). On the dendrogram (Fig. 15) *P. polycaryum* is located much closer to *P. putrinum* than to any species of the "woodruffi" subgroup, but on the MDS plot (Fig. 16) *P. polycaryum* is located quite far from *P. putrinum* as well. According to some biological pecu-liarities (Fokin and Chivilev 1999) *P. polycaryum* should be located somewhere between the "woodruffi" and the "aurelia" subgroups.

The "woodruffi" subgroup thus consists of 4 species: *P. woodruffi*, *P. nephridiatum*, *P. calkinsi* and *P. duboscqui*. Previous morphometric analysis of the subgroup using WPGMA method and a combination of the actual mor-phological measurements and the ratios (Fokin and Chivilev 1999) has shown more morphological similarity between the last three species. However, *P. duboscqui* has a number of biological and morphological traits which are different from the other representatives of the subgroup (Fokin and Chivilev 1999, Fokin *et al.* 1999b). The other species (*P. woodruffi*, *P. nephridiatum* and *P. calkinsi*) are a solid group from the biological point of view (Fokin and Chivilev 1999). Nevertheless, on the MDS plot all these brackishwater species formed a real cluster (Fig. 16).

According to some speculation of Jankowski (1972), *P. putrinum* gave rise to the *P. polycaryum - P. woodruffi*,

P. aurelia line, and *P. woodruffi* was the ancestor of other species within its subgroup.

It seems to us, *P. putrinum* does not to be an ancestor in the scheme because of its morphology. Future work may reveal the real position of *P. putrinum* and the relationship between *P. duboscqui* and others brackishwater *Paramecium* species.

For the higher "aurelia" subgroup of *Paramecium* we used three species of *P. aurelia* complex (Table 1). All of them (*P. biaurelia, P. tetraurelia* and *P. pentaurelia*) have been used for biometrical analysis before (Gates *et al.* 1974, Powelson *et al.* 1975, Gates and Berger 1976), but solely for the purpose of syngenic identification. It is necessary to mention that the variations of cell length for *P. biaurelia* which are given in the different studies was comparatively large from work to work: 145-149 μm (Sonneborn 1957), but 156 -180 μm (Powelson *et al.* 1975) and 133 μm (this study).

Unfortunately, for the morphometry of *P. tetraurelia* we could use only stock 51 which was, apparently, not enough for any conclusion. At the same time, the cells of this stock manifested some special "topographic" peculiarities (Table 4; Figs.15, 16) which caused the separation of the species from the set of the *P. aurelia* complex and even from the "aurelia" species subgroup (Fig.16). Namely, these are the BO position and the distance between proximal edge of the BO and the beginning of the C. Apparently, these characteristics are very stable for the species. Our morphometric data on stock 51 of *P. tetraurelia* are consistent with many literature data (Grandchamp and Beisson 1980, Ng and Tam 1987, Loubresse *et al.* 1988, Iftode *et al.* 1989, Lai and Ng 1991).

The distance between *P. tetraurelia* (stock 51) and other sibling species of the *P. aurelia* complex: *P. primaurelia, P. biaurelia,* and *P. pentaurelia* are the largest (Gates *et al.* 1974, this study). A similar result has been found recently by sequencing 23S rRNA domain for 6 species from the *P. aurelia* complex (Nanney *et al.* 1998). According to the dendrogram and the MDS plot (Figs. 15, 16), the other *P. aurelia* complex species used, *P. biaurelia* and *P. pentaurelia*, are well clustering with the rest of "highest" paramecia. Information about taxonomic and evolutionary relationships within the *P. aurelia* complex can be found in the literature (see: Sonneborn 1957, 1975; Allen *et al.* 1982, 1983; Przyboś 1986a; Wichterman 1986).

As far as we know full morphometric investigations on *P. jenningsi, P. caudatum* and *P. multimicronucleatum* have never been done. Some morphometry was made by Przyboś (1986b) for three stocks of *P. jenningsi.* They

are 171.4 x 86.3 μm - body size, 26.3 x 55.3 μm - MA size, 5.0 x 5.4 μm - MI size (stock I); 159.2 x 82.5 μm-body size, 28.8 x 49.7 μm - MA size, 4.5 x 4.9 μm - MI size (stock U) and 158.7 x 74.1 μm - body size, 29.2 x 49.5 μm - MA size, 4.3 x 4.7 μm - MI size (stock M). Because of the different fixatives which were used (Champy's- in our case and Schaudinn's in Przyboś' work) and the different procedure, the cell's dimensions were found to be different. When we used Bouin's fixative for the same stock cultures we obtained very similar results (Przybos *et al.* 1999). Some morphometric data on *P. jenningsi* were shown in the first description of the species and even earlier (Sonneborn 1957, Diller and Earl 1958).

All of these three species are clustered in a dense group (Figs. 15, 16). According to the dendrogram P. *caudatum* has more similarity to *P. multimicronucleatum* than to *P. jenningsi* (Fig. 15), but on the MDS plot all these species are located very close each other (Fig. 16). Both results have some confirmation in the literature. (Usuki and Irie 1983a, b; Nanney *et al.* 1998). Apparently, the "aurelia" subgroup is a real cluster of species. They have quite uniform morphology, but also manifest some very distinctive morphological traits (Fokin 1997). Using morphometry of 13 cell attributes they can be distinguished confidently by the UPGMA method as well.

The first attempt to divide *Paramecium* on subgroups was made by Woodruff (1921). He wrote, " …the species of *Paramecium*… may be referred to as the 'aurelia group' and the 'bursaria group'. The members of the 'aurelia group' (*P. aurelia, P. caudatum* and *P. multimicronucleata*) are characterized by a relatively long spindle- or cigar-shaped body; those of the 'bursaria group' (*P. bursaria, P. putrinum, P. thichium* and *P. calkinsi*) by a somewhat shorter and broader form, with a tendency, especially prominent in *P. bursaria*, toward a dorsoventral flattening". Jankowski (1969, 1972) recognized three such groups: the "putrinum" (*P. putrinum, P. bursaria*), the "woodruffi" (*P. woodruffi, P. calkinsi, P. polycarium, P. arcticum* and *P. preudotrichium*) and the "aurelia" (*P. aurelia, P. caudatum, P. jenningsi, P. africanum, P. multimicronucleatum* and *P. wichtermani*).

For these subgroups he has proposed a range of taxonomic subgenera, *Helianter, Cypreostoma* and *Paramecium s. str.*, respectively. These subgroups were distinguished by the author mainly by morphological features, but the actual measurements were not presented (Jankowski 1969, 1972).

Later *P. duboscqui* was placed close to or into the "woodruffi" subgroup according to some of its morpho-

logical traits (Fokin 1986, 1989; Fokin and Chivilev 1999). Meantime, *P. arcticum* is still considered to be a doubtful or uncertain species (Fokin 1986, 1997; Wichterman 1986). Any precise data on morphology of *P. peseudotrichium*, other African *Paramecium* species and *P. wichtermani* are also absent (Wichterman 1986). Thus, we have used for the morphometric analysis all valid morphospecies, which exist at present as laboratory cultures. According to the results, neither type of previous taxonomy (the two subgroup of Woodruff and three subgroup taxonomy of Jankowski), is supported by morphometric analysis. We may confidently accept the general separation of the *Paramecium* species that this procedure produced, with the understanding that larger samples will be required to confirm the position of some species.

Of course, the real taxonomy and phylogenetic relationships within the genus must be analyzed by a wide comparative approach. For this purpose, the comparison of not only morphometric, but also biological features of the species and gene sequence analysis using large collections of *Paramecium* species (and stocks) should be done. Some of this work has already been started (Nanney *et al.* 1998, Strueder-Kypke *et al.* 1999, Fokin and Chivilev 1999). The knowledge about phylogenetic relationships within the genus can resolve the evolution of the nuclear reorganization during *Paramecium* conjugation. It is also necessary for a suitable explanation of the differences in mating type systems between some *Paramecium* species.

REFERENCES

Allen S.L., Lau E.T, Nerad T.A., Rushford C.L. (1982) Esterase variants in four species of the *Paramecium aurelia* complex. *J. Protozool.* **29**: 604-611

Allen S.L., Nerad T.A., Rushford C.L. (1983) Comparison of the esterases and acid phosphatases in *Paramecium multimicronucleatum*, syngens 1-5, *Paramecium jenningsi*, *Paramecium caudatum* and the *Paramecium aurelia* complex. *J. Protozool.* **30**: 148-154

Braverman E.M. (1965) On potential function procedure. *Automat. Telemech.* **12**: 21-26

Claparède E., Lachmann J. (1858) Etudes sur les Infusoires et les Rhizopodes. Geneve

Corliss J.O. (1953) Silver impregnation of ciliate protozoa by the Chatton-Lwoff technique. *Stain Technol.* **28**: 97-100

Diller W.F., Earl P.R. (1958) *Paramecium jenningsi*, n. sp. *J. Protozool.* **5**: 155-158

Dujardin M.F. (1841) Histore Naturelle des Zoophytes. Infusoires. Paris

Ehrenberg C.G. (1838) Die Infusionsthierchen als Vollkommene Organismen. L.Voss, Leipzig

Fokin S.I. (1983) Morphology of the *Paramecium bursaria* cells after UV irradiation of the germ nuclei. *Cytology* (St. Petersburg) **35**: 328-333 (in Russian with English summary)

Fokin S.I. (1986) Morphology of the contractile vacuoles in ciliated Protozoa of the genus *Paramecium* (Hymenostomatida, Peniculina) as a species-specific character. *Zool. Zh.* (Moscow) **65**: 5-15 (in Russian with English summary)

Fokin S.I. (1988) *Paramecium caudatum*: morphological analysis of consequences of infection with different nuclear symbionts. *Cytology* (St. Petersburg) **30**: 471-477 (in Russian with English summary)

Fokin S.I. (1989) Infusoria of *Paramecium* genus. Morphology and systematic. In: Ecology of Marin and Fresh-water Protozoans, (Ed. G.I. Poljansky). Yaroslavl, 74 (in Russian with English title)

Fokin S.I. (1997) Morphological diversity of the micronuclei in *Paramecium*. *Archiv. Protistenkd.* **148**: 375-387

Fokin S.I., Chivilev S.M. (1999) Brackishwater *Paramecium* species and *Paramecium polycaryum*. Morphometric analysis and some biological peculiarities. *Acta Protozool.* **38**: 105-117

Fokin S.I., Stoeck T., Schmidt H.J. (1999a) Rediscovery of *Paramecium nephridiatum* Gelei, 1925 and its characteristics. *J. Euk. Microbiol.* **46**: 416-426

Fokin S.I., Stoeck T., Schmidt H.J. (1999b) *Paramecium duboscqui* Chatton, Brachon, 1933. Distribution, ecology and taxonomy. *Europ. J. Protistol.* **35**: 161-167

Gates M.A., Berger J. (1976) Morphometric inseparability of *Paramecium primaurelia* and *Paramecium pentaurelia*. *Trans. Amer. Micros. Soc.* **95**: 507-514

Gates M.A., Powelson E.E., Berger J. (1974) Syngenic ascertainment in *Paramecium aurelia*. *Syst. Zool.* **23**: 482-489

Golikova M.N. (1978) Morphology and viability of amicronucleate clones of the ciliate *Paramecium bursaria*. *Acta Protozool.* **17**: 89-108 (in Russian with English summary)

Grandchamp S., Beisson J. (1980) Positional control of nuclear differentiation in *Paramecium*. *Develop. Biol.* **81**: 336-341

Iftode F., Cohen J., Ruiz F., Rueda A.T., Chen-Shan L., Adoutte A., Beisson J. (1989) Development of surface pattern during division in *Paramecium*. I. Mapping of duplication and reorganization of cortical cytoskeletal structures in the wild type. *Development* **105**: 191-211

Jankowski A.V. (1969) A proposal taxonomy of the genus *Paramecium* Hill 1752 (Ciliophora). *Zool. Zh.* (Moscow) **48**: 30-40 (in Russian with English summary)

Jankowski A.V. (1972) Cytogenetic of *Paramecium putrinum* C. et L., 1858. *Acta Protozool.* **10**: 285-394

Jennings H.S. (1911) Assortative mating, variability and inheritance of size in the conjugation of *Paramecium*. *J. Exp. Zool.* **11**: 1-134

Kent W.S. (1881-1882) Manual of the Infusoria. D. Bogue. London

Kruskal J.B., Wish M. (1978) Multidimensional scaling. Sage publications. Beverly Hills, California

Lai V.C.H., Ng S.F. (1991) Discrimination of cytoskeletal elements of *Paramecium* by heterologous antisera: a preliminary investigation on the presence of intermediate filament-related protein. *Europ. J. Protistol.* **27**: 290-305

Loubresse de N.G., Keryer G., Vigues B., Beisson J. (1988) A contractile cytoskeletal network of *Paramecium*: the infraciliary lattice. *J. Cell Sci.* **90**: 351-364

Maupas E. (1888) Recherches experimentales sur la multiplication des Infusories cilies. *Arch. Zool. Exp. Gen.* **6**: 165-277

Mueller O.F. (1786) Animalcula Infusoria. Fluviatilia et marina. Hauniae, Copenhagen

Nanney D.L., Park C., Preparata R., Simon E.M. (1998) Comparison of sequence differences in a variable 23S rRNA domain among set of cryptic species of ciliated Protozoa. *J. Euk. Microbiol.* **45**: 91-100

Ng S.F., Tam L.-W. (1987) Stomatogenesis in *Paramecium tetraurelia*. The role of the micronucleus and the pre-existing oral apparatus. *Europ. J. Protistol.* **23**: 141-151

Powelson E.E., Gates M.A., Berger J. (1975) A biometrical analysis of 22 stocks of four syngens of *Paramecium aurelia*. *Can. J. Zool.* **53**: 19-32

Przyboś E. (1986a) Species structure in ciliates. *Folia Biol. (Kraków)* **34**: 103-132

Przyboś E. (1986b) Chromosomes in *Paramecium jenningsi* (Diller and Earl, 1958): A serial section study. *Folia Biol. (Kraków)* **34:** 133-160

Przyboś E., Fokin S., Stoeck T., Schmidt H.J. (1999) Occurrence and ecology of *Paramecium jenningsi* strains. *Folia Biol. (Kraków)* **47:** 53-56

Shi X., Jin M., Liu G. (1997) Rediscovery of *Paramecium duboscqui* Chatton & Brachon, 1933, and a description of its characteristics. *J. Euk. Microbiol.* **44:** 134-141

Skovorodkin I.N. (1990) A device for immobilization of biological objects in the light microscope studies. *Cytologia* (Sankt Petersburg) **32:** 515-519 (in Russian with English summary)

Sneath, P.H.A., Sokal, R.R. (1973) Numerical Taxonomy. W. H. Freeman & Co., San Francisco

Sonneborn T.M. (1957) Breeding systems, reproductive methods, and species problems in Protozoa. In: The Species Problem. *Am. Ass. Advanc. Sci.* Washington, D.C., 155-324

Sonneborn T.M. (1975) The *Paramecium aurelia* complex of fourteen sibling species. *Trans. Amer. Micros. Soc.* **94:** 155-178

Strueder-Kypke M.C., Wright A.-D. G., Fokin S., Lynn D.H. 1999. Phylogenetic relationships of Peniculine ciliates, *Paramecium* spp. and *Lembadion bullinum*, inferred from 18S rRNA sequence. *J. Euk. Microbiol.* **46(Suppl.):** 7a

Takahashi T., Okubo M.-A., Hosoya H. (1998) Regional differentiation of cortical structures and their reorganization during cell division in *Paramecium trichium*. *J. Euk. Microbiol.* **45:** 369-380

Takayanagi T. (1960) Cytological and cytogenetical studies on *Paramecium polycaryum* II. Cytological responses of paramecia to mitomycin-C, azan, MEPA and podophyllin. *J. Fac. Sci. Hokkaido Univ.* Ser.VI, Zool. **14:** 453-462

Usuki I., Irie T. (1983a) Inter- and intrasyngenic variation of *Paramecium* hemoglobin- I. *Paramecium aurelia* complex. *Comp. Biochem. Physiol.* **75B:** 415-420

Usuki I., Irie T. (1983b) Inter- and intrasyngenic variation of *Paramecium* hemoglobin-II. *Paramecium caudatum, Paramecium jenningsi* and *Paramecium multimicronucleatum*. *Comp. Biochem. Physiol.* **75B:** 421-424

Viver E. (1974) Morphology, taxonomy and general biology of the genus *Paramecium*. In: *Paramecium*. A Current Survey. (Ed.W.J.Van Wagtendonk). Elsevier Scientific Publishing Company, Amsterdam, London, New York, 1-90

Wenrich D.H. (1928) Eight well-defined species of *Paramecium*. *Trans. Am. Microsc. Soc.* **47:** 274-282

Wichterman R. (1953) The Biology of *Paramecium*. Blakiston, New York, Toronto

Wichterman R. (1986) The Biology of *Paramecium*. 2-nd ed. Plenum Press, New York, London

Woodruff L.L. (1921) The structure, life history and intrageneric relationships of *Paramecium calkinsi* n. sp. *Biol. Bull.* **241:** 171-180

Woodruff L.L. (1945) The early history of the genus *Paramecium* with special reference to *P. aurelia* and *P. caudatum*. *Trans. Conn. Acad. Art. Sci.* **36:** 517-531

Zawoiski E. (1951) A statistical analysis of the size of different clones of *Paramecium calkinsi* and the fate of exconjugants. *Pen. Acad. Sci.* **25:** 66-71

ACTA
PROTOZOOLOGICA

Intra-species Differentiation and Level of Inbreeding of Different Sibling Species of the *Paramecium aurelia* Complex

Thorsten STOECK[1], Ewa PRZYBOŚ[2], Jürgen KUSCH[1] and Helmut J. SCHMIDT[1]

[1]Department of Ecology, University of Kaiserslautern, Kaiserslautern, Germany; [2]Polish Academy of Sciences, Institute of Systematics and Evolution of Animals, Kraków, Poland

Summary. A combination of classical inter- and intra-strain crosses by mating reactions and randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) was used to detect different population specific genotypes within two species of the *Paramecium aurelia* complex. *P. novaurelia* strains originating from Spain, Germany, Scotland, Poland, Czech Republic, Ukraine, Turkey, and *P. pentaurelia* strains from Spain, Hungary, and the USA were studied. A high percentage of surviving clones in both generations, F1 (obtained by conjugation) and F2 (obtained by autogamy), was observed in strain crosses in these two species. The fingerprint method distinguished four genotypes within the studied *P. novaurelia* strains. Genotype I was observed in Spanish strains, genotype II in strains originating from Scotland and Turkey, genotype III was observed in the strain from Germany, and genotype IV described the strains from Central and Eastern Europe (Poland, Czech Republic, Ukraine). In contrast, the studied *P. pentaurelia* strains, originating from Europe (Spain, Hungary) and the USA showed the same genotype, in spite of the geographical isolation of strains. From these results, we conclude that both species, *P. novaurelia* and *P. pentaurelia* may show different degrees of inbreeding. While *P. novaurelia* can be described as a moderate inbreeder consisting of different genotypes which are able to mate under laboratory conditions, *P. pentaurelia* is described as a weak inbreeder which is open to gene flow, as the strains from distant places are characterized by only one single genotype. The species have different life history strategies as a consequence of different degrees of inbreeding. Remarkable ecogenetic differences are also true for strains within the same species.

Key words: breeding system, life history strategies, *Paramecium aurelia* species complex, population structure, RAPD-PCR.

Abbreviations used: bp - base-pairs, c.m.t. - complementary mating types, d.i.l.- daily isolation lines.

INTRODUCTION

For over a century *Paramecium aurelia* was considered to be a single species. Recently, however, it has been described as a species complex consisting of at least 15 different sibling species (Sonneborn 1975,

Aufderheide *et al.* 1983). These sibling species seem morphologically similar, although they are genetically isolated and are therefore true biological species *sensu stricto*, each characterized by two complementary mating types. The species differ significantly with respect to geographical distribution, temperature requirements, culture conditions necessary for conjugation, and system of mating type inheritance. Furthermore the species are characterized by different ecological demands concerning their habitat range and many of their life cycle characteristics.

Address for correspondence: Thorsten Stoeck, Department of Ecology, University of Kaiserslautern, Erwin-Schrödinger-Straße 14, D-67663 Kaiserslautern, Germany; Fax: 0049 631/2052998; E-mail: stoeck@rhrk.uni-kl.de

Paramecia are among the best studied ciliates in the laboratory since researchers of almost all biological fields investigated *Paramecium* for nearly a century. Technical difficulties (Landis 1988) caused that the correlation of these findings to distinct species were of limited success. Thanks to the development of molecular biological methods and their application in *Paramecium* research (Stoeck and Schmidt 1998, Przyboś *et al.* 1999, Fokin *et al.* 1999) this will probably change.

Several studies deal with the population structure of different ciliates investigated by RAPD-fingerprinting (Lynch *et al.* 1995, Kusch and Heckmann 1996, Kusch 1998). Stoeck *et al.* (1998) applied this method and classical strain crosses to study the population structure of two different sibling species of the *Paramecium aurelia* species complex, namely *P. triaurelia* and *P. sexaurelia*. The authors showed that the species have different life history strategies as a consequence of different degrees of inbreeding. Remarkable ecogenetic differences are also true for strains within the same species. This discovery shed new light on the important evolutionary process of nascent speciation.

In the present study, we investigated the population structures of *P. novaurelia* and *P. pentaurelia*. The population structure and life history strategies of the investigated species, related to the data concerning *P. triaurelia* and *P. sexaurelia*, studied previously, will be discussed.

MATERIALS AND METHODS

Strains

The strains which were used for RAPD-analyses and strain crosses are listed in Table 1.

Strain crosses

The methods of culturing the strains, in order to obtain the F1 generation by conjugation and F2 by autogamy in intra- and inter-strain crosses, were those described by Sonneborn (1950, 1970). The complementary mating types (c.m.t.) were mixed and about 5 h later the closely united pairs, 50 in each cross, were isolated for observations on the survival of the F1 generation clones. The existence of a strong union of conjugating cells as well as the stage of conjugation was checked on preparations stained with acetocarmine. The next day, exconjugants were separated and 100 established clones cultured for 3 days at 24°C. As a criterion of clone survival we assumed its capability of undergoing 6 fissions, according to Chen's method (Chen 1956). To obtain F2 generation by autogamy five additional pairs were also isolated for daily isolation lines (d.i.l.), i. e., 10 exconjugant lines. Within these lines autogamy was found. Autogamous paramecia were isolated at

Table 1. Strains of *Paramecium novaurelia* and *P. pentaurelia* which were used for RAPD-analyses and strain crosses

Strains	Geographical sources	Reference
P. novaurelia		
SO	Castille, Spain	Przyboś 1991
SB	Pyrenees, Spain	Przyboś 1991
TB	Beysahir, Turkey	Przyboś 1998a
UC	Carpathians, Ukraine	Przyboś and Chornobai 1994
UH	Horodnica, Ukraine	Przyboś 1997
UK	Krutiliv, Ukraine	Przyboś 1997
PR	Rożnów Plateau, Poland	Przyboś and Komala 1984
PP	Pieniny Mts, Poland	Przyboś *et al.* 1979
PT	Tyniec, Poland	Kościuszko *et al.* 1961
PS	Western Sudetes, Poland	Komala and Przyboś 1990
CS	Orlickie Plateau, Czech Republic	Komala and Przyboś 1992
CVH	Nizky Jesenik, Czech Republic	Przyboś and Komala 1992
GM	Münster, Germany	Przyboś and Fokin 1997
510	Edinburgh, Scotland	Beale and Schneller 1954
P. pentaurelia		
HB	Balatonfuzfo, Hungary	Kościuszko 1964
SH	Castille, Spain	Przyboś 1993
87	Pennsylvania, USA	Sonneborn 1974

the stage of two macronuclear anlagen, defined on the preparations stained with acetocarmine. Fifty autogamous specimens were isolated, then 100 postautogamous karyonids separated, and the survival of F2 clones evaluated after 3 days of culturing. Paramecia cultured by the method of d.i.l. at 24°C in a medium assuming a constant rate of 3 fissions daily underwent autogamy, giving rise to F2, after 3 to 4 days of starvation of the clone in a given line and thus after having gone through 9 to 12 fissions. Paramecia were cultivated on a lettuce medium inoculated with *Enterobacter aerogenes*. The possibility of selfing reaction (homotypic pairs) within the complementary mating types was excluded before isolating the conjugating pairs (heterotypic) in the studied crosses. The unmixed part of cultures representing the c.m.t. were observed several hours to avoid selfing reaction within c.m.t.

RAPD-fingerprinting

Randomly amplified polymorphic DNA fingerprint analyses of the investigated *Paramecium aurelia* species were performed according to Stoeck and Schmidt (1998). Briefly, for polymerase chain reaction (PCR) ciliates were cultivated at 20°C on hay medium inoculated with *Enterobacter aerogenes*. Cells were washed in sterile SMB medium (distilled water plus 1.5 mM NaCl, 0.05 mM KCl, 0.4 mM $CaCl_2 \cdot 2H_2O$, 0.05 mM $MgCl_2 \cdot 6H_2O$, 0.05 mM $MgSO_4 \cdot 7H_2O$, 2.0 mM sodium phosphate buffer, pH 6.8). Then two or three of the ciliates were isolated in a total volume of 2.5 µl SMB wash medium under a dissecting microscope and transferred into PCR-tubes. 22.5 µl sterile filtered lysis buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.45% Tween 20, 0.45% Nonidet P 40 and 100 ng Proteinase K/ml) were added to the probes. The reaction mixtures

Table 2. Genetical Similarity Index between the four different *P. novaurelia* genotypes (PnI - PnIV) as revealed by the 10mer random primer Ro460-04, calculated according to Wetton *et al.* (1987)

	Pn I	Pn II	Pn III	Pn IV
Pn I	1.00	0.914	0,944	0.971
Pn II	0.914	1.00	0.857	0.941
Pn III	0.944	0.857	1.00	0.914
Pn IV	0.971	0. 941	0.914	1.00

were incubated at 56°C for 90 minutes and afterwards at 95°C for 15 minutes.

The following polymerase chain reactions were performed exactly according to the description of Stoeck and Schmidt (1998) including the oligonucleotide 10mer random primer (Ro 460-04, Roth, Karlsruhe, Germany) characterized by the sequence 5'-GCAGAGAAGG -3'.

We routinely analyzed 15 µl PCR product along with the pGEM DNA molecular weight marker distributed by Promega/Serva Heidelberg, Germany, by electrophoresis in 1.8% TAE-agarose midi gels, stained with 0.5 µg/ml ethidium bromide.

Reproducibility of resulting banding patterns was verified by repeating the experiments several times with other paramecia from appropriate stocks or clones.

Similarity-index S of the DNA-band pattern was calculated as described by Wetton *et al.* (1987) with the equation $S = 2NAB / (NA + NB)$. NAB describes the number of DNA-bands shared by sample A and sample B and NA and NB is the number of DNA-bands which occur in sample A and in sample B, respectively.

RESULTS

RAPD-fingerprinting of *P. novaurelia*

In spite of their widely different geographic origins the 14 investigated *P. novaurelia* strains are characterized by an identical basic diagnostic fingerprint DNA-pattern (Fig. 1). This pattern was described as specific for the *P. novaurelia* sibling species (Stoeck and Schmidt 1998). On top of their basic pattern, a number of differences were noted. A comparison of all band patterns of the investigated strains revealed additional population specific DNA bands which allow the differentiation of four different genotypes. Consequently, in this case the term "genotype" is solely and arbitrarily used to describe paramecia of different populations of either *P. novaurelia* or *P. pentaurelia* with minor but typical differences in their RAPD-patterns. The different genotypes are schematically represented in Fig. 2. An additional band at about 540 bp characterizes the genotype *P. novaurelia* (Pn) I, whereas a lack of a DNA-band at about 1050 bp but an

Table 3. Distribution of the observed genotypes within the investigated strains of *P. novaurelia* (Pn)

Genotype				Strain	Geographical source
PnI	PnII	PnIII	PnIV	*P. novaurelia*	
*				SO	Spain
*				SB	Spain
	*			TB	Turkey
	*			510	Scotland
		*		GM	Germany
			*	PS	Poland
			*	PR	Poland
			*	PT	Poland
			*	PP	Poland
			*	UC	Ukraine
			*	UH	Ukraine
			*	UK	Ukraine
			*	CS	Czech Republic
			*	CVH	Czech Republic

additional band at about 360 bp characterizes the genotype Pn II. The genotype Pn III shows two additional bands at about 540 bp and 475 bp but a DNA-band at 490 bp is missing. The genotype Pn IV resembles the genotype Pn I with a similarity of 97.1%. These two genotypes differ in only one single DNA-band at 540 bp. The genetical similarity between the patterns of the different strains as revealed by the 10mer oligonucleotid primer Ro460-04 ranges between 85.7% and 97.1% with an overall mean of 92.3% (Table 2).

Concerning the geographical distribution of the different *P. novaurelia* genotypes (Table 3) the genotype Pn I can be observed in Western Europe only and the genotype Pn IV seems to be restricted to the Central and Eastern Europe. The genotype II probably is widely distributed and can be found in Turkey as well as in Scotland. The genotype Pn III was observed only in Germany.

Strain crosses of *P. novaurelia*

A high percentage of clones of F1 and F2 generations survived in intra-strain crosses of the 8 chosen strains from Spain, Germany, Czech Republic, Poland, Ukraine, Turkey, as well as in inter-strain crosses between them and with the standard strain 510 (at present designated in ATCC number 30745, strain 323) of *P. novaurelia* from Scotland (Table 4). The rate of fissions in intra- and inter-strain crosses was similar in the F1 lines leading to the F2 generation, cultured by the method of d.i.l. Autogamy in all the studied hybrid lines appeared after a similar number of fissions. No disorder occurred in the course of the life cycle of the hybrids.
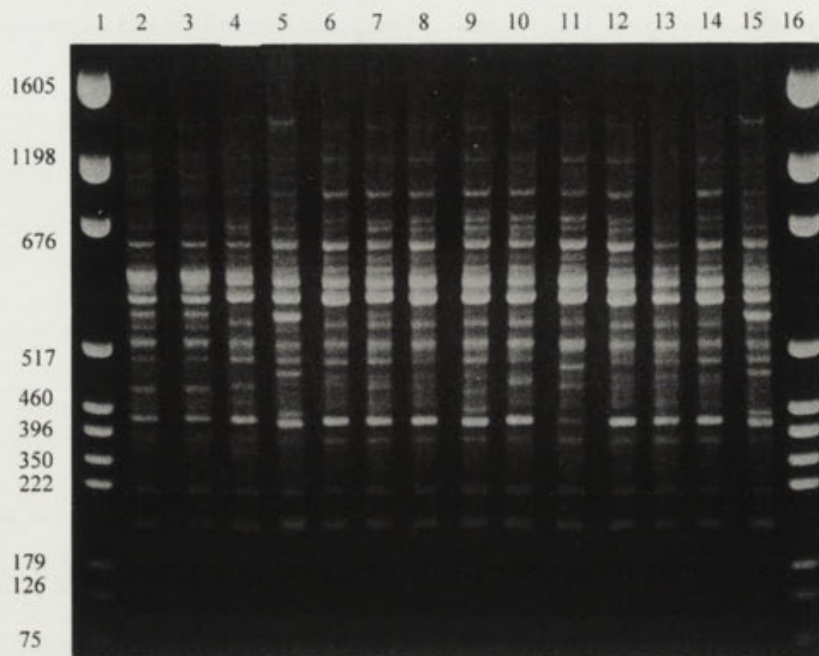
Fig. **1**. RAPD-fingerprint band pattern of the investigated *Paramecium novaurelia* strains in a 1.8% TAE-agarose gel. 1 pGEM-marker (molecular weight of the DNA-bands is given in bp); 2 SO (Spain), 3 SB (Spain), 4 TB (Turkey), 5 GM (Germany), 6 PS (Poland), 7 PR (Poland), 8 PT (Poland), 9 PP (Poland), 10 UC (Ukraine), 11 UH (Ukraine), 12 UK (Ukraine), 13 CS (Czech Republic), 14 CVH (Czech Republic), 15 Standard 510 (Scotland), 16 pGEM-marker

Table 4. Percentage of surviving clones in crosses of *P. novaurelia* strains. Numbers before brackets F1 generation, numbers in brackets F2 generation, * no data available

strains	510	SO	TB	UK	PR	PS	CVH	GM
510	100	100	100	100	100	100	100	100
	(100)	(94)	(98)	(100)	(100)	(94)	(100)	(100)
SO	100	100	100	*	*	100	*	*
	(94)	(100)	(100)			(92)		
TB	100	100	100	*	*	100	*	*
	(98)	(100)	(98)			(100)		
UK	100	*	*	100	100	*	98	100
	(100)			(98)	(100)		(95)	(100)
PR	100	*	*	100	100	*	100	98
	(100)			(100)	(100)		(95)	(90)
PS	100	100	100	*	*	100	*	*
	(94)	(92)	(100)			(98)		
CVH	100	*	*	98	100	*	100	100
	(100)			(95)	(95)		(100)	(96)
GM	100	*	*	100	98	*	100	100
	(100)			(100)	(90)		(96)	(100)

RAPD-fingerprinting of *P. pentaurelia*

The fingerprint band patterns of the strains isolated from Spain and from Hungary are characterized by the same band pattern as we could observe for the standard strain 87, collected in the USA (Fig. 3). The DNA-pattern is identical to the *P. pentaurelia* specific pattern as described by Stoeck and Schmidt (1998). In contrast to the results of *P. novaurelia* the different *P. pentaurelia* strains show a pattern which is 100% identical between
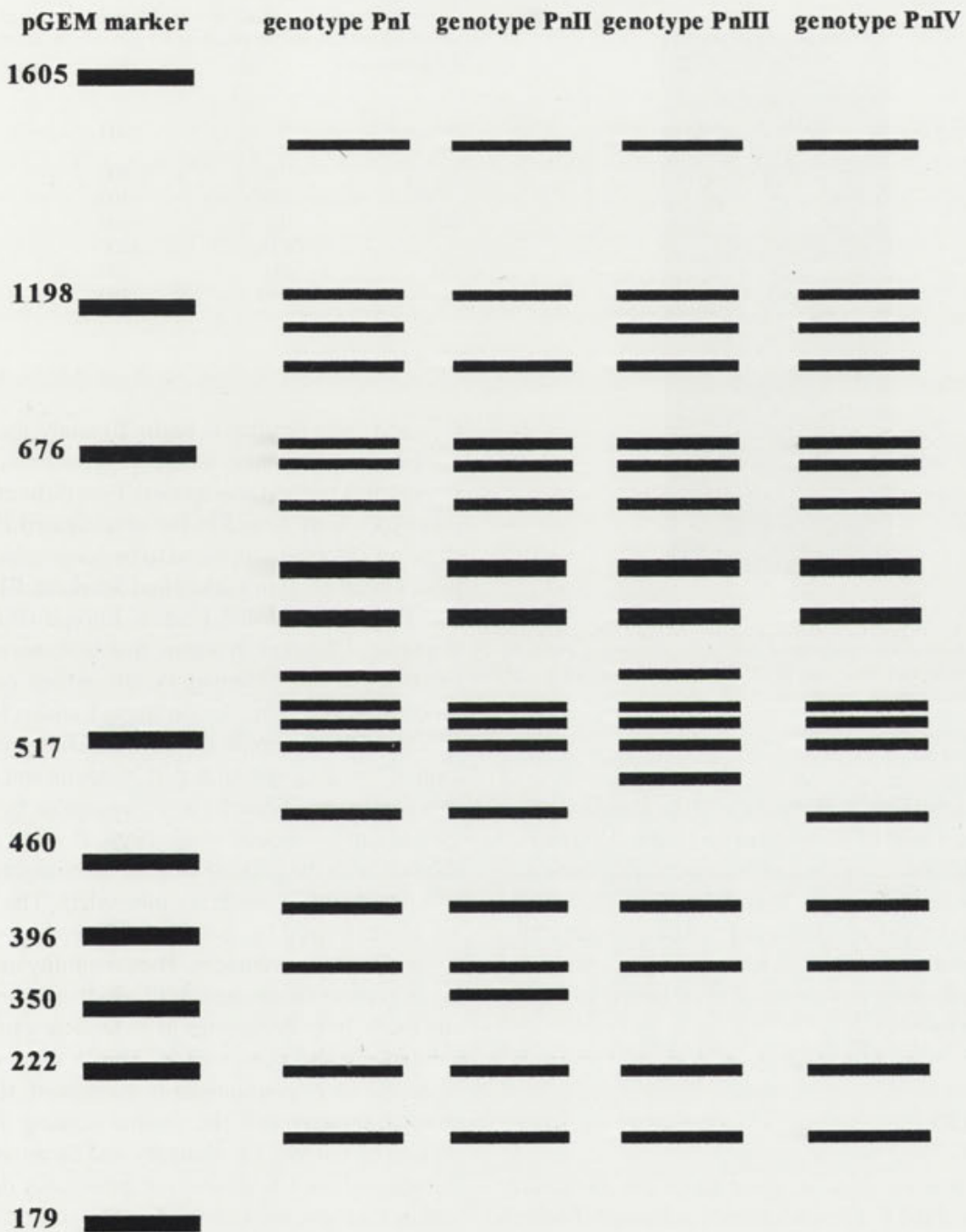
Fig. 2. Schematical representation of the different *Paramecium novaurelia* genotypes (Pn 1-Pn IV) as revealed by RAPD-fingerprinting using the 10mer random primer Ro460-04

the strains as calculated by the similarity index. No matter if *P. pentaurelia* was isolated in Central Europe, Western Europe or on the Northern American continent, we only could observe one single genotype (Fig. 3).

Strain crosses of *P. pentaurelia*

A high percentage of surviving clones of F1 and F2 generations was observed in intra- and inter- strain crosses
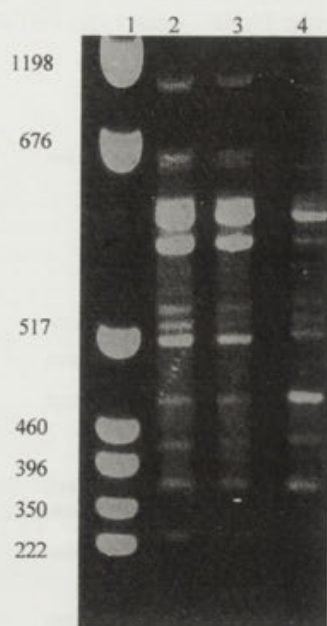
Fig. **3**. RAPD-fingerprint band pattern of the investigated *Paramecium pentaurelia* strains in a 1.8% TAE-agarose gel. 1 pGEM-marker (molecular weight of the DNA-bands is given in bp); 2 SH (Spain); 3 HB (Hungary), 4 standard 87 (USA)

Table 5. Percentage of surviving clones in crosses of *P. pentaurelia* strains. Numbers before brackets F1 generation, numbers in brackets F2 generation

Strains	87	SH	HB
87	100	100	100
	(100)	(100)	(100)
SH	100	100	100
	(100)	(100)	(100)
HB	100	100	100
	(98)	(100)	(100)

of 3 strains from Spain, Hungary, and the standard strain 87 (from the USA) of *P. pentaurelia* (Table 5). The rate of clone fissions in intra- and inter-strain crosses was similar in the F1 lines leading to the F2 generation, cultured by the method of daily isolation lines. Autogamy in all the studied hybrid lines appeared after a similar number of fissions. No disorder was observed in the course of the life cycle of the hybrids.

DISCUSSION

Paramecium novaurelia seems to be the most common species of the *P. aurelia* complex in Europe (Przyboś 1998b), and was regarded as restricted to this continent (Sonneborn 1975). The species was, however, recorded recently in Asia (Turkey, Anatolian Upland), (Przyboś 1998a). It would seem interesting to study within this species the genetic relations of strains originating from distant localities in Europe and Turkey. The results of crosses showed that strains of *P. novaurelia* coming from different regions of Europe and from Asia were not genetically differentiated within the species. The studied strains can interbreed in spite of their geographical isola-

tion and their fertility is high. To study the problem of intra-species structure within *P. novaurelia*, the RAPD fingerprints method was applied. Four different fingerprint genotypes were found in the investigated *P. novaurelia* strains. Genotypes appeared to be geographically distributed, I in Spain, II in Turkey and Scotland, III in Germany and IV in Central and Eastern Europe (Poland, Czech Republic, Ukraine). It seems that in *P. novaurelia* there exists a type of breeding system which can be called moderate inbreeding. According to Landis (1986) several species of the *P. aurelia* complex are characterized by that kind of breeding system, e.g. *P. biaurelia* and *P. triaurelia*. It was also confirmed for *P. triaurelia* by the studies carried out by Stoeck *et al.* 1998. *P. novaurelia*, as we showed with the present studies, also might be included into the group of moderate inbreeders. The species that are characterized by that kind of breeding system have some selective advantages. The possibility of inter-breeding of remote strains results in a higher genetic diversity and might help the species in its tendency to invade new habitats, as also discussed by Stoeck *et al.* (1998).

As far as *P. pentaurelia* is concerned, the results of crosses showed that the strains coming from distant regions of Europe, i.e. Hungary and Spain, or even from Europe and the USA, were not genetically differentiated within that species. Comparing the genetic diversity of *P. pentaurelia* strains, collected in geographically isolated regions, with the genetic diversity of the until now investigated species *P. novaurelia* (this study), *P. triaurelia* and *P. sexaurelia*, it is astonishing that the fingerprint band patterns of the studied strains are 100% identical. In fact only one single genotype was observed.

According to Sonneborn (1975) the species of the *P. aurelia* complex show inbreeding, in general, but to a varying degrees. Some of them manifest extreme inbreeding, such as *P. tetraurelia*. The other studied species show

different degrees of inbreeding, e. g. the genetic differentiation of strains within *P. novaurelia* (Pringle 1956) was less pronounced than within *P. primaurelia* (Kościuszko 1965). A series of works concerned the genetic differentiation of strains within *P. triaurelia* (Przyboś 1998c) and it was found that the studied species manifested a lack of such differentiation which characterizes species with a more or less open gene flow between individuals or different genotypes of the species. The investigations carried out on *P. triaurelia* and *P. sexaurelia* (Stoeck *et al.* 1998) in which a combination of classical crosses of strains with modern molecular biological technique (RAPD-PCR) was used, showed that those species differ in their degree of inbreeding. *P. triaurelia* was confirmed to be a moderate inbreeder and *P. sexaurelia* was discovered to be an extreme inbreeder.

The species of the *P. aurelia* complex can be arranged in some order as far as degree of inbreeding is concerned, from extreme inbreeders (*P. tetraurelia*, *P. sexaurelia* and to some extent *P. primaurelia*), moderate inbreeders (*P. novaurelia*, *P. triaurelia*), and at the other end, weak inbreeders (*P. pentaurelia*).

With a combination of a molecular biological method and a classical technique we showed that the various degrees of inbreeding within the same species complex might play a decisive role in life history and evolutionary strategies. With an increasing degree of inbreeding the specialization of a species increases, too. *P. sexaurelia* an extreme inbreeder was characterized by four different genotypes between them a gene flow is blocked, since a mating reaction with an exchange of genetic material between these strains hardly occurred (Stoeck *et al.* 1998). In fact the different genotypes of this species are characterized by a lower genetic diversity since *P. sexaurelia* is much more dependent on autogamy to renew its clonal life cycle. Since autogamy is described as the most extreme form of inbreeding (Nyberg 1974) gene flow within a population is blocked (Landis 1986). But on the other hand, Antonovics (1968a, 1968b) and Lefebure (1970), showed on different plant species that an increased self fertilization could well be an adaptive mechanism for maintaining certain alleles or groups of alleles responsible for the adaptation to special environmental conditions. Consequently, Stoeck *et al.* (1998) describes *P. sexaurelia* as a relative specialist with an increased efficiency in utilizing a particular resource. But the cost is an assumed decrease in genetic variety as was also described by Landis (1988). This strategy is hypothesized by the author to be a higher rate of reproduction under

a comparatively narrow range of conditions, compared with a less specialized species such as *P. triaurelia* (Stoeck *et al.* 1998).

According to such considerations the species investigated in this study are characterized as less specialized than extreme inbreeders are. The relative generalists *P. novaurelia* as well as *P. pentaurelia* are characterized by an open gene flow system which increases the genetic diversity. With an increased genetic diversity these species are assumed to be able to live under a broader range of environmental conditions compared with relative specialists such as *P. sexaurelia* and *P. tetraurelia*. The costs might be a decrease of the reproduction rate as also described by Stoeck *et al.* (1998).

Since *P. novaurelia* was shown to be a moderate inbreeder and *P. pentaurelia* only a weak inbreeder the extent of this strategy is more pronounced concerning *P. pentaurelia*. On the other hand, it might be also possible that the high genetic similarity among strains of the latter species was caused by rather recent spreading of its strains.

REFERENCES

Antonovics J. (1968 a) Evolution in closely adjacent plant population. V. Evolution of self-fertility. *Heredity* **23**: 219-238
Antonovics J. (1968 b) Evolution in closely adjacent plant population. VI. Manifold effects of gene flow. *Heredity* **23**: 507-524
Aufderheide K. J., Daggett P.- M., Nerad T. A. (1983) *Paramecium sonneborni* n.sp., a new member of the *Paramecium aurelia* species-complex. *J. Protozool.* **30**: 128-131
Beale G. H., Schneller M. (1954) A ninth variety of *Paramecium aurelia*. *J. Gen. Microbiol.* **11**: 57-58
Chen T. T. (1956) Varieties and mating types in *Paramecium bursaria*. II. Variety and mating types found in China. *J. exp. Zool.* **132**: 255-268
Fokin S., Stoeck T., Schmidt H. J. (1999) Rediscovery of *Paramecium nephridiatum* Gelei, 1925 and its characteristics. *J. Euk. Microbiol.* **46**: 416-426
Komala Z., Przyboś E. (1990) Studies on the distribution of species of the *Paramecium aurelia* species complex in the Western Sudetes of Poland. *Folia Biol. (Kraków)* **38**: 35-42
Komala Z., Przyboś E. (1992) Species of the *Paramecium aurelia* complex in the Middle Sudetes of Czecho-Slovakia. *Folia Biol. (Kraków)* **40**: 129-135
Kościuszko H. (1964) Syngens of *Paramecium aurelia* in Hungary. *Folia Biol. (Kraków)* **12**: 17-22
Kościuszko H. (1965) Karyologic and genetic investigations in syngen 1 of *Paramecium aurelia*. *Folia Biol. (Kraków)* **13**: 339-368
Kościuszko H., Komala Z., Humiczewska M. (1961) Investigations on the occurrence of varieties of *Paramecium aurelia*. *Folia Biol. (Kraków)* **9**: 43-46
Kusch J. (1998) Local and temporal distribution of different genotypes of pond-dwelling *Stentor coeruleus*. *Protist* **149**: 147-154
Kusch J., Heckmann K. (1996) Population structure of *Euplotes* ciliates revealed by RAPD fingerprinting. *Écoscience* **3**: 378-384
Landis W. G. (1986) The interplay among ecology, breeding systems, and genetics in the *Paramecium aurelia* and *Paramecium bursaria* complexes. In: Progress in Protistology, (Ed. J.O. Corliss and P. Patterson). Biopress, Bristol, **1**: 287-307

Landis W.G. (1988) Ecology. In: *Paramecium*, (Ed. H.-D. Görtz). Springer-Verlag, Berlin, Heidelberg, 419-436

Lefebure C. (1970) Self-fertility in maritime and zinc mine populations of *America maritima* (Mill) *Evolution* 24: 571-577

Lynch T.J., Brickner J., Nakano K.J., Orias E. (1995) Genetic map of randomly amplified DNA polymorphisms closely linked to the mating type locus of *Tetrahymena thermophila*. *Genetics* 141: 1315-1325

Nyberg D. (1974) Breeding systems and resistance to environmental stress in ciliates. *Evolution* 28: 367-380

Pringle C. R. (1956) Antigenic variation in *Paramecium aurelia*, variety 9. *Zeitsch. Abst. Vererbsl.* 87: 421-423

Przyboś E. (1991) Studies on the *Paramecium aurelia* species complex in Spain (Ciliophora). *Arch. Protistenkd* 140: 151-156

Przyboś E. (1993) Species of the *Paramecium aurelia* complex in Spain. *Microbiol. SEM* 9: 113-117

Przyboś E. (1997) The *Paramecium aurelia* species complex on the Volyn-Podolian Upland. *Folia Biol. (Kraków)* 45: 59-63

Przyboś E. (1998a) The first habitat of *Paramecium novaurelia* of the *P. aurelia* spp. complex in Asia (Turkey). *Folia Biol. (Kraków)* 46: 91-95

Przyboś E. (1998b) Frequency of occurrence of species of the *Paramecium aurelia* complex in Europe. *Folia Biol. (Kraków)* 46: 83-86

Przyboś E. (1998c) Intraspecies differentiation of *Paramecium triaurelia* (Ciliophora, Protista). IV. Strains from Ukraine. *Folia Biol. (Kraków)* 46: 105-108

Przyboś E., Chornobai J. M. (1994) The *Paramecium aurelia* species complex in the Chornohora Mts. *Folia Biol. (Kraków)* 42: 109-114

Przyboś E., Fokin S. (1997) Species of the *Paramecium aurelia* complex Sonneborn in Germany. *Arch. Protistenkd* 148: 167-172

Przyboś E., Komala Z. (1984) Species of the *Paramecium aurelia* complex in the Roznow Reservoir. *Folia Biol. (Kraków)* 32: 175-188

Przyboś E., Komala Z. (1992) The occurrence of the *Paramecium aurelia* species complex in the Eastern Sudetes. *Folia Biol. (Kraków)* 40: 57-60

Przyboś E., Komala Z., Kościuszko H. (1979) Species of the *Paramecium aurelia* complex in the Pieniny region. *Folia Biol. (Kraków)* 27: 231-233

Przyboś E., Fokin S., Stoeck T., Schmidt H. J. (1999) Occurrence and ecology of *Paramecium jenningsi* strains. *Folia Biol. (Kraków)* 47: 53-59

Sonneborn T. M. (1950) Methods in the general biology and genetics of *Paramecium aurelia*. *J. exp. Zool.* 113: 87-148

Sonneborn T. M. (1970) Methods in *Paramecium* research. In: Methods in Cell Physiology, (Ed. M. Prescott). Academic Press, New York, London, 4: 241-339

Sonneborn T. M. (1974) *Paramecium aurelia*, In: Handbook of Genetics, (Ed. R. C. King) Plenum Press, New York, 2: 469-594

Sonneborn T. M. (1975) The *Paramecium aurelia* complex of fourteen sibling species. *Trans. Amer. Micros. Soc.* 94: 155-178

Stoeck T., Schmidt H. J. (1998) Fast and accurate identification of European species of the *Paramecium aurelia* complex by RAPD-fingerprints. *Microb. Ecol.* 35: 311-317

Stoeck T., Przyboś E., Schmidt H. J. (1998) A combination of genetics with inter- and intra-strain crosses and RAPD-fingerprints reveal different population structures within the *Paramecium aurelia* species complex. *Europ. J. Protistol.* 34: 348-355

Wetton J. H., Carter R. E., Parkin D. T., Walters D. (1987) Demographic study of a wild house sparrow population by DNA fingerprinting. *Nature* 327: 147-149

ACTA
PROTOZOOLOGICA

Rapid Assessments of Microbial Biodiversity Using Relationships between Genus and Species Richness. Studies on Testate Amoebae

David M. WILKINSON and **Stephen R. DAVIS**

Biology and Earth Sciences, Liverpool John Moores University, Liverpool, UK

Summary. This study demonstrates a high correlation between species richness and genera richness for testate amoebae, both for modern biogeographical data sets and a paleontological data set. Such patterns have previously been described for many groups of multicellular organisms, such a relationship may not have been expected for testate due to the plasticity of morphological phenotypes. It suggests a possible method for rapid assessments of microbial diversity which could be of particular use to general ecologists working in environmental assessment or paleoecological studies.

Key words: biodiversity, higher taxa, microbial biogeography, microbial ecology, paleoecology, rhizopod, testate amoebae.

INTRODUCTION

One of the most easily interpreted measures of biodiversity is species richness. However for many groups it has proved difficult to produce full species lists for most sites. This has lead to the suggestion of using surrogate measures such as higher taxa richness. This approach has been widely used in paleontology where "the vagaries of the fossil record, and problems concerning what constitutes a fossil species, often make it difficult to work at the species level." (Gaston and Spicer 1998). The idea is that there is a correlation between species richness and higher taxa richness (e.g. Genera or Fami-

lies), this provides a more rapid method of comparing sites as it does not require the identification of all individuals to species level (a problem with species rich and/or poorly known groups). This approach has been found to work for a number of groups of multicellular organisms (Gaston and Williams 1993, Williams and Gaston 1994, O'Brien *et al*. 1998, Wilkinson 1999). However this relationship is not universal; for example Anderson (1995) working on genus richness as a surrogate for species richness in Australian ants, found that 'except in limited circumstances, genus richness ... appears to be an unreliable surrogate for species richness in local Australian ant faunas. This may apply more generally to taxa in which relatively small numbers of genera can contribute a large proportion of the species'.

Academic ecology has tended to concentrate on animals and plants with few studies of microorganisms (Wilkinson 1998a). Clearly it is of some interest to ask if

---

Address for corespondence: David M. Wilkinson, Biology and Earth Sciences, Liverpool John Moores University, Byrom Street, Liverpool L3 3AF, UK; Fax: 0151 207 3224; E-mail: D.M.Wilkinson@livjm.ac.uk

statistical relationships developed for multicellular organisms also apply to microbes. At a more practical level many microbial groups suffer from uncertain taxonomies (e.g. Finlay *et al.* 1996), or are largely asexual (including testate amoebae) or can swap genes between even distantly related types (e.g. between Archaea and Bacteria, Mayr 1998). These latter two problems rule out the biological species concept for many groups and so lead to similar problems over species definition to those experienced by paleontologists. This could make the use of higher taxa attractive for comparing biodiversity of microbes between sites.

This study tests the higher taxa, species richness rule developed for multicellular organisms on three testate amoebae data sets. Testate amoebae are amoeboid protozoa in which the cytoplasm is enclosed in a shell ("test") and which extrude filose, lobose or reticulose pseudopodia; while polyphyletic they can for practical purposes be regarded as a functional ecological group (Smith 1996). Their large scale biodiversity is best explained by climate variables while at the scale of individual sites local hydrology is very important (Wilkinson 1994).

In addition to being polyphyletic testate amoebae can exhibit a large amount of morphological plasticity (Schonborn and Peschke 1990, Schonborn 1992, Bobrov *et al.* 1995, Wanner 1999). This causes problems as test morphology is a major criteria used in their identification, indeed it is the only practical approach for sub fossil testate (Charman 1999). These problems suggest that the clear relationship between species richness and higher taxa richness described for many multicellular organisms may not be found in testate amoebae.

METHODS

Three data sets were used in the analysis, two spatial and one temporal data set from a peat core. These were:

(1) a large scale geographical data set, listing the testate amoebae at 13 terrestrial regions in the Arctic (Beyens and Chardez 1995);

(2) a smaller scale geographical data set for 15 sites in the Faroe Islands, between Iceland and the European mainland (Beyens *et al.* 1988);

(3) a paleontological data set from a Holocene peat core from Chat Moss a lowland peatland in north west England (Davis and Wilkinson, unpubl.). The shells of testate amoebae are well preserved by the anaerobic conditions in peats (Warner 1989). This data set comprised counts of sub fossil tests from 32 levels down a 2 m core and is therefore a single geographical site sampled at 32 different time intervals. Radiocarbon dating has shown that the base of this core is approximately 4500 years old (Davis and Wilkinson, unpubl.).

In the following analysis species richness is compared with genus richness using Spearman's rank correlation. Family richness was not used in this analysis as many of the testate families contain few genera. For example in the British Isles the mean (± Standard deviation) number of genera per family is 2.1 ± 1.5 (calculated from data in Ogden and Hedley 1980). Most families only contained one genera while the Hyalospheniidae contained six. This gives a similar situation to Andersons (1995) Australian ants (described above). Using the same data set the mean number of species per genus is 5.5 ± 7.3 (n=31). There are two large genera; *Nebela* (25 species) and *Difflugia* (31 species). The possible effects of these large genera on the analysis are described below.

RESULTS AND DISCUSSION

The results (Table 1) show highly significant correlations ($p < 0.001$) between species richness and genus richness in all three data sets. It should be stressed that these data sets cover two very different spatial scales (the Arctic and the Faroe Islands) and a paleontological data set (temporal scale). While the sample sizes are small (especially in the two modern data sets) the correlation coefficients are large (around 0.9) and highly significant, suggesting confidence can be placed in these results.

This study is the first of which we are aware that demonstrated that relationships between species richness and higher taxa richness developed for multicellular organisms also apply to a microbial group. The correlation coefficients are very similar to those for some multicellular groups, for example Wilkinson (1999) obtained a correlation coefficient of 0.97 between species and genus richness in the British flora. This suggests that the problems caused by the polyphyletic nature of the testate along with their morphological plasticity are not large enough to effect the statistical relationship between species and genus richness. The small number of large genera also do not appear to be a problem, this may be due to the large genera occurring in almost all data sets (at least in peatlands) and so not being a cause of variation in richness of genera between sites.

This work suggests that studies which only describe testate amoebae to genus level can still provide useful data on testate biodiversity, albeit not as detailed as species level identifications. For example Hingley (1993) provides a key to testate for non specialists, which identifies them to genus level. Such data could be collected in paleoecological studies which are mainly focusing on other evidence such as fossil pollen. This study also provides support for the common paleontological practice of using changes in higher taxa over time as a surrogate for changes

Table 1. Correlations between species richness and genus richness in three testate amoebae data sets

Data Set	Number of locations in space or time	Number of species	Number of genera	r (Spearman's) between species and genus richness
Arctic	13	114	30	r = 0.947 p< 0.001
Faroe Islands	15	42	19	r = 0.969 p< 0.001
Chat Moss peat core	32	33	15	r = 0.895 p< 0.001

in species richness (see Ellison 1995, Fig. 2 for an example using testate amoebae).

It is plausible that further work may show that it is possible to use regression techniques to extrapolate an approximate value of species richness using genera richness data. It is likely that such regression equations, if feasible, would have to be constructed from data sets from similar habitat types. For example it is unlikely that data on testate communities from beach sand (e.g. Golemansky 1998) or salt-marsh (e.g. Charman et al. 1998) could be used for the construction of reliable regression equations for peat bogs. The habitat type for which any such regression could be applied may turn out to be very restrictive; for example in a comparison of lake floras it was found that emergent vegetation exhibited different trends in species richness to the submerged plants in the same lakes (Wilkinson 1998b).

This approach could also be applied in studies which use testate as environmental indicators, an area in which there is growing interest (e.g. Warner and Chmieleski 1992, Buttler et al. 1996, Foissner 1997). Clearly most information would be obtained by full species lists. This is illustrated by the genus *Difflugia* which contains species with a range of different behaviors in relation to water table depth in peat bogs (Charman 1997, Woodland et al. 1998). However testate genera richness could easily be recorded alongside other environmental data by non specialists and this study shows that even such limited data could be useful. Soil Nematodes recorded at only the family level have been shown to give useful environmental data (Bongers and Ferris 1999), testate genera may turn out to be of similar use.

REFERENCES

Anderson A.N. (1995) Measuring more of biodiversity: Genus richness as a surrogate for species richness in Australian ant faunas. *Biol. Conserv.* **73**: 39-43

Beyens L., Chardez D., De Baere D. (1988) Some data on the testate amoebae from the Shetland Islands and the Faeroer. *Arch. Protistenkd.* **136**: 79-96

Beyens L., Chardez D. (1995) An annotated list of testate amoebae observed in the Arctic between longitudes 27E and 168 W. *Arch. Protistenkd.* **146**: 219-233

Bobrov A.A., Yazvenko S.B., Warner B.G. (1995) Taxonomic and ecological implications of shell morphology of three testaceans (Protozoa: Rhizopoda) in Russia and Canada. *Arch. Protistenkd.* **145**: 119-126

Bongers T., Ferris H. (1999) Nematode community structure as a bioindicator in environmental monitoring. *Trends Ecol. Evol.* **14**: 224-228

Buttler A., Warner B.G., Grosvernier P., Matthey Y. (1996) Vertical patterns of testate amoebae (Protozoa: Rhizopoda) and peat-forming vegetation in a cutover bog in the Jura, Switzerland. *New Phytol.* **134**: 371-382

Charman D.J. (1997) Modelling hydrological relationships of testate amoebae (Protozoa: Rhizopoda) in New Zealand peats. *J. R. Soc. New Zeland* **27**: 465-483

Charman D.J. (1999) Testate amoebae and the fossil record: issues in biodiversity. *J. Biogeogr.* **26**: 89-96

Charman D.J., Roe H.M., Gehrels W.R. (1998) The use of testate amoebae in studies of sea-level change: a case study from the Taf Estury, south Wales, UK. *Holocene* **8**: 209-218

Ellison R.L. (1995) Paleolimnological analysis of Ullswater using testate amoebae. *J. Paleolimnol.* **13**: 51-63

Finlay B.J., Corliss J.O., Esteban G., Fenchel T. (1996) Biodiversity at the microbial level: the number of free-living Ciliates in the Biosphere. *Q. Rev. Biol.* **71**: 221-237

Foissner W. (1997) Protozoa as bioindicators in agroecosystems, with emphasis on farming practices, biocides and biodiversity. *Agric. Ecosys. Environ.* **62**: 93-103

Gaston K.J., Spicer J.I. (1998) Biodiversity, an Introduction. Blackwell, Oxford

Gaston K.J., Williams P.H. (1993) Mapping the worlds species- the higher taxon approach. *Biodiversity Lett.* **1**: 2-8

Golemansky V. (1998) Interstitial testate amoebae (Rhizopoda: Testacea) from the Italian coast of the Mediterranean sea. *Acta Protozool.* **37**: 139-143

Hingley G. (1993) Microscopic life in *Sphagnum*. Richmand Publishing Co., Slough

Mayr E. (1998) Two empires or three? *Proc. Nat. Acad. Sci, USA* **95**: 9720-9723

O'Brien E.M., Whittaker R.J., Field R. (1998) Climate and woody plant diversity in southern Africa: relationships at species, genus and family levels. *Ecography* **21**: 495-509

Ogden C.G., Hedley R.H. (1980) An Atlas of Freshwater Testate Amoebae. Oxford University Press, Oxford

Schonborn W. (1992) Adaptive polymorphism in soil-inhabiting testate amoebae (Rhizopoda): its importance for delimitation and evolution of asexual species. *Arch. Protistenkd.* **142:** 139-155

Schonborn W., Peschke T. (1990) Evolutionary studies on the *Assulina-Valkanovia* complex (Rhizopoda, Testacearfilosia) in Sphagnum and soil. *Biol. Fertil. Soils* **9:** 95-100

Smith H.G. (1996) Diversity of Antarctic terrestrial protozoa. *Biodiversity Conserv.* **5:** 1379-1394

Wanner M. (1999) A review on the variability of Testate Amoebae: methodological approaches, environmental influences and taxonomical implications. *Acta Protozool.* **38:** 15-29

Warner B.G. (1989) Methods in Quaternary ecology 5. Testate Amoebae (Protozoa). *Geoscience, Canada* **15:** 251-260

Warner B.G., Chmielewski J.G. (1992) Testate amoebae (Protozoa) as indicators of drainage in a forested mire, Northern Ontario, Canada. *Arch. Protistenkd.* **141:** 179-183

Wilkinson D.M. (1994) A review of the biogeography of the protozoan genus *Nebela* in the southern temperate and Antarctic zones. *Area* **26:** 150-157

Wilkinson D.M. (1998a) Fragments of an entangled bank: do ecologists study most of ecology? *Oikos* **82:** 393-394

Wilkinson D.M. (1998b) Relationship between species richness and rarity in Welsh aquatic floras. *Watsonia* **22:** 29-32

Wilkinson D.M. (1999) Correlations between higher-taxon richness and species richness in the British Isles flora. *Watsonia* **22:** 269-273

Williams P.H., Gaston K.J. (1994) Measuring the more of biodiversity: can higher-taxon richness predict wholesale species richness *Biol. Conserv.* **67:** 211-217

Woodland W.A., Charman D.J., Sims P.C. (1998) Quantitative estimates of water tables and soil moisture in Holocene peatlands from testate amoebae. *Holocene* **8:** 261-273

ACTA
PROTOZOOLOGICA

Testate Amoebae Communities from Terrestrial Moss Habitats in the Zackenberg Area (North-East Greenland)

Andy VAN KERCKVOORDE[1], Koen TRAPPENIERS[1], Didier CHARDEZ[2], Ivan NIJS[3] and Louis BEYENS[1]

[1] Polar Ecology, Limnology and Paleobiology Unit, Department of Biology, University of Antwerp (RUCA), Antwerp; [2] Laboratoire de Zoologie Générale et Faunistique, Faculté des Sciences Agronomiques de l'Etat, Gembloux[3] Laboratory of Plant Ecology, Department of Biology, University of Antwerp (UIA), Wilrijk, Belgium

Summary. In total 45 taxa, belonging to 12 genera, were recorded from moss samples, collected in Zackenberg (Northeast Greenland). The highest number of taxa belongs to the genera *Nebela* and *Euglypha*. The genus *Trinema* showed the highest mean relative abundance, this is due to *Trinema lineare* Penard. Other important genera were *Euglypha*, *Corythion*, *Assulina* and *Nebela*. A hierarchic-agglomerate cluster analysis reveals 2 assemblages. The first assemblage has higher abundance of taxa typical for wetter conditions (*Trinema lineare* Penard, *Euglypha rotunda* Wailes and *Centropyxis aerophila* Deflandre). Taxa typical for drier conditions like *Assulina muscorum* Greeff, *Corythion dubium* Taranek, *Nebela collaris* Leidy and *Nebela minor* Penard were found in the other assemblage. These assemblages are also known from other sites in the Arctic e.g. the Søndre Strømfjord Region (West-Greenland) and Devon Island (Canadian Arctic).

Key words: Arctic, Greenland, Testaceae, testate amoebae.

INTRODUCTION

The Danish Polar Center started in 1995 a long term research program in the Arctic, by establishing the Zackenberg Ecological Research Station at 74°28'12" N and 20°34'23" W. The aim of the scientific program includes basic quantitative documentation of ecosystem structure and processes (Meltofte and Thing 1996).

Address for correspondence: Andy Van Kerckvoorde, Polar Ecology, Limnology and Paleobiology Unit, Department of Biology, University of Antwerp (RUCA), Groenenborgerlaan 171, B-2020 Antwerp, Belgium; Fax.: (+32) 03/218.02.95; E-mail: andker@ruca.ua.ac.be; koentrap@ruca.ua.ac.be

Our contribution focuses among others on testate amoebae living in soil, mosses and aquatic habitats in the Zackenberg area. Zackenberg is situated in the High Arctic of Northeast Greenland (Fig. 1) according to the definition of Aleksandrova (1980). The mean July-temperature in 1998 was 4.7°C and the mean annual temperature was -10.1°C in 1997. The period with frequent temperatures above 0°C starts at the end of May and ends in the beginning of September (Meltofte and Rasch 1998). The mean annual precipitation is about 223 mm water equivalents.

Papers on the testate amoebae of Greenland are not numerous. Decloître (1954) and Beyens *et al.* (1992)

studied testate amoebae in West Greenland, while Dixon (1939), Stout (1970) and Beyens *et al.* (1986a, b) conducted similar studies in East Greenland.

The aim of this study is to evaluate the local biodiversity by determining the composition and distribution of testacean assemblages in the different moss habitats of the Zackenberg area. When possible the relation with some environmental conditions will be addressed. In this way, more information will be added to the "Arctic Database", which is built up by our research group in the perspective of assessing testate amoebae diversity and geography in Polar Regions.

MATERIALS AND METHODS

Sampling. Field sampling was conducted between the 25th of July and 1st of September 1998. The mosses were stored in polythene bags and fixed with 3% formaldehyde. The humidity of the mosses was estimated using the field method and classification of Jung (1936). This scale is based on the water content of the moss samples and is determined as follows: FI - submerged mosses; FII - free-floating mosses; FIII - very wet, water drips out without pressure; FIV - wet, water drips out after only slight pressure; FV - quasi-wet, water drips out after moderate pressure; FVI - moist, water drips out after strong pressure; FVII - quasi-dry, only a few drops of water can be squeezed out; FVIII - dry, no water drips out. Although this seems to be a somewhat subjective method, it has proved to be reliable and is as such used in several studies (Jung 1936; Schönborn 1962; Laminger 1972; Meisterfeld 1977; Beyens *et al.* 1986a, 1990; Charman 1997). The mosses were not identified.

Some characteristics of the samples are given in Table 1. The location of the sample sites is given in Fig. 1.

Slide preparation and counting. The mosses were shaken thoroughly in distilled water. The suspension was washed on two sieves with mesh diameters of 850 μm and 300 μm. The testate amoebae in the filtrate were concentrated by centrifugation (1000 rpm). At least 100 individuals per sample were counted. Identifications were based mainly on Deflandre (1928, 1929, 1936), Grospietsch (1964), Decloître (1961, 1962), Corbet (1973) Ogden (1983) and Hoogenraad and de Groot (1940). The species *Difflugia globulosa* Dujardin probably includes the species *Phryganella hemisphaerica* Penard. These two species can only be distinguished by the shape of the pseudopodia which are only visible in living cells (Hoogenraad and de Groot 1940). Since our material was fixed it was impossible to observe this.

Data analysis. The Shannon-Wiener index (log-based) was calculated for diversity. The data set was first screened for rare taxa. If a taxon was not present in at least one sample with a relative abundance of minimum 2%, it was removed from further statistical analysis (down-weighting of rare taxa). A hierarchic-agglomerate cluster analysis based on minimum variance strategy, with the squared Euclidian distances as dissimilarity measure was used to classify the samples. The classification was performed using the computer program MVSP (multivariate statistical package version 2.1; Kovach 1993).

RESULTS AND DISCUSSION

A total of 45 taxa, belonging to 12 genera, were recorded in 36 samples. The complete taxonomic list is given in Appendix 1. This number is comparable with the number of taxa of other studies dealing with moss inhabiting testate amoebae. On Devon Island (NWT, Canada) Beyens *et al.* (1990) found 46 taxa (45 samples). Beyens *et al.* (1992) observed 53 taxa (21 samples) in a study of the Søndre Strømfjord region (West-Greenland). According to Beyens and Chardez (1995) terrestrial mosses in the Arctic harbour more taxa then can be found in water and soil samples. However, a previous study of water samples in the Zackenberg area revealed a higher number of taxa (67 in 44 samples) (Trappeniers *et al.* 1999). Three taxa (*Difflugia pristis* Penard, *Euglypha strigosa* f. *heterospina* Wailes and *Heleopera rosea* Penard) that were found for the first time in the Arctic in the latter study are also recorded in the moss samples.

The relative abundance of the different genera is shown in Fig. 2. The importance of the genus *Trinema* is almost entirely due to the taxon *Trinema lineare* Penard. This species was found in every sample and has a mean relative abundance of 42.9% ± 16.7. Other important species are (ordered by decreasing importance) *Euglypha rotunda* Wailes, *Corythion dubium* Taranek and *Assulina muscorum* Greeff. Previous studies from Greenland (Beyens *et al.* 1986a, 1992) also noticed the dominance of cosmopolite, ubiquistic taxa. In our study the species *Centropyxis aerophila* Deflandre occurred only in low numbers (2.7%). Beyens and Chardez (1994) and Schönborn (1966) noted that this species is a dominant one for the Arctic in water, moss and soil habitats. In the present study a low number of taxa of the genus *Centropyxis* (5 taxa) is observed when compared with e.g. data from moss studies from Devon Island, Canadian Arctic (10 taxa) (Beyens *et al.* 1990) and alpine Austria (11 taxa) (Laminger 1972). In sub-Antarctic regions *Corythion dubium* Taranek occupies the position of dominant or co-dominant species (Decloître 1962, Grospietsch 1971).

The average number of taxa in the samples was 16.1 ± 3.7. The highest numbers of taxa were found in the samples ZM24 (24 taxa) and M453 (22 taxa), the lowest number (9 taxa) in ZM11. These numbers are high when compared to Beyens *et al.* (1990). The number of taxa of the different genera is represented in Fig. 3. Most taxa were found in the genera *Euglypha* (10 taxa) and *Nebela* (10 taxa).
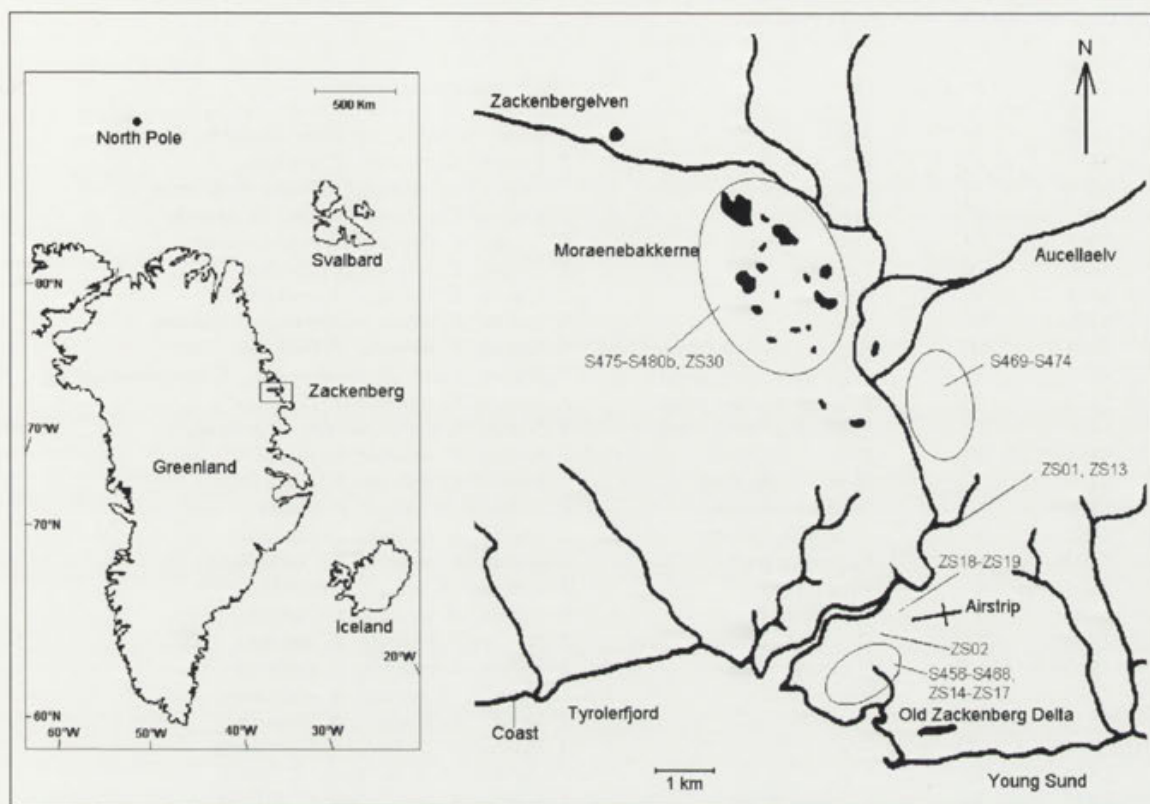
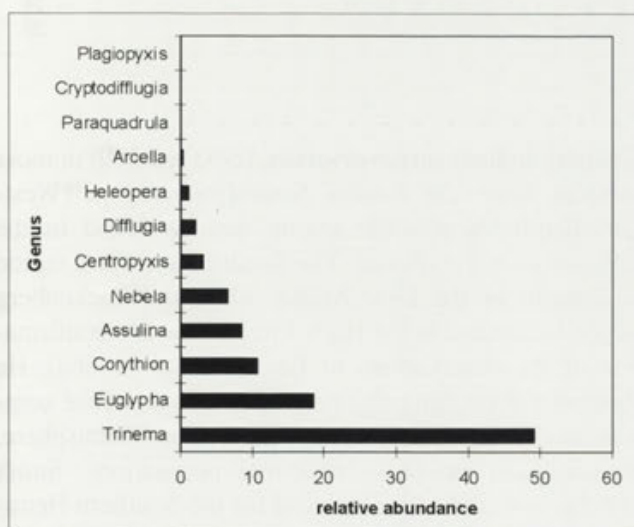Fig. **1**. Location of Zackenberg and the sample sites

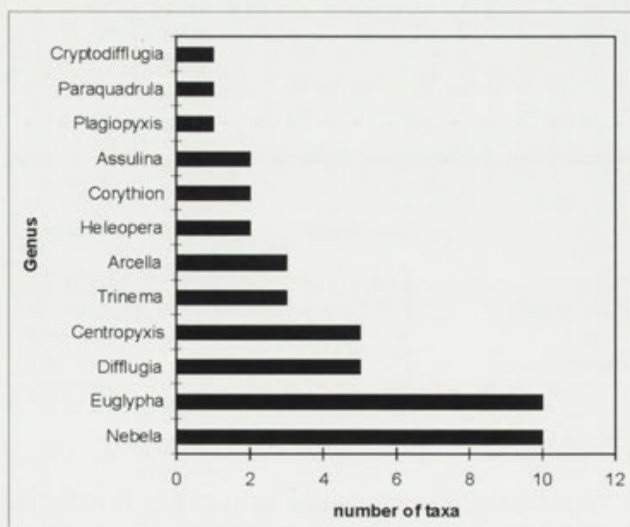Fig. **2**. The relative abundance of the different genera

Fig. **3**. The number of taxa occurring in the different genera

Table 1. List of some characteristics of the samples

Sample	Site	F-value	Habitat	Three dominating species	No species
M446	S456	IV	Fen	*Trinema lineare; T. enchelys; Euglypha rotunda*	19
M448	S456	III	Fen	*T. lineare; E. rotunda; T. enchelys*	18
M450	S459	VI	Near a stony pond	*T. lineare; E. rotunda; Assulina muscorum*	14
M452	S463	V	Fen	*T. lineare; Corythion dubium; E. rotunda*	14
M453	S464	IV	Fen	*T. lineare; C. dubium; Nebela collaris*	22
M455	S464	IV	Fen	*T. lineare; E. rotunda; C. dubium*	18
M456	S465	V	Fen	*T. lineare; E. rotunda; N. collaris*	18
M459	S468	IV	Fen	*T. lineare; Assulina muscorum; C. dubium*	19
M460	S469	III	Edge of a small stream	*T. lineare; E. rotunda; T. enchelys*	12
M464	S471	V	Edge of a stony pond	*T. lineare; Centropyxis aerophila; T. complanatum*	16
M466	S472	III	Edge of a stony pond	*T. lineare; E. rotunda; T. enchelys*	20
M467	S472	IV	Edge of a stony pond	*T. lineare; T. complanatum; E. rotunda*	11
M469	S474	VI	Fen	*T. lineare; E. rotunda; E. laevis*	11
M470	S475	V	Near a stony lake	*T. lineare; C. dubium; E. rotunda*	10
M471	S479	V	Near a stony lake	*C. dubium; N. minor; T. lineare*	21
M472	S480	V	Near a stony lake	*T. lineare; E. rotunda; T. enchelys*	17
M473	S480b	III	Edge of a pool	*T. lineare; E. rotunda; Ce. aerophila*	16
ZM01	ZS01	V	Near a river	*E. rotunda; E. laevis; T. enchelys*	13
ZM03	ZS02	V	*Vaccinium* heath	*T. lineare; E. rotunda; C. dubium*	12
ZM04	ZS02	VII	*Vaccinium* heath	*T. lineare; E. rotunda; E. strigosa*	16
ZM06	ZS13	VII	Near a peat lake	*T. lineare; E. rotunda; T. enchelys*	9
ZM11	ZS14	V	Fen	*C. dubium; T. lineare; A. muscorum*	11
ZM12	ZS14	IV	Fen	*T. lineare; E. rotunda; A. muscorum*	20
ZM13	ZS14	VI	Fen	*A. muscorum; T. lineare; T. enchelys*	18
ZM14	ZS14	V	Fen	*C. dubium; A. muscorum; T. lineare*	15
ZM15	ZS15	VI	*Cassiope* heath	*T. lineare; A. muscorum; C. dubium*	17
ZM16	ZS15	V	*Cassiope* heath	*T. lineare; A. muscorum; E. rotunda*	14
ZM17	ZS15	V	*Cassiope* heath	*T. lineare; A. muscorum; T. complanatum*	14
ZM18	ZS16	IV	*Cassiope* heath	*T. lineare; A. muscorum; C. dubium*	18
ZM19	ZS17	III	*Cassiope* heath	*T. lineare; E. rotunda; C. pulchellum*	10
ZM20	ZS18	V	Near a muddy pond	*T. lineare; E. laevis; E. rotunda*	18
ZM21	ZS18	III	Near a muddy pond	*T. lineare; Difflugia pristis; E. rotunda*	19
ZM22	ZS19	IV	Near a muddy pond	*C. dubium; T. lineare; A. muscorum*	17
ZM23	ZS19	V	Near a muddy pond	*T. lineare; A. muscorum; E. rotunda*	17
ZM24	ZS19	IV	Near a muddy pond	*T. lineare; C. dubium; E. laevis*	24
ZM25	ZS30	V	Near a stony lake	*T. lineare; E. laevis; E. rotunda*	20

Table 2. Some characteristics of the two assemblages

	Assemblage 1	Assemblage 2
F-value	4.67 ± 1.24	4.73 ± 0.70
Diversity-idex	0.752 ± 0.143	0.896 ± 0.162
Evenness	0.639 ± 0.087	0.728 ± 0.09
Number of taxa	14.7 ± 3.5	17.9 ± 3.2

The overall diversity-index is 0.775 ± 0.133. The highest diversity-indices were found in ZM24 (1.229) and M453 (1.117), the lowest in ZM06 (0.534) and ZM19 (0.537). The latter two show a very high abundance of *Trinema lineare* Penard (65.2% and 62.4% respectively). Beyens *et al.* (1992) found much higher

diversity-indices (mean diversity 1.993 ± 0.360) in moss samples from the Søndre Strømfjord region (West-Greenland). A possible reason can be found in the differences in the climate. The Søndre Strømfjord region is situated in the Low Arctic, while the Zackenberg region is situated in the High Arctic. This is a confirmation of the observations of Beyens *et al.* (1986a). He showed a decreasing diversity of testate amoebae communities towards the North in the Northern Hemisphere, especially in the moss-inhabiting populations. Smith (1982a, b) found a similar trend for the Southern Hemisphere.

According to the MVSP classification, two major assemblages can be distinguished (Fig. 4):

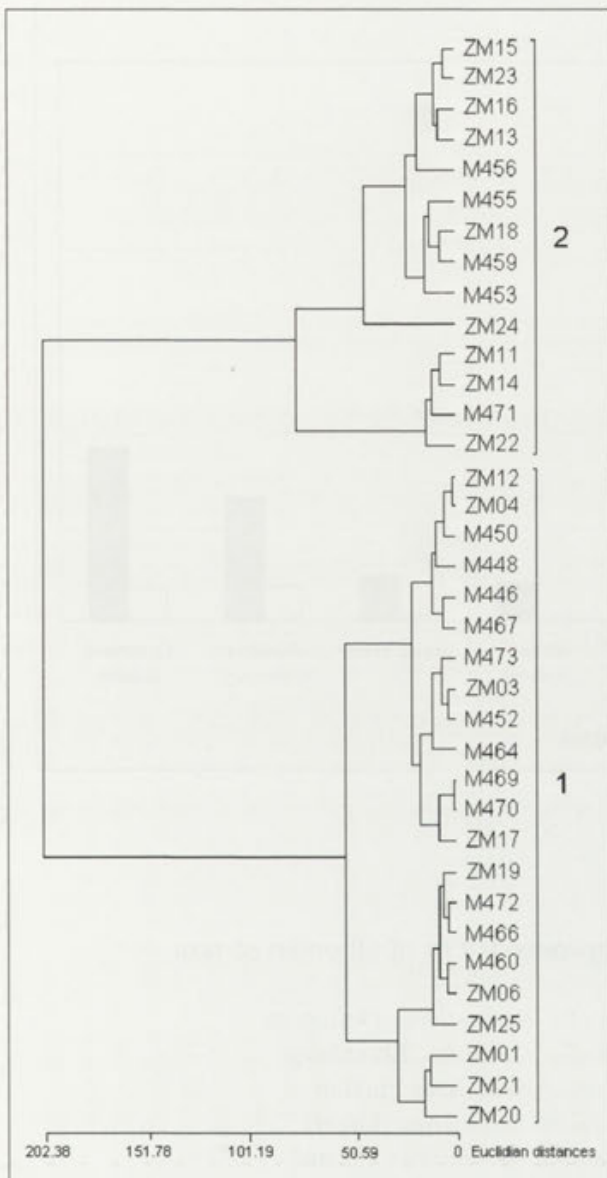assemblage 1: the *Trinema lineare-Euglypha rotunda* assemblage,

Fig. **4**. Result of the MVSP-classification of the samples

(0.6% / 4.6%). Both assemblages can further be divided, based on a sociological character, in two sub-assemblages each.

The relative abundance of some taxa in the different assemblages is illustrated in Fig. 5. Some characteristics of the different assemblages are shown in Table 2. A slightly higher diversity-index is observed in assemblage 2 in contrast with the other assemblage.

These assemblages were also encountered in other arctic sites. Beyens and Chardez (1994) found assemblages in moss habitats on Devon Island (Canadian Arctic) characterized by *Assulina muscorum - Corythion dubium* and others by *Euglypha rotunda*. The dominating species however was not *Trinema lineare* but *Centropyxis aerophila*. An overall classification of water, moss and soil habitats revealed that these two assemblages are typical for mosses in that region. The same assemblages were also found, among others, in the Søndre Strømfjord region (Beyens *et al.* 1992). Beyens *et al.* (1990) suggest that the *Corythion dubium-Assulina muscorum* assemblage inhabits the dry, more acidic mosses.

Moisture is an important factor determining the occurrence of different testate amoebae (Schönborn 1962, Meisterfeld 1977). However there are no clear differences concerning the mean F-value of the two assemblages in this study (Table 2). This lack of difference concerning the F-values can be due to the fact that the measured moisture degree is a one time-point measurement, while the microscopic analysis deals with an accumulation of tests over a longer time. However there are some observations that can be made concerning the moisture content of the samples.

The six moss samples with F-value III are all classified in assemblage 1. This group has the highest abundance of *Trinema lineare* Penard (51.7%). Beyens *et al.* (1986a) mention for this species a preference for more humid conditions (optimal F-range III-IV). The highest concentration of taxa of the genus *Difflugia* is also found in this assemblage. Based on the pseudostome-type, Bonnet (1975) stated that the genus *Difflugia* mainly occurs in aquatic or very wet habitats. Also the occurrence of *Arcella* species indicates that this assemblage groups taxa living in wetter mosses (Meisterfeld 1977, Beyens *et al.* 1992).

The dominance of *Corythion dubium* and *Assulina muscorum* in assemblage 2 points towards a drier state of the sampled mosses in this group. According to Schönborn (1962), Laminger (1972), Meisterfeld (1977), Beyens *et al.* (1986a, 1990) and Charman (1997) they
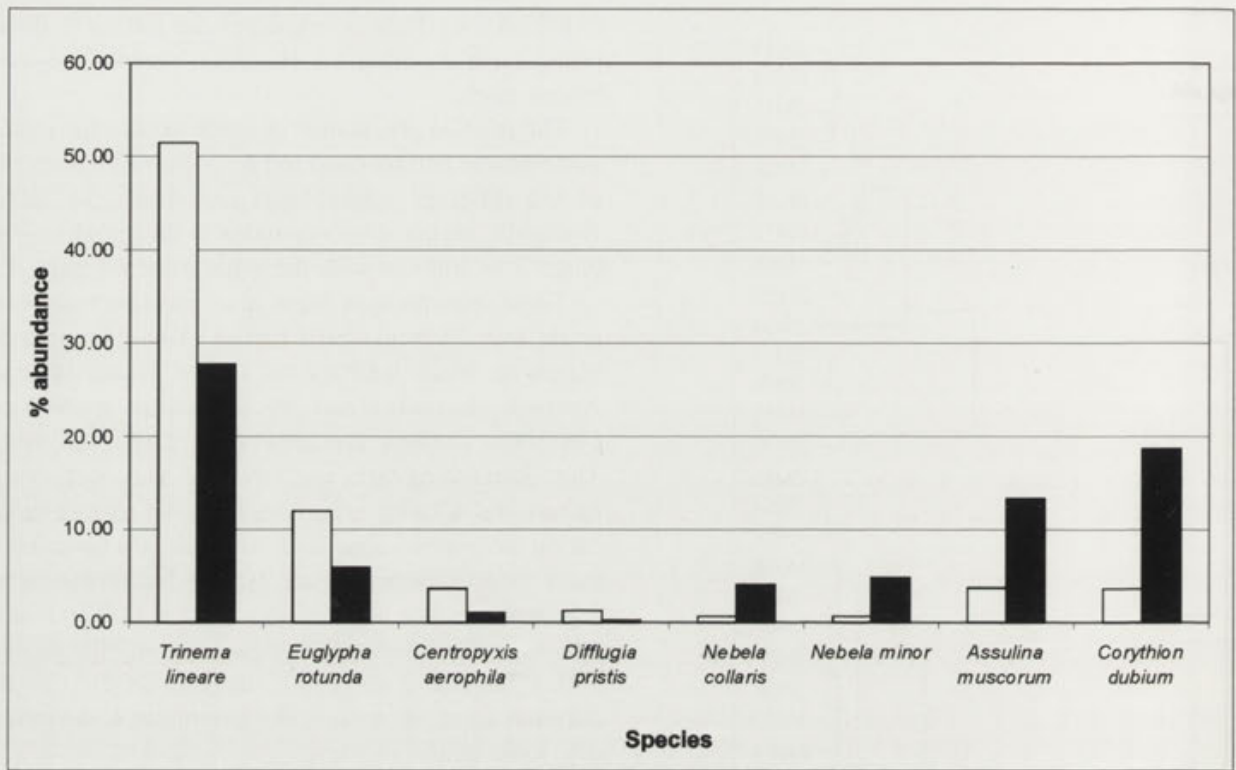
assemblage 2: the *Corythion dubium-Assulina muscorum* assemblage.

Taxa with higher abundance in assemblage 1 were *Trinema lineare* Penard (51.7% in assemblage 1 versus 29.1% in assemblage 2), *Euglypha rotunda* Wailes (12.5% / 5.6%) and *Centropyxis aerophila* Deflandre (3.8% / 0.9%). Taxa mainly occurring in assemblage 2 were *Assulina muscorum* Greeff (3.0% in assemblage 1 / 13.7% in assemblage 2), *Corythion dubium* Taranek (3.5% / 17.8%) and *Nebela minor* Penard

Fig. **5**. Mean relative abundance of some taxa in the two assemblages

have an optimal F-value of VI or higher. We also notice higher abundances of taxa from the genus *Nebela*. Meisterfeld (1977) gives some optimal F-values: an F-value of V for *Nebela collaris* Leidy and *Nebela tincta* Awerinzew and an F-value of VII for *Nebela militaris* Penard.

Beyens *et al.* (1990) warn however that species ecology is not an constant fact but should be revised in relation to the region studied. The possible occurrence of geographical races with slightly different environmental optimums should be kept in mind.

As mentioned already, these samples represent an overall picture of the content of some moss habitats. We tried to link the assemblages to the moisture content of the mosses at the very moment of sampling. This resulted in a first evaluation of the moss dwelling rhizopod fauna in the sense that we assessed its species diversity and were able to recognize two main assemblages. However, at the moment we have no insight in the succession, which may take place during a high arctic vegetation season. To resolve this question we will follow up different sites during the following summer field season.

Appendix 1. List of all observed taxa

Arcella bathystoma Deflandre
Arcella discoides Ehrenberg
Arcella rotundata Playfair
Assulina muscorum Greeff
Assulina seminulum Penard
Centropyxis aerophila Deflandre
Centropyxis aerophila var. *sphagnicola* Deflandre
Centropyxis minuta Deflandre
Centropyxis platystoma Deflandre
Centropyxis sylvatica Thomas
Corythion dubium Taranek
Corythion pulchellum Penard
Cryptodifflugia compressa Penard
Difflugia bryophila Jung
Difflugia globulosa Dujardin
Difflugia globulus Hopkinson
Difflugia penardi Hopkinson
Difflugia pristis Penard
Euglypha ciliata Ehrenberg
Euglypha compressa Carter
Euglypha filifera Penard

Euglypha laevis Perty
Euglypha polylepis Bonnet & Thomas
Euglypha rotunda Wailes
Euglypha rotunda var. *dorsalis* Decloitre
Euglypha strigosa f. *glabra* Wailes
Euglypha strigosa f. *heterospina* Wailes
Euglypha tuberculata Dujardin
Heleopera petricola Leidy
Heleopera rosea Penard
Nebela bigibbosa Penard
Nebela collaris Leidy
Nebela dentistoma Penard
Nebela lageniformis Penard
Nebela militaris Penard
Nebela minor Penard
Nebela penardiana Deflandre
Nebela tincta Awerinzew
Nebela tubulosa Penard
Nebela wailesi Deflandre
*Paraquadrula irregulari*s Deflandre
Plagiopyxis declivis Thomas
Trinema complanatum Penard
Trinema enchelys Leidy
Trinema lineare Penard

REFERENCES

Aleksandrova V. (1980) The Arctic and Antarctic: Their Divisions into Geobotanical Areas. Cambridge University Press, Cambridge London New York

Beyens L., Chardez D. (1994) On the habitat specificity of the Testate Amoebae Assemblages from Devon Island (NWT, Canadian Arctic), with the description of a new species: *Difflugia ovalisina. Arch. Protistenkd.* **144**: 137-142

Beyens L., Chardez D. (1995) An Annotated List of Testate Amoebae Observed in the Arctic between the Longitudes 27°E and 168°W. *Arch. Protistenkd.* **146**: 219-233

Beyens L., Chardez D., De Baere D. (1990) Ecology of Terrestrial Testate Amoebae Assemblages from Coastal Lowlands on Devon Island (NWT, Canadian Arctic). *Polar Biol.* **10**: 431-440

Beyens L., Chardez D., De Baere D. (1992) The Testate Amoebae from the Søndre Strømfjord Region (West-Greenland): Their Biogeographic Implications. *Arch. Protistenkd.* **142**: 5-13

Beyens L., Chardez D., De Landtsheer R. (1986a) Testate Amoebae Populations from Moss and Lichen Habitats in the Arctic. *Polar Biol.* **5**: 165-173

Beyens L., Chardez D., De Landtsheer R. (1986b) Testate Amoebae communities from aquatic habitats in the Arctic. *Polar Biol.* **6**: 197-205

Bonnet L. (1975) Types morphologiques, écologie et évolution de la thèque chez les thécamoebiens. *Protistologica* **11**: 363-378

Charman D. (1997) Modelling hydrological relationships of testate amoebae (Protozoa: Rhizopoda) on New Zealand peatlands. *J. R. Soc. New Zealand* **27**: 465-483

Corbet S.A. (1973) An illustrated introduction to the testate rhizopods in Sphagnum, with special reference to the area around Malham Tarn, Yorkshire. *Fld. Stud.* **3**: 801-838

Decloître L. (1954) Les Thécamoebiens de l'Eqe (Groenland). Hermann & Cie, Paris.

Decloître L. (1961) Le genre Paraquadrula (Thekamoebina). *Int. Revue ges. Hydrobiol.* **46**: 321-330

Decloître L. (1962) Le genre *Euglypha* Dujardin. *Arch. Protistenkd.* **106**: 51-100

Deflandre G. (1928) Le genre *Arcella. Arch. Protistenkd.* **64**: 152-288

Deflandre G. (1929) Le genre *Centropyxis. Arch. Protistenkd.* **67**: 322-375

Deflandre G. (1936) Etude monographique sur le genre *Nebela* Leidy. *Ann. Protistol.* **5**: 201-327

Dixon A. (1939) The protozoa of some east Greenland soils. *J. Animal Ecol.* **8**: 162-167

Grospietsch T. (1964) Die gattungen *Cryptodifflugia* und *Difflugiella* (Rhizopoda, Testacea). *Zool. Anz.* **172**: 243-257

Grospietsch T. (1971) Beitrag zur Ökologie der Testaceen Rhizopoden von Marion Island. In: Marion and Prince Edward Islands (Eds. E.M. Van Zinderen Bakker, J.M. Winterbottom, R.A. Dyer). Balkema, Cape Town, 411-423

Hoogenraad H.R., de Groot A.A. (1940) Zoetwaterrhizopoden en - heliozoën. In: Fauna van Nederland (Ed. H. Boschma), aflevering IX. Sijthoff's Uitgeversmij, Leiden.

Jung W. (1936) Thecamöben urspründchliger lebender deutscher Hochmoore. *Abh. Landesmus Naturkd. Muenster Westfalen* **7**: 1-87

Kovach W.L. (1993) Multivariate Statistical Package Version 2.1 Users manual. Kovach Computing services, Pentraeth, Wales, U.K.

Laminger H. (1972) Ein Beitrag zur Kenntnis der Hochgebirgs-Testaceen Österreichs. *Arch. Protistenkd.* **114**: 101-151

Meisterfeld R. (1977) Die horizontale und vertikale verteilung der testaceen (Rhizopoda, Testacea) in Sphagnum. *Arch. Hydrobiol.* **79**: 319-356

Meltofte H., Rasch M. (Eds.) (1998) Zackenberg Ecological Research Operations, 3rd Annual Report, 1997. Danish Polar Center, Ministry of Research and Information Technology.

Meltofte H., Thing H. (Eds.) (1996) Zackenberg Ecological Research Operations, 1st Annual Report, 1995. Danish Polar Center, Ministry of Research & Technology.

Ogden C.G. (1983) Observations on the systematics of the genus *Difflugia* in Britain (Rhizopoda, Protozoa). *Bull. Br. Mus. (Nat. Hist.), Zool. ser.* **44**: 1-73

Schönborn W. (1962) Die Ökologie der Testceen im oligotrophen See, dargesellt am Beispiel des Großen Stechlinsees. *Limnologica (Berlin)* **1**: 111-182

Schönborn W. (1966) Beitrag zur Ökologie und Sytematik der Testaceen Spitzbergens. *Limnologica (Berlin)* **4**: 784-792

Smith H.G. (1982a) A comparative study of the terrestrial Protozoa inhabiting moss turf peat on Iles Crozet, South Georgia and the South Orkney Islands. *C. N. F. R. A.* **51**: 137-145

Smith H.G. (1982b) The terrestrial protozoan fauna of South Georgia. *Polar Biol.* **1**: 173-179

Stout J.D. (1970) The bacteria and protozoa of some soil samples from Scoresby land, East Greenland. *Medd. Grønland* **184**: 1-22

Trappeniers K., Van Kerckvoorde A., Chardez D., Nijs I., Beyens L. (1999) Ecology of Testate Amoebae communities from aquatic habitats in the Zackenberg area (North-East Greenland). *Polar Biol.* **20**: 271-278

ACTA
PROTOZOOLOGICA

Protozoa in the Breast Muscle of Raptors in Germany

Oliver KRONE, Michael RUDOLPH and Willi JAKOB

Institute for Zoo Biology and Wildlife Research, Berlin, Germany

Summary. Seventy-nine raptors from Germany were examined for protozoan parasites in the breast muscle (*musculus pectoralis*). One Eurasian buzzard (n = 3) and two long-eared owls (n = 8) were found to be infected with three different types of *Sarcocystis* spp. The sarcocyst found in a long-eared owl is the first proof for a *Sarcocystis* sp. with a wall structure characterised by hair-like protrusions in a bird as intermediate host. The name *Sarcocystis otus* sp. n. is suggested with reference to the host *Asio otus*. Megaloschizonts of haematozoa were found in the skeletal muscle of a marsh harrier (n = 4) and a Eurasian buzzard (n = 3).

Key Words: birds of prey, megaloschizonts, owls, pectoralis muscle, raptors, *Sarcocystis otus* sp. n., *Sarcocystis* spp.

INTRODUCTION

Since Henry (1932) described oocysts (*Isospora buteonis*) from the small intestine of various hawks from California and an owl from Seattle much research has been conducted on the existence of coccidia in raptors as definitive hosts (Nieschulz 1935, Yakimoff and Matchoulsky 1936/37, Gottschalk 1972, Cerna 1976). Later, taking predator-prey relationship into consideration, life cycles of coccidia were investigated firstly on

rodents (Rommel and Krampitz 1975; Cerna and Louckova 1976; Munday 1977; Cerna and Senaud 1977; Rommel *et al.* 1977; Cerna *et al.* 1978 a, b), secondly on birds (Ashford 1975, Svobodova 1996) as intermediate hosts. These intermediate hosts of the parasite at the same time represent the prey of the raptor.

Isolated cases of sarcocystosis suggested birds of prey to be intermediate host for *Sarcocystis*. Crawley *et al.* (1982) found numerous sarcocysts in the myocardium of a bald eagle (*Haliaeetus leucocephalus*), Munday *et al.* (1979) in an Australian goshawk (*Accipiter fasciatus*), a brown falcon (*Falco berigora*) and an Australian black-shouldered kite (*Elanus axillaris*) but not in the barn owl (*Tyto alba*) and other owls (Strigidae), Vande Vusse (1966) in the muscles of the

Address for correspondence: Oliver Krone, Institute for Zoo Biology and Wildlife Research, P.O. Box 601103, D-10252 Berlin, Germany; E-mail: krone@izw-berlin.de

wings and legs of a great-horned owl (*Bubo virginianus*) and Darling (1915) in the muscles of the leg in a South American hawk (*Leucopterni*s sp.).

Other protozoan parasites can occur in the muscle tissue of birds. Additionally to the *Sarcocystis* sp. Crawley *et al.* (1982) found a cyst in the breast muscle of a bald eagle which was not described sufficiently. Experimental studies have indicated that the heart and breast muscles are often infected with *Toxoplasma gondii*, but tissue cysts were never found (Dubey *et al.* 1992; Lindsay *et al.* 1991, 1993). Together with *Hammondia* sp. these parasites can produce cysts in the skeletal muscles of mice (Mehlhorn and Frenkel 1980). More often asexual stages of haematozoa known as megaloschizonts are found in the muscles of birds. Atkinson *et al.* (1986) demonstrated the development of *Haemoproteus meleagridis* megaloschizonts in capillary endothelial cells and myofibroblasts in the skeletal muscle of domestic turkeys. The pre-erythrocytic development of *Leucocytozoon* sp. includes hepatocytes in the first stage. Organs such as spleen, lung, heart, brain and liver can be infected with megaloschizonts in the second stage (Atkinson and Van Riper 1991). Some authors (Frank 1965, Schüppel and Kronberger 1976) found megaloschizonts in the breast muscle of psittacine birds that they believed to be *Leucocytozoon* sp.

The aim of this work is to show the occurrence of protozoan parasites in the breast muscle of raptors. Furthermore this study should throw some light on the ecological function of *Sarcocystis* cysts in birds of prey and owls. Can they be classified as accidental cases, reaching a cul-de-sac in the raptor, or do they occur more often in small raptors taking advantage of the fact that large raptors frequently prey on smaller ones (Newton 1979)? To answer these questions a wide variety of raptors were examined for protozoan cysts in their breast muscle.

MATERIALS AND METHODS

The raptors examined in this study had been found dead or were submitted to rehabilitation stations where they died or were euthanized because of an unfavourable prognosis. 31 birds were submitted to the "Zentrum für Vögel bedrohter Arten" in the Land of Baden-Württemberg, 11 birds to the "Naturschutzstation Woblitz" in the Land of Brandenburg and 37 birds to the "Naturschutzbund (NABU) Artenschutzzentrum Leiferde" in Lower Saxony. A total of 79 birds were examined for protozoan parasites in their pectoralis muscle. In particular 48 birds of prey belonging to 8 species and 31 owls from 7 species were examined for protozoan parasites (Table 1).

Of the *musculus pectoralis* a one-cubic-centimetre piece (from the angle between *furcula* and *carina sterni*) was taken out and fixed in 10% neutral-buffered formalin. Tissues were embedded in paraffin and sectioned at 5 µm. Serial cuts were made every 50 µm, repeatedly for 5 procedures. For light microscopy the tissue was stained with haematoxylin and eosin (HE). The tissue of various organs (lung, liver, kidney) from the Eurasian buzzard with haematozoa was processed in the same way as the muscle tissue.

For examination by transmission electron microscope (TEM), the tissue samples containing cysts were fixed with 3% glutaraldehyde. Following repeated washing with 0.1 M phosphate buffer, they were post-fixed or pre-contrasted in 2% osmium tetroxide solution, dehydrated in ethanol and embedded in Epon 812. After polymerisation for 3 days, semithin sections were stained following Richardson *et al.* (1960). The TEM examinations were carried out using a Zeiss EM 902 A.

Cyst-containing tissue samples were also processed for immunohistological examination using a streptavidin-biotin-peroxidase method, with diaminobenzidine (DAB) as the chromogen. A primary rabbit polyclonal antibody against *Toxoplasma gondii* (Quartett®, Berlin) was used in a pre-diluted solution.

The sarcocysts were measured using a calibrated ocular micrometer and the cystozoites were measured on electron micrographs.

RESULTS

Out of 79 birds of prey and owls examined one Eurasian buzzard and two long-eared owls were infected with *Sarcocystis* spp. The first *Sarcocystis* type from a long-eared owl had a round appearance with no protrusions detectable (Figs. 1-3). The *Sarcocystis* sp. (type II) from the Eurasian buzzard had a longitudinal appearance and no protrusions, but normal invaginations were visible (Figs. 4-7). The third *Sarcocystis* type with a longitudinal appearance found in another long-eared owl is regarded to be a new species showing hair-like protrusions (Figs. 8-9). Measurements of the three different types are described in Table 2.

Apart from the infections with *Sarcocystis* sp. in the Eurasian buzzard and in the long-eared owls another protozoan parasite was found in the Eurasian buzzard and the marsh harrier. The immunohistological examination for *Toxoplasma gondii* in both birds was negative. The measurements of the cysts (Figs. 10, 11) in the skeletal muscle of the marsh harrier and the Eurasian buzzard are described in Table 2. Due to the small size of the bradyzoites and the lack of metrocytes, *Hammondia* sp. can be excluded (Mehlhorn and Frenkel, 1980). In the erythrocytes in the HE stained tissue of the marsh harrier *Haemoproteus* sp. was found. In addition, *Leucocytozoon toddi* and *Haemoproteus* sp. were found in a previously made bloodsmear (air dried, fixed

Table 1. Birds of prey and owls examined for protozoan parasites in the breast muscle

Bird species		Examined	Infected
Black kite	*Milvus migrans*	1	0
Marsh harrier	*Circus aeruginosus*	4	1 x megaloschizonts
Sparrow-hawk	*Accipiter nisus*	15	0
Goshawk	*Accipiter gentilis*	6	0
Eurasian buzzard	*Buteo buteo*	3	1 x mixed infection (*Sarcocystis* and megaloschizonts)
Common kestrel	*Falco tinnunculus*	9	0
Hobby	*Falco subbuteo*	3	0
Peregrine falcon	*Falco peregrinus*	7	0
Barn owl	*Tyto alba*	14	0
Eagle owl	*Bubo bubo*	2	0
Long-eared owl	*Asio otus*	8	2 x *Sarcocystis*
Short-eared owl	*Asio flammeus*	2	0
Pygmy owl	*Glaucidium passerinum*	1	0
Little owl	*Athene noctua*	2	0
Tawny owl	*Strix aluco*	2	0
Total		79	
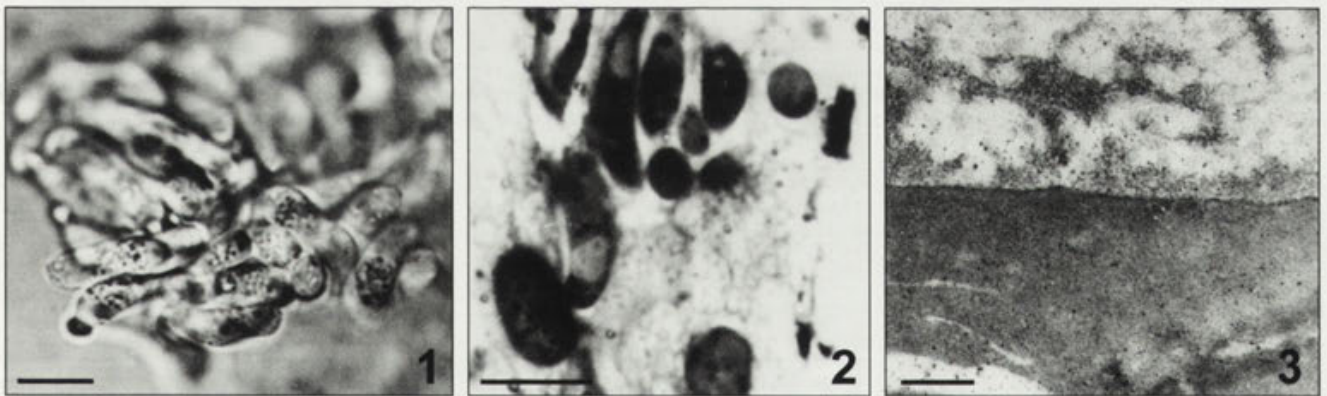
Figs. **1-3**. *Sarcocystis* sp. type I from a long-eared owl. **1** - released bradyzoites, **2** - semithin section of oval to banana-shaped cystozoites. **3** - transmission electron microscope micrograph of the same cyst, wall without invaginations or protrusions. Scale bars: **1, 2** - 10 µm; **3** - 0.25 µm

in pure methanol and stained with Giemsa´s solution) of the Eurasian buzzard. The examination of kidney, liver and lung on megaloschizonts *from L. toddi* was negative in those organs.

DISCUSSION

Sarcocystis spp. which use birds as intermediate hosts belong to a bird-bird- or bird-mammal-system.

Two types of sarcocysts are known using birds as both intermediate and definitive hosts: *Sarcocystis accipitris* and *S. alectoributeonis*. Five more species of *Sarcocystis* are known from birds as intermediate with mammals as definitive hosts: *S. falcatula*, *S. rileyi*, *S. alectorivulpes*, *S. wenzeli*, *S. peckai*. The definitive hosts of five earlier described *Sarcocystis* spp. are unknown, but for *S. nontenella*, *S. horvathi*, *S. garzettae*, *S. kaiserae*, *S. spaldingae* birds are the intermediate hosts (Odening 1998).
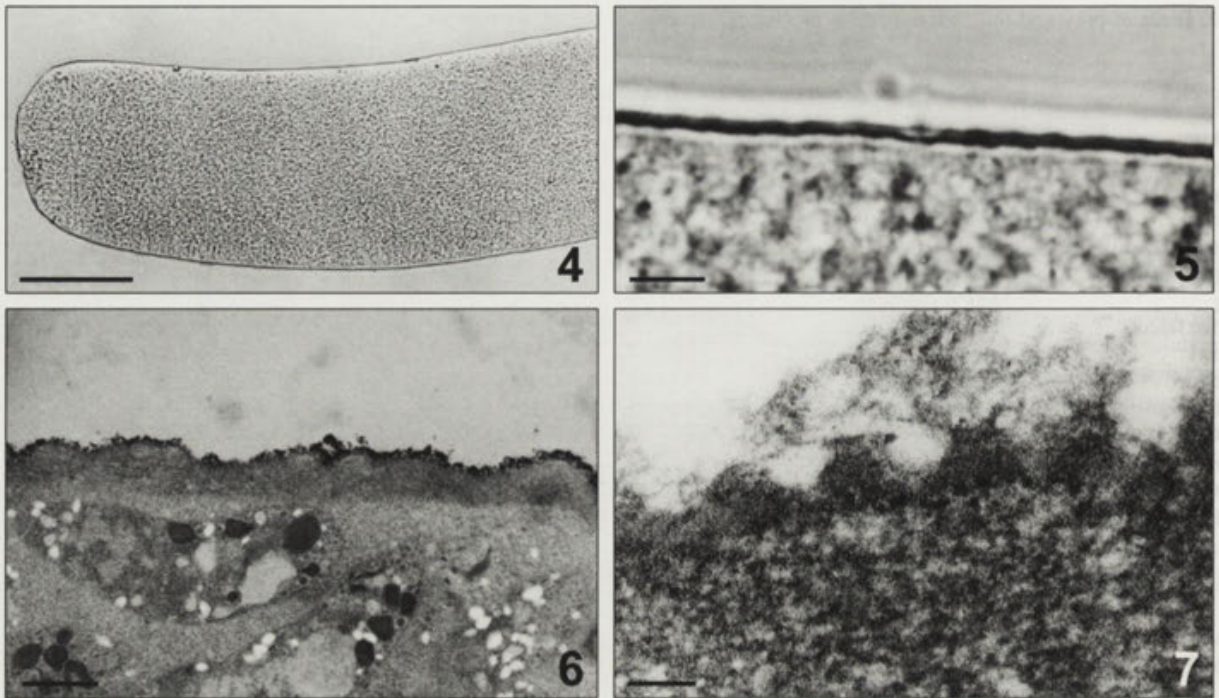
Figs. 4-7. *Sarcocystis* sp. type II from a Eurasian buzzard. **4** - view of a 1.4 mm long sarcocyst from a fresh sample, **5** - smooth appearing cyst wall of a fresh sample. **6, 7** - transmission electron microscope micrographs. **6** - irregular surface of the cyst containing cystozoites, **7** - high power magnification reveals the normal invaginations (0.9 x 1.2 µm). Scale bars: **4** - 100 µm, **5** - 5 µm, **6** - 1.1 µm, **7** - 0.09 µm

Table 2. Average, standard deviation, minimum-maximum of three *Sarcocystis* types and the megaloschizonts. Measurements in µm

	Sarcocystis type			Megaloschizont	
	I	II	III		
Host	*Asio otus*	*Buteo buteo*	*Asio otus*	*Circus aeruginosus*	*Buteo buteo*
Length	1064.6 ± 646.1 (658-1470)	1147.4 ± 442.3 (694-1850)	612.3 ± 177.4 (380-905)	50.7 (48.6-52.8)	65.8 ± 17.1 (41.2-80.3)
Width	202.6 ± 279.1 (36-1000) (n=13)	125.5 ± 79.6 (42-235) (n=8)	93 ± 43.8 (42-180) (n=6)	45.7 (44.3-47.7) (n=2)	92.8 ± 17.7 (76.6-117.5) (n=4)
Protrusions	none	none	hair-like		
Length of cystozoites	6.1 ± 1.2 (4.1-7.6) (n=10)	5.7 ± 1.1 (4.6-8.2) (n=11)	3.9 ± 0.7 (2.9-5.1) (n=9)	1.9 ± 0.5 (1.2-2.8) (n=14)	1.7 ± 0.4 (1.2-2.7) (n=13)
Width of cystozoites	3.2 ± 0.4 (2.6-4.0) (n=10)	1.5 ± 0.16 (1.25-1.7) (n=12)	2.3 ± 0.31 (1.9-2.8) (n=9)	1.4 ± 0.2 (1.2-2.0) (n=14)	1.1 ± 0.3 (0.6-1.6) (n=10)
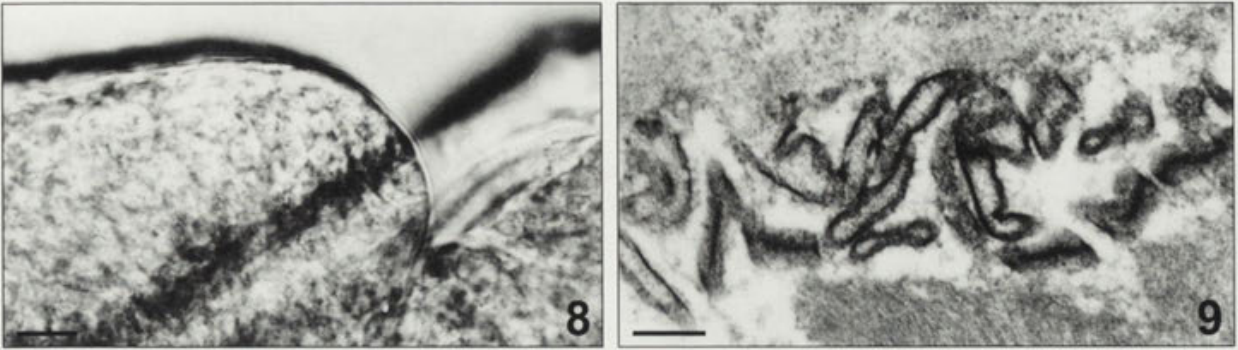
Figs. **8**, **9**. *Sarcocystis otus* sp. n. from a long-eared owl. **8** - section of the cyst wall; **9** - transmission electron microscope micrograph, hair-like protrusions becoming apparent under high power magnification. Scale bars: **8** - 10 µm, **9** - 0.4 µm
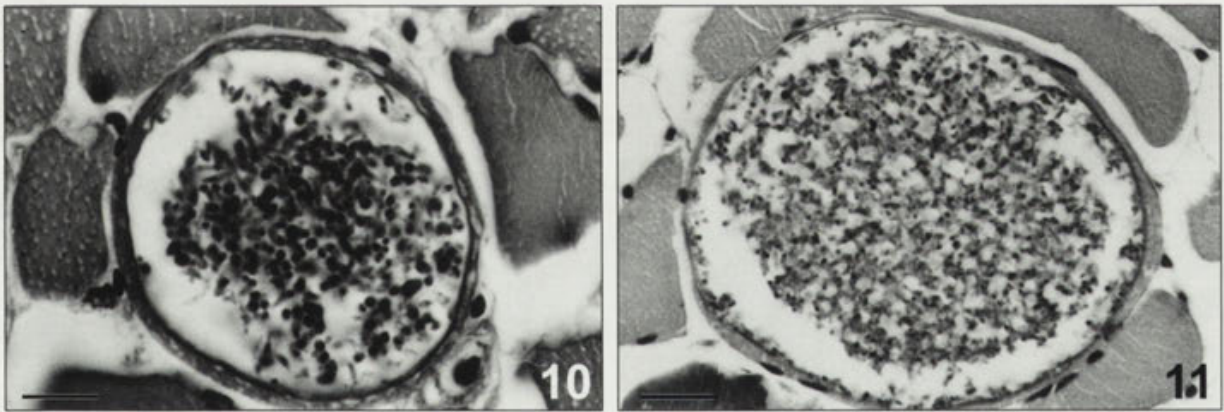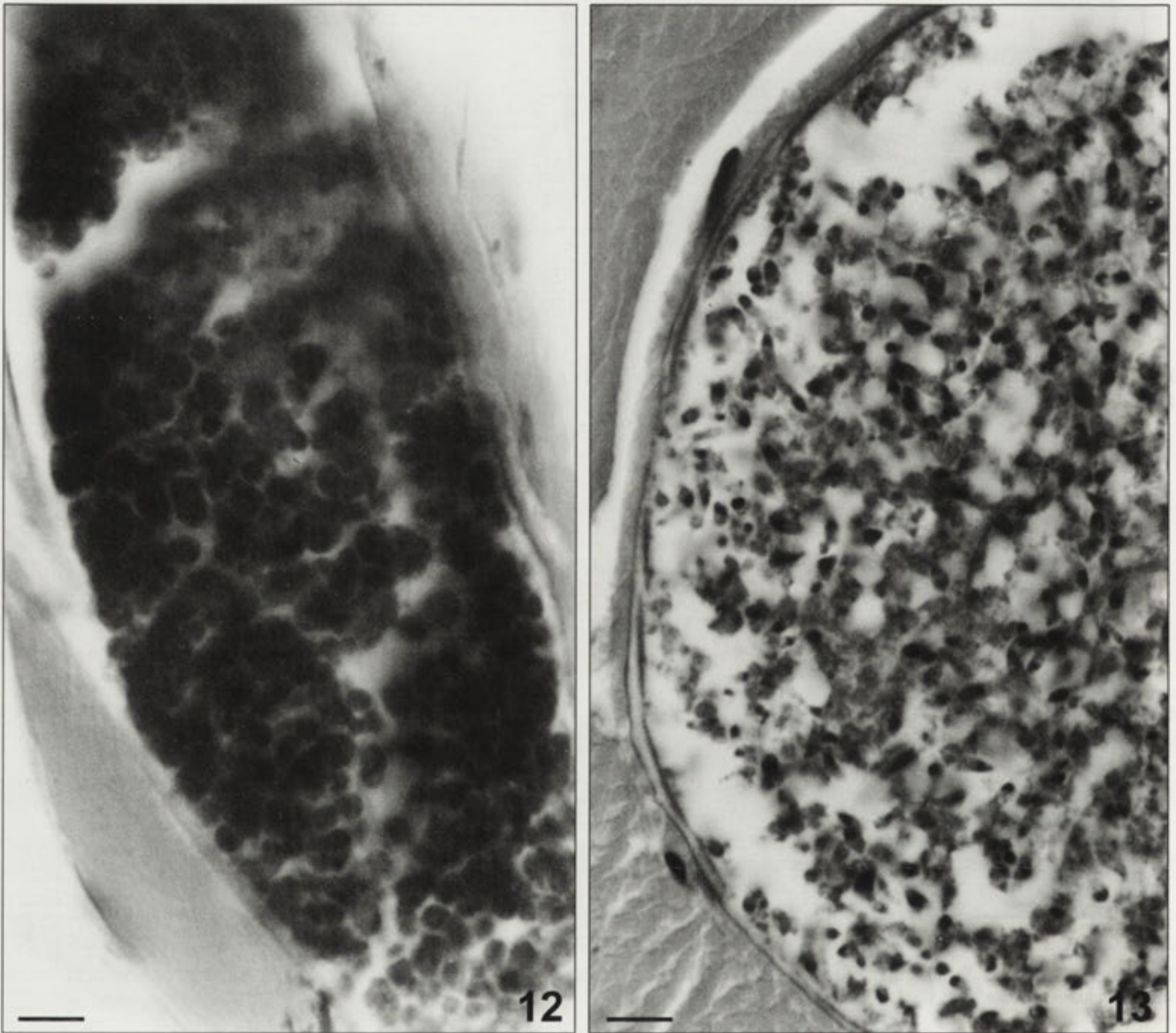

Figs. **10**, **11**. Haematoxylin and eosin stained megaloschizont in the skeletal muscle from a marsh harrier; **10** - early megaloschizont; **11** - mature megaloschizont. Scale bars: **10**, **11** - 10µm

Subsequently the results are compared with description of the sarcocysts of the bird-bird-system. With reference to the descriptions given by Cerna and Kvasnovska (1986) it can neither be excluded nor confirmed that one of the sarcocysts found belongs to *Sarcocystis accipitris*. Their description is based on the light microscopy only, showing a very thin wall (less than 1 µm) without any characteristic structure. For the second species *S. alectoributeonis* a detailed description was not given.

The five species belonging to the bird-mammal-system have been described in greater detail using the wall structure for species identification (Odening 1997). The wall structure can only be evaluated sufficiently from TEM micrographs. The classification by Dubey *et al.* (1989) of sarcocysts by their wall structure provides a good tool to distinguish the different types. The wall structure (TEM-type 11) of *S. falcatula* shows villar protrusions containing microtubules which extend from the villar tips to the plasmalemma of the bradyzoites (Dubey *et al.* 1989). The wall structure (TEM-type 23) of *S. rileyi* consists of anatomising, cauliflower-like protrusions containing fine granules and microfilaments. *S. alectorivulpes* is characterised by finger-like villar protrusions (TEM-type 9 or 10). None of the remaining *Sarcocystis* spp. in birds as intermediate hosts is

Figs. **12**, **13**. Two different haematoxylin and eosin stained megaloschizonts in the skeletal muscle from a Eurasian buzzard; **12** - early megaloschizont; **13** - mature megaloschizont. Scale bars: **12**, **13** - 10μm

characterised by hair-like protrusions of the sarcocyst wall. Therefore, the sarcocyst (type III) found in a long-eared owl provides the first proof for a *Sarcocystis* sp. with this wall structure in a bird as intermediate host. The name *Sarcocystis otus* sp. n. is proposed with reference to the host *Asio otus*. The *Sarcocystis* sp. (type II) found in the Eurasian buzzard is considered to be the same as *S. nontenella*, lacking any specific wall structures. Even the sarcocyst (type I) found in another long-eared owl can not be distinguished from the other species with a simple wall structure.

The low prevalence of *Sarcocystis* cysts in raptors indicates that these birds only occasionally function as intermediate hosts for these parasites. No evidence was found to substantiate the idea of smaller raptors such as sparrowhawks and common kestrels being more often infected by sarcocysts than larger ones. For Eurasian buzzards and long-eared owls the prevalence for *Sarcocystis* needs to be proved on a larger scale.

Lindsay and Blagburn (1999) used the acid-pepsin digestion technique for the detection of muscle cysts and found a prevalence of 45.6% (n = 114) in 16 bird of prey

and owl species from the Southeastern United States. The muscle cysts found were identified as apicomplexa and combine both megaloschizonts of haemosporidia and *Sarcocystis* muscle cysts.

The cysts found in the skeletal muscle of a marsh harrier and a Eurasian buzzard are most likely megaloschizonts of haematozoa. Due to the fact that both birds are hosting *Haemoproteus* sp. in their erythrocytes, it can be assumed that these megaloschizonts belong to this genus. Comparison with the morphometrics of the megaloschizonts, cytomeres and merozoites of *Haemoproteus meleagridis* (Atkinson *et al.*, 1986) confirmed the identity of the megaloschizonts in this study.

Crawley *et al.* (1982) already found an unidentified cyst in the skeletal muscle of a white-tailed sea eagle, which may well be a megaloschizont of a haematozoan such as *Haemoproteus* sp.

Even Atkinson and Van Riper (1991) believed that many of the unidentified protozoan megaloschizonts in the muscle tissue of a wide variety of birds which were described as aberrant *Leucocytozoons* in abnormal hosts, are in fact *Haemoproteus* spp.

REFERENCES

Ashford R. W. (1975) Preliminary observations in the life cycle of *Isospora buteonis*. *J. Protozool.* **22**: 48A
Atkinson C.T., Van Riper C. (1991) Pathogenicity and epizootiology of avian hematozoa: *Plasmodium, Leucocytozoon* and *Haemoproteus*. In: Bird-Parasite Interactions. (Eds. J.E. Loye and M. Zuk) Oxford University Press, Oxford, New York, Tokyo, 19-48
Atkinson C.T., Greiner E. C., Forrester D. J. (1986) Pre-erythrocytic development and associated response to *Haemoproteus meleagridis* (Haemosporina: Haemoproteidae) in experimentally infected domestic turkeys. *J. Protozool.* **33**: 375-381
Cerna Z. (1976) Relationship of oocysts of "*Isospora buteonis*" from the Barn-owl (*Tyto alba*) to muscle cysts of Sarcosporidians from the House mouse (*Mus musculus*). *Folia Parasitol.* **23**: 285
Cerna Z., Kvasnovska Z. (1986) Life cycle involving bird-bird relation in *Sarcocystis* coccidia with the description of *Sarcocystis accipitris* sp. n. *Folia Parasitol.* **33**: 305-309
Cerna Z., Louckova M. (1976) *Microtus arvalis* as the intermediate host of a coccidian from the Kestrel (*Falco tinnunculus*). *Folia Parasitol.* **23**: 110
Cerna Z., Senaud J. (1977) Sur un type nouveau de multiplication asexuée d'une Sarcosporidie, dans le foie de la souris. *C. r. seances Acad. Sci. Paris,* série D **285**: 347-349
Cerna Z., Kolarova I., Sulc P. (1978a) Contribution to the problem of cyst-producing coccidians. *Folia Parasitol.* **25**: 9-16
Cerna Z., Kolarova I., Sulc P. (1978b) *Sarcocystis cernae* Levine, 1977, excystation, life-cycle and comparison with other heteroxenous coccidians from rodents and birds. *Folia Parasitol.* **25**: 201-207
Crawley R. R., Ernst J. V. ,Milton J. L. (1982) Sarcocystis in a bald eagle (*Haliaeetus leucocephalus*). *J. Wildl. Dis.* **18**: 253-255
Darling S. T. (1915) Sarcosporidia encountered in Panama. *J. Parasitol.* **1**: 113-120
Dubey J. P., Speer C. A., Fayer R. (1989) *Sarcocystis* of Animals and Man. CRC Press, Boca Raton, Florida
Dubey J. P., Porter S. I., Tseng F., Shen S. K., Thulliez P. (1992) Induced toxoplasmosis in owls. *J. Zoo Wildl. Med.* **23**: 98-102
Frank W. (1965) Eine *Leukozytozoon*-Infektion bei Pennantsittichen (*Platycercus elegans*). *Gefied. Welt* **89**: 193-194
Gottschalk C. (1972) Beitrag zur Faunistik der Vogelkokzidien Thüringens und Sachsens. *Beitr. Vogelkd.* **18**: 61-69
Henry D. P. (1932) *Isospora buteonis* sp. nov. from the hawk and owl, and notes on *Isospora lacazii* (Labbe) in birds. *Univ. Calif. Publ. Zool.* **37**: 291-300
Lindsay D. S., Blagburn B. L. (1999) Prevalence of encysted apicomplexans in muscles of raptors. *Vet. Parasitol.* **80**: 341-344
Lindsay D. S., Dubey J. P., Blagburn B. L. (1991) *Toxoplasma gondii* infections in red-tailed hawks inoculated orally with tissue cysts. *J. Parasitol.* **77**: 322-325
Lindsay D. S., Smith P. C., Hoerr F. J., Blagburn B. L. (1993) Prevalence of encysted *Toxoplasma gondii* in raptors from Alabama. *J. Parasitol.* **79**: 870-873
Mehlhorn H., Frenkel J. K. (1980) Ultrastructural comparison of cysts and zoites of *Toxoplasma gondii, Sarcocystis muris* and *Hammondia hammondi* in skeletal muscle of mice. *J. Parasitol.* **66**: 59-67
Munday B. L. (1977) A species of *Sarcocystis* using owls as definitive host. *J. Wildl. Dis.* **13**: 205-207
Munday B. L., Hartley W. J., Harrigan K. E., Presidente P. J. A., Obendorf D. L. (1979) Sarcocystis and related organisms in Australian wildlife: II. Survey findings in birds, reptiles, amphibians and fish. *J. Wildl. Dis.* **15**: 57-73
Newton I. (1979) Population Ecology of Raptors. T & AD Poyser, London
Nieschulz O. (1935) Über Coccidien der Schleiereule. Zentralblatt für Bakteriologie, Parasitenkunde und Infektionskrankheiten. Zweite Abteilung: Allgemeine, landschaftliche, technische, Nahrungsmittel-Bakteriologie und Mykologie (einschließlich der Gärungsphysiologie und Enzymologie), Protozoologie, Pflanzenkrankheiten und Pflanzenschutz, sowie Tierkrankheiten (ausschließlich der in das Gebiet der Medizin gehörenden) **92**: 279-280
Odening K. (1997) Die *Sarcocystis*-Infektion: Wechselbeziehungen zwischen freilebenden Wildtieren, Haustieren und Zootieren. *Zool. Gart. N. F.* **67**: 317-340
Odening K. (1998) The present state of species-systematics in *Sarcocystis* Lankester, 1882 (Protista, Sporozoa, Coccidia). *Syst. Parasitol.* **41**: 209-233
Richardson K. C., Jarrett L., Finke H. (1960) Embedding in epoxy resins for ultrathin sectioning in electron microscopy. *Stain Technol.* **35**: 313-323
Rommel M., Krampitz H. E. (1975) Beiträge zum Lebenszyklus der Frenkelien. I. Die Identität von *Isospora buteonis* aus dem Mäusebussard mit einer Frenkelienart (*F. clethrionomyobuteonis* spec. n.) aus der Rötelmaus. *Berl. Münch. Tierärztl. Wochenschr.* **88**: 338-340
Rommel M., Krampitz H. E., Geisel O. (1977) Beiträge zum Lebenszyklus der Frenkelien. III. Die sexuelle Entwicklung von *F. clethrionomyobuteonis* im Mäusebussard. *Z. Parasitenkd.* **51**: 139-146
Schüppel K. F., Kronberger H. (1976) Leukozytozoeninfektion bei Australischen und Neuseeländischen Sittichen. Verhandlungsbericht des 18. Internationalen Symposiums über die Erkrankungen der Zootiere. Innsbruck 69-72

Svobodova M. (1996) A *Sarcocystis* species from goshawk (*Accipiter gentilis*) with great tit (*Parus major*) as intermediate host. *Acta Protozool.* **35:** 223-226

Vande Vusse F. J. (1966) *Sarcocystis* (Protozoa: Sarcocystidae) from three new avian hosts. *J. Parasitol.* **52:** 22

Yakimoff W. L., Matschoulsky S. N. (1936/37) *Isospora strigis* n. sp., Eine neue Eulencoccidie. *Arch. Protistenkd.* **88:** 286-288

ACTA
PROTOZOOLOGICA

A Redescription of *Pseudovorticella patellina* (O. F. Müller, 1776) nov. comb., a Peritrichous Ciliate (Protozoa: Ciliophora: Peritrichida) Isolated from Mariculture Biotopes in North China

Weibo SONG[1] and Alan WARREN[2]

[1]Laboratory of Protozoology, Ocean University of Qingdao, People's Republic of China; [2]Department of Zoology, Natural History Museum, London, UK

Summary. *Pseudovorticella patellina* (O.F. Müller, 1776) nov. comb. (formerly *Vorticella patellina*), was isolated from eutrophic waters in shrimp-farming ponds off the coast of Haiyang, near Qingdao, China. Its morphology, infraciliature and silverline system were studied from living and silver-impregnated specimens. Zooids of individuals in the Haiyang population measured 55-110 x 50-100 μm *in vivo* and were characterized by having two small, dorsally located contractile vacuoles, a short epistomial membrane and a wide peristomial lip. Unlike most of its congeners, the cell surface of this species was generally smooth with inconspicuous, widely spaced pellicular striations. The number of silverlines from the oral area to the aboral wreath of cilia (AWC) was 19-22, and from the AWC to the scopula, 13-16. Another vorticellid, *Vorticella nebulifera*, was isolated from the same location. According to our observations the new combination, *Pseudovorticella nebulifera* (O.F. Müller, 1786) established by Jankowski (1976), was probably a misidentification and the original name, *Vorticella nebulifera*, should be maintained.

Key words: marine ciliate, morphology, new combination, Peritrichida, *Pseudovorticella, Vorticella nebulifera*.

Abbreviations: AWC - aboral wreath of cilia, CV - contractile vacuole, EM - epistomial membrane, FV - food vacuole, G - germinal row of kineties, H - haplokinety, Ma - macronucleus, Mi - micronucleus, My - myoneme, P_{1-3} - peniculus 1-3, Po - polykinety, PD - persitomial disc, PL - peristomial lip, Sa - scopula, Sp - spasmoneme, St - stalk

INTRODUCTION

Peritrich ciliates are common and abundant in both marine and freshwater biotopes, and include numerous nominal taxa (Greeff 1870; Calkins 1901; Wang and Nie

Address for correspondence: Weibo Song, College of Fisheries, Ocean University of Qingdao, Qingdao 266003, P. R. China; Fax: +86 532 203 2283; E-mail: wsong@ouqd.edu.cn

1932; Kahl 1933; Precht 1935; Stiller 1935, 1946; Uyemura 1938; Šrámek-Hušek 1946; Sommer 1951; Lom 1961; Biernacka 1963; Dietz 1964; Küsters 1974; Foissner and Schiffmann 1974,1979; Foissner *et al.* 1985; Song 1986). Among the stalked, solitary peritrichs, *Vorticella* and its morphological relatives are notoriously difficult to identify to species level, partly because of the large number of taxa that have been described (Noland and Finley 1931; Kahl 1935; Stiller 1971; Warren 1986, 1987; Foissner *et al.* 1992). This problem is compounded

by a lack of detail in many of the older, and occasionally in some of the more recent, species descriptions.

The genus *Pseudovorticella* was established by Foissner and Schiffmann (1974) for *Vorticella*-like organisms which possess a reticulate silverline system. To date, about 19 species have been recognized and described (Foissner and Schiffmann 1974, 1979; Jankowski 1976; Foissner 1979; Schödel 1987; Warren 1987; Song and Wilbert 1989; Foissner *et al.* 1992; Song 1997). As noted in most of these studies, the critical features for species identification are the appearance of the pellicle and the stalk, the shape and position of the macronucleus, the number and position of contractile vacuoles, the number and distribution of silverlines (striations) and the habitat.

During the summer, 1995, ciliate communities in marine culture waters off the coast of Haiyang, near Qingdao, China, were examined. During the survey, a large peritrichous ciliate was observed attached to both inorganic material and to the body surface of larvae of the cultured shrimp, *Penaeus chinensis*. We believe this peritrich to be a poorly described species of *Pseudovorticella* which, for over 200 years, has been known as *Vorticella patellina* O. F. Müller, 1776. In this paper we formally transfer this taxon to the genus *Pseudovorticella* and present a redescription based on morphological studies. The taxonomic position of another vorticellid, *Vorticella nebulifera*, is briefly discussed.

MATERIALS AND METHODS

Pseudovorticella patellina was collected on 2nd September, 1995 from a shrimp-breeding pond off the coast of Haiyang near Qingdao (Tsingtao). Cells were isolated in the laboratory and examined *in vivo* using a high-power oil immersion objective and differential interference contrast microscopy.

The following silver methods were used to reveal the infraciliature and cytological details: the Chatton-Lwoff silver nitrate method as described by Corliss (1953) and the protargol impregnation according to Wilbert (1975).

The drawings of impregnated specimens were made with the help of a camera lucida at 1250-fold magnification.

Terminology is mainly according to Lom (1961) and Warren (1986).

Vorticella nebulifera, isolated from the same location, was also examined.

RESULTS AND DISCUSSION

In Corliss' classification schemes (1979, 1994), which are widely adopted by many taxonomists, the systematic position of the genus *Pseudovorticella* is given as follows:

Phylum: Ciliophora Doflein, 1901
Class: Oligohymenophorea de Puytorac *et al.*, 1974
Order: Peritrichida Stein, 1859
Family: Vorticellidae Ehrenberg, 1838
Genus: *Pseudovorticella* Foissner & Schiffmann, 1974

Pseudovorticella patellina (O.F. Müller, 1776) nov. comb. (Figs. 1-18; Table 1) syn. *Vorticella patellina* O.F. Müller, 1776

Improved diagnosis

Zooid inverted bell-shaped, length *in vivo* 42-110 µm; length:width ratio 1:1, up to 1.2:1. Macronucleus vermiform with strongly twisted ends. Two CVs near dorsal wall of vestibulum, one more active than the other. Pellicle smooth with inconspicuous, widely-spaced silverline system; number of striations from peristome to aboral wreath of cilia (AWC), 19-22 (mean 20.3); from AWC to scopula, 13-16 (mean 14.6). Stalk 3-5 fold zooid length. Marine.

Deposition of slides

Three microscope slides of silver nitrate and protargol impregnated specimens are deposited in the collection of the Laboratory of Protozoology, Ocean University of Qingdao, P.R. China, with registration numbers HY-95-0601, -0602, and -0603.

Living morphology

Cell size in the Haiyang population mostly 55-110 µm long x 50-100 µm wide. Body shape relatively constant, typically inverted bell-shaped, widest at peristomial area with highly everted, thick and relatively rigid border (peristomial lip, Pl, Figs. 1,4,5). Cell length (including peristomial lip) about equal to width, but in elongated zooids the ratio of length : width may be 1.2 : 1 (Fig. 5). Peristomial disc (PD) broad, flat and slightly obliquely elevated when cell fully extended (Figs. 1, 4-6). Pellicle generally smooth, especially at low magnification, and without tubercles; striations could be observed only under high magnification (Fig. 4).

Cytoplasm colourless or greyish, usually containing many large (*ca* 3 µm in diameter) food vacuoles containing densely packed bacteria (Figs. 1, 5). Contractile vacuoles (CV) in the Haiyang population exhibited apparent diversity in terms of number, size and position: in some zooids only one medium-sized CV was observed, positioned dorsally below the centre of the funnel-shaped vestibulum, while in others two small contractile vacuoles were identified lying close to each other and located near the dorsal wall of the vestibulum. In the latter case, the
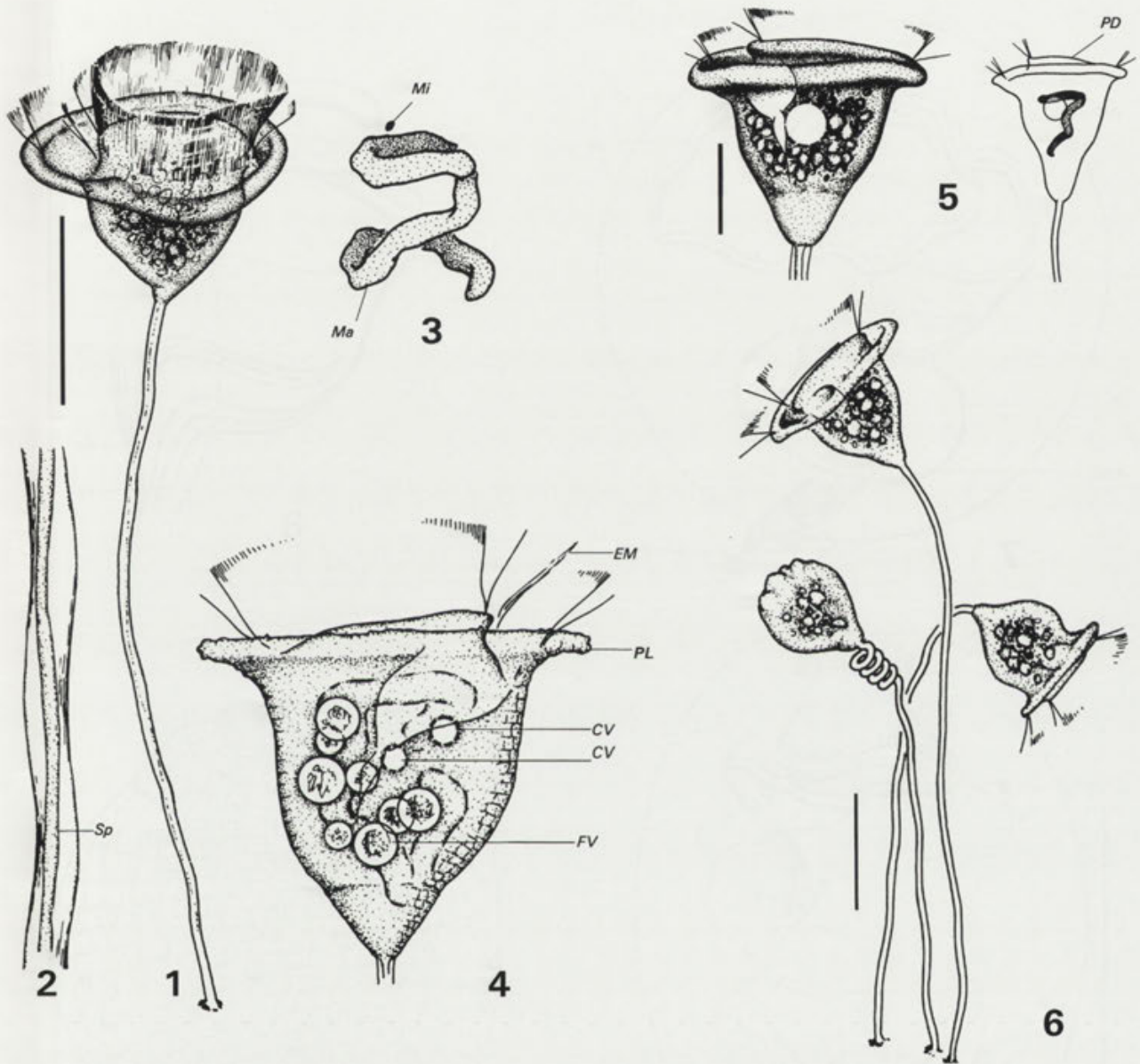
Figs. **1-6**. General morphology of *Pseudovorticella patellina* nov. comb. from life. **1** - typical zooid at low magnification; **2** - a part of stalk, note spasmoneme without conspicuous the thecoplasmic granules; **3** - macro- and micronucleus; **4** - zooid at high magnification, showing the appearance of pellicle and the position of contractile vacuoles; **5** - two zooids of Qingdao-population (after Song 1991) at low magnification; note the smaller size, smooth pellicle and each cell with one relatively large contractile vacuole; **6** - small pseudocolony with 3 individuals. Abbreviations: CV - contractile vacuoles, EM - epistomial membrane, FV - food vacuoles, Ma - macronucleus, Mi - micronucleus, PD - peristomial disc, PL - peristomial lip, Sp - spasmoneme. Scale bars: **1-4, 6** - 80 µm; **5** - 20 µm

CVs contracted at different rates: one every *ca* 10-20 s, the other at much longer (often more than 60 s) intervals.

Macronucleus (Ma) band-like, longitudinally positioned, with both ends strongly twisted or coiled (Fig. 3). Micronucleus small, near to anterior end of macronucleus (Figs. 3, 18)

Telotroch (swarmer) thick cylindrical, about 60 x 40 µm in size.

In culture, individuals often closely spaced, thus forming pseudocolonies. Stalk slender, usually 3-5 fold zooid length. Myoneme (spasmoneme) smooth, without conspicuous thecoplasmic granules (Fig. 2).

Infraciliature and silverline system

Buccal apparatus of similar structure to most congeners. Haplokinety (H) and polykinety (Po) describing
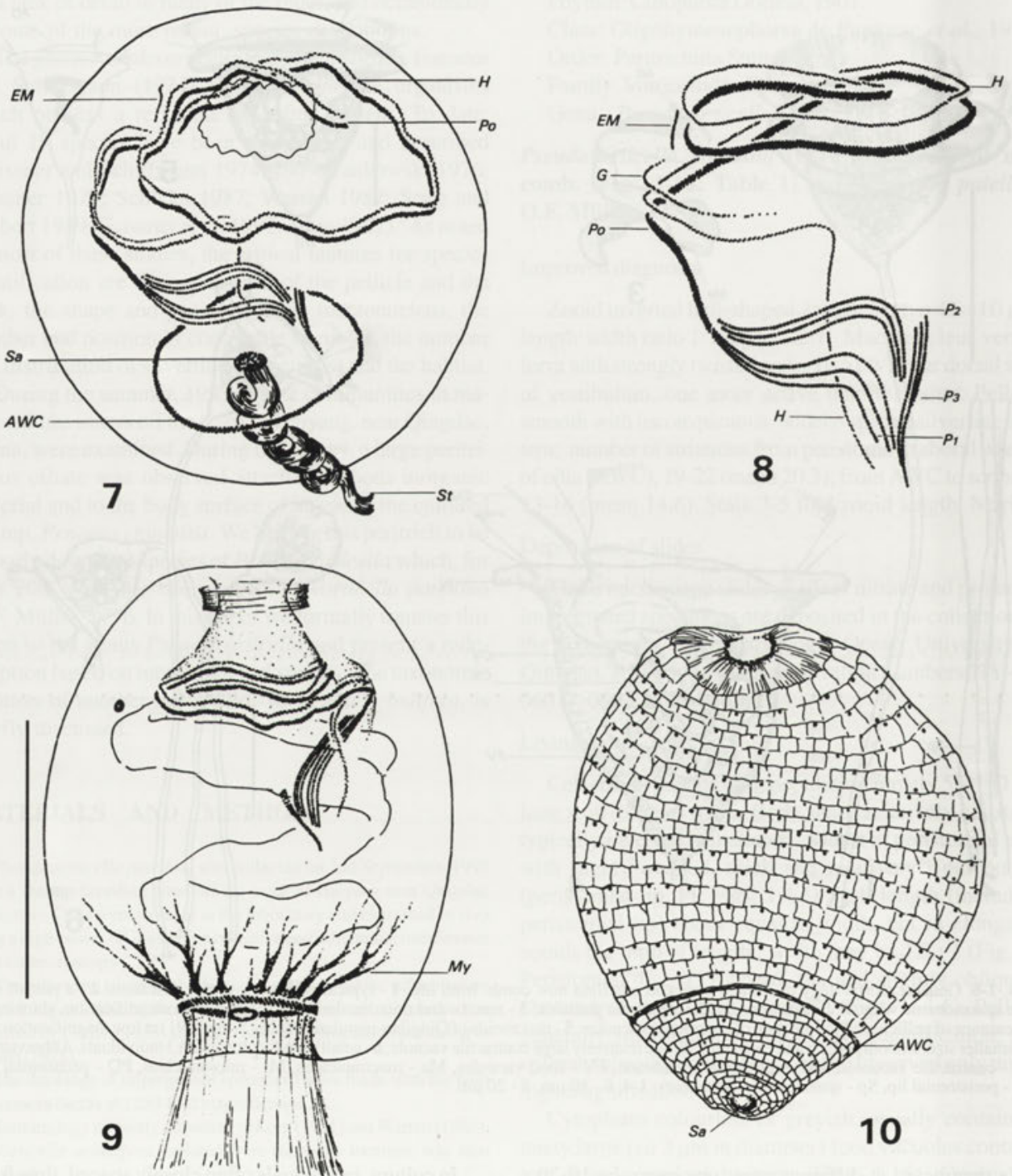
Figs. **7-10**. Infraciliature and silverline system of *Pseudovorticella patellina* nov. comb. after protargol (**7-9**) and silver nitrate (**10**) impregnations. **7** - apical-lateral view, a contracted cell, note that the aboral wreath of cilia is relatively small; **8** - buccal infraciliature, note the peniculus 3 is remarkably lower positioned; **9** - a telotroch; **10** - side view, to show the silverline system. Abbreviations: AWC - aboral wreath of cilia, EM - epistomial membrane, G - germinal row of kineties, H - haplokinety, My - myoneme, Po - polykinety, P_{1-3} - peniculus 1-3, Sa - scopula, St - stalk. Scale bar - 40 μm
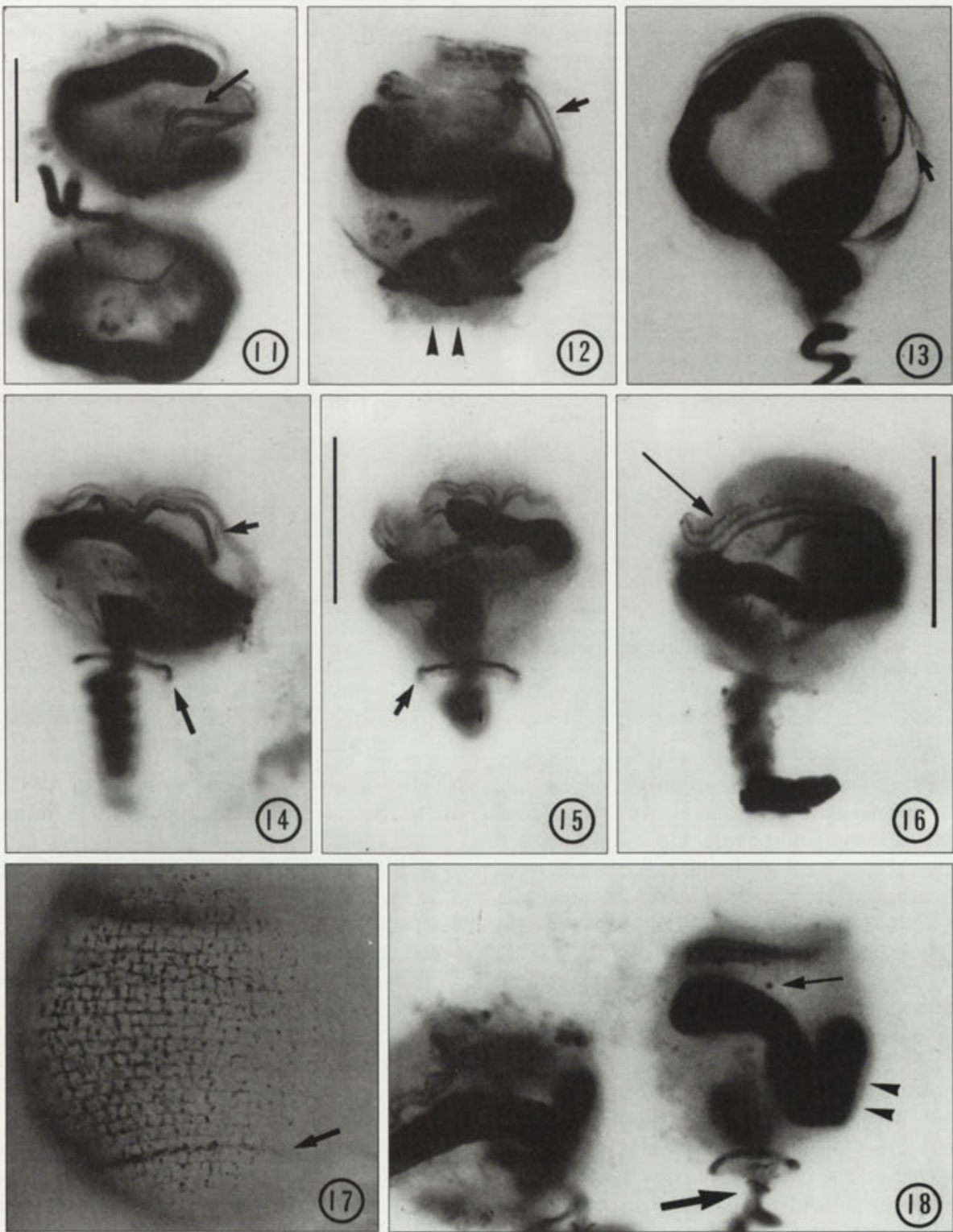
Figs. **11-18**. Photomicrographs of *Pseudovorticella patellina* nov. comb. from protargol (**11-16,18**) and silver nitrate (**17**) impregnated specimens. **11** - two zooids, arrow indicates the peniculi; **12** - a telotroch, arrowheads mark the aboral pole of cell, arrow indicates the haplokinety and polykinety; **13** - to show the epistomial membrane; **14** - to show the aboral wreath of cilia (large arrow) and the epistomial membrane (small arrow); **15**, **16** - to show the oral apparatus, arrow in 15 exhibits the aboral wreath of cilia, while in 16 marks the germinal row of kineties; **17** - silverline system, arrow indicates the aboral wreath of cilia; **18** - to show the macro- (arrowheads) micronucleus (small arrow) and the scopula (large arrow). Scale bars: **11** - 40 μm; **15**, **16** - 50 μm

Table 1. Morphometrical characterizations of *Pseudovorticella patellina*. Measurements in μm. Max - maximum, Min - minimum, Mean - arithmetic mean, n - sample size, SD - standard deviation, SE - standard error of the mean, Vr - coefficient of variation

Character	Min	Max	Mean	SD	SE	Vr	n
Body length *in vivo*	55	110	81.0	22.6	8.54	27.9	7
Body width *in vivo*	50	100	70.6	19.9	7.56	28.3	7
Number of silvelines from oral area to aboral wreath of cilia	19	22	20.3	0.87	0.25	403	12
Number of silvelines from aboral wreath of cilia to scopula	13	16	14.6	0.97	0.31	6.6	10
Number of contractile vacuoles	1 (?)	2	-	-	-	-	-

Table 2. Morphological comparison of related *Vorticella-Pseudovorticella*-species inhabiting marine biotopes. Measurements in μm. Source of data as indicated. ND - not determined

Species	Cell length *in vivo*	Number of contractile vacuoles	Pellicle appearance	Feature of silverline system	Body shape	Data resource
Pseudovorticella patellina (Haiyang-population)	55-110	1(?)-2	smooth	reticulate	inverted bell	original
(*Vorticella patellina*) (Qingdao-population)	42-47	1(?)	smooth	ND	inverted bell	Song 1991
Vorticella fornicata	24-32	1	smooth	ND	wide conical	Song 1991
Vorticella nebulifera Müller, 1786	30-67	1	finely striated	transverse striations	high inverted bell	Song 1991

about 1,5 turns around peristomial disc before entering vestibulum, where they make about one turn (Fig. 7). Polykinety forming three peniculi in lower half of vestibulum. Peniculus 1 (P_1) and 2 (P_2) about equal length, reaching end of vestibulum and converging with short peniculus 3 (P_3) (Figs. 7, 8, 11). Haplokinety passing around vestibulum on opposite wall to peniculi (Fig. 8). Germinal kinety (G) consisting of "zig-zag" structure of kinetosomes, extending to upper 1/4 of vestibulum where it passes parallel to haplokinety (Figs. 8, 16). Epistomial membrane (EM) short, located near opening of vestibulum (Figs. 7, 8, 13). Aboral wreath of cilia (AWC) composed of close-set fragments of kineties, each of which has *ca* 3-4 kinetosomes (Figs. 7, 9).

Myoneme system consisting of thick spasmoneme within stalk (St). Around scopula (Sa), myoneme extends anteriorly towards central region of the cell as commonly seen in other peritrichs (Fig. 9).

Silverline system typical of the genus with reticulate pattern as shown in figures 10, 17, transverse lines anterior to AWC more widely spaced than posteriad, and with sparsely distributed pellicular pores (Figs. 10, 17; Table 1).

Number of silverlines from peristome to AWC, 19-22 (mean 20.3); from AWC to scopula, 13-16 (mean 14.6).

Vorticella nebulifera O.F. Müller, 1786 (Table 2). Syn. *Pseudovorticella nebulifera* (O.F. Müller, 1786) Jankowski, 1976

Individuals which correspond precisely to the redescriptions by Kahl (1935) and Noland and Finley (1931) of *Vorticella nebulifera* were also isolated from the same location. Careful observation revealed that these had a fine *Vorticella*-like, rather than a reticulate, silverline system.

Discussion and comparison

Pseudovorticella patellina is an exemplary pseudovorticellid being solitary with a spirally-contractile stalk and a reticulate silverline pattern on an otherwise smooth zooid pellicle. However, one feature in particular that is worthy of further discussion is the apparent variation in the number of contractile vacuoles. Solitary, stalked peritrichs may possess one or two CVs per zooid. On first inspection of the Haiyang population, both character states appeared to be present; some zooids clearly had two

CVs while in others, which were otherwise morphologically indistinguishable, only one CV could be observed. In those with two discernible CVs, one CV pulsed far more actively and was slightly larger than the other. In these cases, the smaller CV was often difficult to observe, either failing to expand for relatively long periods following the expulsion of water or, more commonly, remaining somewhat inactive during an extended (often >60 s) systole phase. Where only one CV could be observed, the vacuole was usually somewhat larger than those in cells with two CVs. This is consistent with observations reported previously (Song 1991) for a population of the same taxon isolated from Qingdao (Fig. 5). The reason for this variation in observable CV number is unclear but may be due to either environmental or physiological factors, whereby under certain circumstances only one CV may be active while the other remains dormant and therefore difficult to observe. Unlike many other ciliates, it is not possible to observe the CV pore in silverstained peritrichs. An apparent variation in CV number has also been observed in certain marine hypotrich ciliates, with 'additional' CVs appearing when the cells are placed either in lower salinities (10-25‰) or under a coverslip (Song, unpublished). Similar apparent variations in CV number have yet to be reported in freshwater (soil or aquatic) peritrichs. Indeed, CV number and position are regarded as reliable characters for species level identification among these forms (Foissner, pers. comm.). In the light of our observations, the same confidence cannot be placed on the CV as a useful species character for marine peritrichs.

Compared with other *Pseudovorticella* spp, the present form is most similar to *P. monilata* (Tatem, 1870) Foissner & Schiffmann, 1974, which it resembles in several important characteristics: body shape and size, number of contractile vacuoles, and the position and shape of the macronucleus. However, *P. patellina* can be clearly distinguished from *P. monilata* by its habitat (marine *vs.* freshwater), appearance of the pellicle (smooth *vs.* with small tubercles) and the number of striations between the peristome and the AWC (19-22 *vs.* 31-41) (Foissner and Schiffmann 1974).

Considering its general morphology, comparisons should be also made with some *Vorticella*-species. *Pseudovorticella patellina* corresponds very well *in vivo* with *Vorticella fornicata* Dons, 1915. Although this latter form has never been described in detail using modern techniques it was considered by Kahl (1935) to be a synonym of *P. (Vorticella) patellina*. Several individuals

of a smaller, much flattened conical (i.e. *fornicata*-like) *Vorticella,* but with definitely only one contractile vacuole, have been observed by Song (1991), suggesting *V. fornicata* should remain a valid species. *Vorticella fornicata* can hence be distinguished from *P. patellina in vivo* by zooid length (24-32 *vs.* 42-110 μm), the number of contractile vacuoles (1 *vs.* 2) and body shape (wide conical- *vs.* inverted bell-shaped).

In 1976, Jankowski transferred *Vorticella nebulifera* to the genus *Pseudovorticella,* thus raising a new combination *P. nebulifera* (O.F. Müller, 1786) Jankowski, 1976. However, Jankowski (1976) offered no further description of this taxon and we believe that his decision was almost certainly mistaken. Vorticellids were isolated from the Haiyang site which exhibit the exact characters of *V. nebulifera* as redescribed by Kahl (1935), and Noland and Finley (1931), i.e. high inverted bell-shaped zooid with very fine *Vorticella*-type striations (number of striations from peristome to AWC, 35-39; from AWC to scopula, 8-12). Furthermore, similar individuals have been observed by the senior author on many previous occasions in samples collected from the Yellow Sea, North China (Song 1991). We thus conclude that *Vorticella nebulifera* should remain within the genus *Vorticella*.

REFERENCES

Biernacka I. (1963) Die Protozoenfauna in untersuchten Biotopen der Seekuste. *Pol. Arch. Hydrobiol.* **11:** 17-75
Calkins G. N. (1901) Marine protozoa from Woods Hole. *Bull. U. S. Fish. Comm.* **21:** 415-468
Corliss J. O. (1953) Silver impregnation of ciliated protozoa by the Chatton-Lwoff technic. *Stain Tech.* **28:** 97-100
Corliss J. O. (1979) The Ciliated Protozoa: Characterization, Classification and Guide to the Literature, 2 ed. Pergamon Press, New York
Corliss J.O. (1994). An interim utilitarian ("user-friendly") hierarchical classification and characterization of the protists. *Acta Protozool.* **33:** 1-51
Dietz G. (1964) Beitrag zur Kenntnis der Ciliatenfauna einiger Brackwassertümpel (Etangs) der französischen Mittelmeerküste. *Vie Milieu* **15:** 47-93
Foissner W. (1979) Peritriche Ciliaten (Protozoa: Ciliaten) aus alpinen Kleingewässern. *Zool. Jb. Syst.* **106:** 529-558
Foissner W., Schiffmann H. (1974) Vergleichende Studien an argyrophilen Strukturen von vierzehn peritrichen Ciliaten. *Protistologica* **10:** 489-508

Foissner W., Schiffmann H. (1979) Morphologie und Silberlinien-system von *Pseudovorticella sauwaldensis* nov. spec. und *Scyphidia physarum* Lachmann, 1856 (Ciliophora, Peritrichida). *Ber. Naturwiss.-Mediz. verein. Salzburg* **3-4:** 83-94

Foissner W., Hoffmann G. H., Mitchell A. J. (1985) *Heteropolaria colisarum* Foissner & Schubert, 1977 (Protozoa: Epistylididae) of North American freshwater fishes. *J. Fish. Dis.* **8:** 145-160

Foissner W., Berg, H., Kohmann H. (1992) Taxonomische und Ökologische Revision der Ciliaten des Saprobiensystems - Band II: Peritrichida, Heterotrichida, Odontostomatida. Informations berichte der Bayer. Landesamtes für Wasserwirtschaft **5/92,** 1-502.

Greeff R. (1870) Untersuchungen über den Bau und die Naturgeschichte der Vorticellen. *Arch. Naturgesch.* **37:** 353-384

Jankowski A. W. (1976) Revision of the order Sessilida (Peritricha). *Mater. Pub. Meet. Protozool.,* Kiev, 168-170 (in Russian)

Kahl A. (1933) Ciliata librae et ectocommensalia. In: Die Tierwelt der Nord- und Ostsee (Eds G. Grimpe & E. Wagler). Lief. 23, Leipzig, 147-183

Kahl A. (1935) Urtiere oder Protozoa I: Wimpertiere oder Ciliata (Infusoria) 4. Peritricha und Chonotricha. *Tierwelt Dtl.* **30:** 651-886

Küsters E. (1974) Ökologische und systematische Untersuchungen der Aufwuchsciliaten im Königshafen bei List/Sylt. *Arch. Hydrobiol.* **45(Suppl.):** 121-211

Lom J. (1961) On the buccal apparatus of peritrichous ciliates. *Prog. Protozool. 1st Intern. Conf. Protozool. Prague,* 91-95

Müller O.F. (1776) Zoologiae Danicae Prodromus, seu Animalium Daniae et Norvegiae Indigenarum Characteres, Nomina, et Synonyma Imprimis Popularium. Havniae

Müller O.F. (1786) Animalcula Infusoria Fluviatilia at Marina. Havniae et Lipsae

Noland L. E., Finley H. E. (1931) Studies on the taxonomy of the genus *Vorticella. Trans. Am. Microsc. Soc.* **50:** 81-123

Precht H. (1935) Epizoen der Kieler Bucht. *Nova Acta Leopol.* **3:** 405-474

Schödel H. (1987) Sebhafte Wimpertiere (Peritricha, Chonotricha, Suctoria) auf *Asellus aquaticus* und Gammariden. *Limnologica (Berlin)* **18:** 83-166

Sommer G. (1951) Die peritrichen Ciliaten des groben Plöner Sees. *Arch. Hydrobiol.* **44:** 349-440

Song W. (1986) Descriptions of seven new species of peritrichs on *Penaeus orientalis* (Peritricha: Zoothamnidae, Epistylididae). *Acta Zootax. Sin.* **11:** 225-235 (in Chinese with English summary)

Song W. (1991) Contribution to the commensal ciliates on *Penaeus orientalis.* I. (Ciliophora, Peritrichida). *J. Ocean Univ. Qingdao* **21:** 119-128 (in Chinese with English summary)

Song W. (1997) A new genus and two new species of marine peritrichous ciliates (Protozoa, Ciliophora, Peritrichida) from Qingdao, China. *Ophelia* **47:** 203-214

Song W., Wilbert N. (1989) Taxonomische Untersuchungen an Aufwuchsciliaten (Protozoa, Ciliophora) im Poppelsdorfer Weiher, Bonn. *Lauterbornia* **3:** 2-221

Šrámek-Hušek R. (1946) Some epizoic ciliates from the river Vltava. *Vést. čsl. Zool. Spol.* **10:** 241-244

Stiller J. (1935) Peritrichen der Gewässer des Berges Lázhegy in Ungarn. *Acta Biol. Szeged* **3:** 145-148

Stiller J. (1946) Beitrag zur Kenntnis der Peritrichenfauna der Schwefelthermen von Split. *Ann. Hist.-Nat. Mus. Nation. Hung.* **39:** 19-56

Stiller J. (1971) Szájkoszorús Csillósok-Peritricha. *Fauna Hung.* **105:** 1-245

Uyemura M. (1938) Studies on ciliates from marine mussels in Japan II. *Madsenia cyclina* n. sp. and *Epistylis elongata* Stokes. *Ann. Zool. Japan* **17:** 365-371

Wang C.- C., Nie D. (1932) A survey of the marine protozoa of Amoy. *Zool. Ser.* **8:** 285-385

Warren A. (1986) A revision of the genus *Vorticella* (Ciliophora: Peritrichida). *Bull. Br. Mus. nat. Hist. (Zool.)* **50:** 1-57

Warren A. (1987) A revision of the genus *Pseudovorticella* Foissner & Schiffmann, 1974 (Ciliophora: Peritrichida). *Bull. Br. Mus. nat. Hist. (Zool.)* **52:** 1-12

Wilbert N. (1975) Eine verbesserte Technik der Protargolimprägnation für Ciliaten. *Mikrokosmos* **64:** 171-179

Acta Protozool. (2000) 39: 51 - 60

ACTA
PROTOZOOLOGICA

Cholamonas cyrtodiopsidis gen. n., sp. n. (Cercomonadida), an Endocommensal, Mycophagous Heterotrophic Flagellate with a Doubled Kinetid

Martin FLAVIN[1], Charles J. O'KELLY[2], Thomas A. NERAD[3] and Gerald WILKINSON[4]

[1] Laboratory of Cell Biology, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD; [2] Bigelow Laboratory for Ocean Sciences, McKown Point, West Boothbay Harbor, ME; [3] Protistology Collection, American Type Culture Collection Manassas, VA; [4] Zoology Department, University of Maryland, College Park, MD, U.S.A.

Summary. The name *Cholamonas cyrtodiopsidis* gen. n., sp. n., is created for a mycophagous heterotrophic flagellate isolated and cultivated from the intestine of a diopsid fly. Flagellates were subanteriorly biflagellate, uninucleate, and naked. Golgi dictyosomes were anterior to the nucleus, and a reticulate paranuclear body posterior to it. Two groups of refractile bodies were present, one at the anterior end of the cell, the other in the vicinity of the nucleus. Numerous elongate, unbranched mitochondria with tubular cristae were distributed around the periphery and parallel to the long axis of the cell. The kinetid consisted of two symmetrical subunits, each with two basal bodies (one of which was associated with a stubby flagellum), a compound microtubular root, and associated fibers and bands. Cytoskeletal microtubules emanated from the cell anterior and were not associated with any kinetid element. Feeding, on yeast, was accomplished by rapid pseudopodial action at the posterior end of the cell. *Cholamonas cyrtodiopsidis* is referred to Cercomonadida because it possesses a paranuclear body and has a kinetid architecture similar to some species of *Cercomonas*. It differs from all other cercomonads in its endocommensal habitat, mycophagy, doubled kinetid, distribution of refractile granules and mitochondria, and minimal production of pseudopodia.

Key words: *Cercomonas*, cercomonads, *Cholamonas cyrtodiopsidis* gen. n., sp. n., kinetid architecture, paranuclear body.

Abbreviations: A - anterior basal body, AL - left anterior basal body, AR - right anterior basal body, f - food vacuole, g - Golgi, gl - apical refractile granules, gm - median refractile granules, m - mitochondria, MR - microtubular roots, MRL - left microtubular roots, MRR - right microtubular roots, n - nucleus, nu - nucleolus, p - paranuclear body (parabasal body), P - posterior basal body, PL - left posterior basal body, PR - right posterior basal body, r - ring structure, sb - striated band, w - wings, y - yeast cell.

INTRODUCTION

Cercomonads (order Cercomonadida) are common and widespread heterotrophic protists in aquatic and soil

Address for correspondence: Martin Flavin, Laboratory of Cell Biology, NHLBI, National Institutes of Health, Bldg.3, Rm. B1-22, 9000 Rockville Pike, MD 20892 - 0301, U.S.A.; Fax: 301-402-1519; E-mail: flavinM@gwgate.nhlbi.nih.gov

environments (Sandon 1927, Hänel 1979, Larsen and Patterson 1990, Patterson and Zölffell 1991). Despite their frequency of occurrence, little is yet known of the biodiversity or systematic biology of these protists, and their taxonomy is uncertain.

At present, organisms referred to this group are biflagellate, tubulocristate (having mitochondria with tubular cristae; Patterson 1994) free-living, mostly

bacterivorous flagellates or amoeboflagellates that move by gliding or swimming, take in prey by means of pseudopodia and have a conspicuous, homogeneous paranuclear body. Hairs and paraxial rods are lacking from the flagella, and cytoskeletally-defined cytostome are absent from the cell body. Patterson and Zölffel (1991) recognized three genera: *Cercomonas* Dujardin, 1841; *Heteromita* Dujardin, 1841; *Massisteria* Larsen and Patterson, 1990. These authors suggested that *Cercobodo* Krassilstchick, 1886, is a synonym of *Cercomonas*, and that several lesser-known genera of flagellates are likewise synonyms of *Cercomonas* or *Heteromita*. Cavalier-Smith (1993) added *Discocelis* Vørs, 1988 to the order.

The morphological concepts of the order and its included genera are supported, to a greater or lesser extent, by ultrastructural studies on selected species of *Cercomonas* (Mignot and Brugerolle 1975; Mylnikov 1985, 1986a, b), *Heteromita* [(MacDonald *et al.*, 1977; Mylnikov 1985 (as *Bodomorpha* Mylnikov, 1990)], *Massisteria* (Patterson and Fenchel, 1990), and *Discocelis* (Vørs, 1988), and by molecular sequence investigations on selected species especially in the first two genera (Cavalier-Smith and Chao 1996-97).

In this report, an organism is described that has several features in common with the cercomonads that have previously been examined, but also has many novel features including an endocommensal habitat, feeding on yeast instead of bacteria, and a "doubled" kinetid. The organism is named *Cholamonas cyrtodiopsidis* gen. n., sp. n.

MATERIALS AND METHODS

Collection, isolation and cultivation

The protist was isolated from the intestinal tract of the diopsid fly *Cyrtodiopsis dalmanni* Wiedemann, 1830, collected by GW in 1989 from Kuala Lumpur, Malaysia. This fly species is common in Indonesia, Malaysia and Thailand, where it feeds on decaying forest litter (de la Motte and Burkhardt 1993). The fly was maintained in the laboratory using pureed corn. Clonal monoprotist cultures were established by serial dilution and maintained in ATCC medium 802 (Nerad 1992) with the addition of the yeast *Saccharomyces cerevisiae* ATCC 48894; bacteria also were present. One clone was cryopreserved (Nerad and Daggett 1992, Poynton *et al.* 1995) and deposited in the American Type Culture Collection with the strain number ATCC 50325.

Light microscopy

Light microscopical observations were made on live cells and on those fixed in Bouin's fluid and stained using the quantitative protargol procedure (Lynn 1992). They were viewed on a Zeiss Axioskop light microscope equipped with bright-field, phase-contrast and differential interference contrast optics. Micrographs were made on Kodak Technical Pan 35 mm film, exposed at ISO 25 and processed with Kodak Technidol developer.

Scanning electron microscopy

Cells to be fixed were harvested from a 2-day culture. Several drops of a culture suspension were allowed to settle for 10 min on a poly-l-lysine coated Thermanox (EMS) coverslip. After removing the supernatant by touching the edge of the grid with a piece of torn filter paper, a few drops of fixative was added and left for 10 to 40 min. The fixative was either the one used for TEM (below) or Parducz's fixative; the latter yielded cells with artefactual deep longitudinal fissures. The coverslips were washed with either water or 0.1 M sodium phosphate, pH 7.4, and at once dehydrated by dipping successively for 5 min in two changes of 50, 70, and 95% ethanol and then for 5 min in three changes of 100% ethanol. Coverslips were then dipped for 10 min each in two changes of 100% hexamethyldisilazane in a vacuum hood and then placed on filter paper in a Petri dish. Coverslips were mounted on a metal stub (Pella) with silver conducting paint, and kept in a dessicator overnight. The next morning they were sputter coated for 90 seconds with gold using a Hummer-V instrument filled with argon, and then examined in a JEOL JSM 35C microscope.

Transmission electron microscopy - harvest and fixation.

Twenty ml of 8% glutaraldehyde was added to 20 ml of a 2 or 3 day (late log) culture. The mixture was centrifuged at once for 5 min at 1000 x g, the supernatant was aspirated from a small loose pellet, and the latter was resuspended by injecting, through a 22 ga. needle, 4 ml of chilled 0.1 M sodium phosphate buffer, pH 7.25, containing 3 % glutaraldehyde, 1.5% osmium tetroxide and sometimes also 2 mM $MgCl_2$. The suspension was allowed to rise to room temperature while standing for 45 min. The pellet was then recovered and washed twice with PBS by centrifugation for 5 min at 500 x g.

Transmission electron microscopy - enrobing and Epon embedding

After suspending the pellet in 0.9 ml of the same phosphate buffer in a conical plastic tube, 1 ml of chilled 20% bovine serum albumin was added followed by 0.3 ml of 8% glutaraldehyde, and the cold mixture was immediately centrifuge for 5 min at 5°C at 3000 rpm in a swinging bucket Sorvall RT6000B centrifuge. The tube was kept for 20 min at 37° C to solidify the contents. The bottom portion of the tube containing fixed cells was separated with a razor blade, and the contents emptied into a Petri dish containing the same phosphate buffer. The plug was minced finely with a clean razor blade. Small fragments were transferred to a scintillation bottle with 3 ml of the sodium phosphate buffer, to which 1 ml of osmium tetroxide was

added. The post-fixation was for 1h stationary at room temperature in a vacuum hood. The blocks of cells were dehydrated in a graded alcohol series followed by propylene oxide, and incubated on a rotary platform in an uncapped scintillation vial overnight in a mixture of equal volumes of propylene oxide and Epon. After again rotating overnight in Epon alone blocks were embedded in 8.5 mm gelatin capsules containing Epon and kept at 60° C for 3 days. Serial silver to pale gold sections were cut with a DDK diamond knife.

Transmission electron microscopy - staining

The serial sections were collected on formvar coated Nochnum slot grids (Pella 4518), and ten grids' were placed on the Pella grid-stick which had been coated with this companies' adhesive to keep the metal edges of each grid firmly adherent. The grid stick was inserted in a small Pasteur pipette, and sections were stained for 15 min with 2% aqueous uranyl acetate. To avoid stain artifacts (possibly resulting from the two adhesives present - grid stick and Tackiwax used for sectioning), extensive washing in deionized water was necessary before the 10 min in lead citrate: besides repeated dipping, water was allowed to flow through the pipette for 15 min.

RESULTS

Trophic cells of *Cholamonas cyrtodiopsidis* were cylindrical to narrowly ellipsoidal when swimming unimpeded (Fig. 1), but the cells were easily flattened under coverslip pressure (Figs. 2, 9) and could bend and twist to get around objects. No pseudopodia were observed. Cylindrical cells measured 5.0 - 8.0 μm in length and 2.0 - 5.0 μm in width at the widest point; cells deformed by flattening were shorter and wider. The cells bore two subapically-inserted, posteriorly-directed emergent flagella that usually were equal in length, 1 - 1.5 times as long as the cell body, and had tapering tips (Figs. 1, 3). The flagella contained the standard 9 + 2 axoneme except at their tips, and lacked hairs, scales and paraxial rods. The attenuated tips were seen only by SEM. Fig. 1 shows a cell with a very long tip on one flagellum, and a typical tip on the other. Nothing that could correspond to the tips has been seen by TEM; perhaps they are easily broken off.

Two flagellar stumps were present anterior to the emergent flagella (Figs. 1, 3). The stumps contained a very short axoneme consisting of nine singlet microtubules (Fig. 4).

The single nucleus was found in the anterior third of the cell, always closely associated with the flagellar insertion (Figs. 2, 3). Each interphase nucleus contained a single, prominent, central nucleolus (Fig. 5).

The Golgi apparatus, which consisted of two dictyosomes, one on either side of the kinetid, was located at the anterior surface of the nucleus (Fig. 5). Also anterior to the nucleus, typically forming a cap at the anterior end of the cell, was a population of refractile granules (Fig. 5). These granules, not demonstrably membrane-bound and having electron-transparent contents (Figs. 5, 11), were presumably responsible for a bright apical spot in living cells which might aid identification with a light microscope.

A second population of refractile granules formed a looser aggregation around and posterior to the nucleus (Figs. 2, 6). These granules were larger than the apical cap granules, were bounded by a single membrane, and had electron-dense contents (Fig. 6). Also associated with the posterior surface of the nucleus was a large and complex paranuclear body (Figs. 7, 8). Reconstruction revealed a reticulate organelle, with lobes often extending well away from the nucleus towards the posterior end of the cell.

Much of the posterior two-thirds of the cell was taken up by food vacuoles, which contained only yeast cells when full (Fig. 8). The cells did not take up, or survive on, media containing only bacteria or other species of yeast. All protist cells appeared dead within 24 h of yeast depletion, and we were not able to induce spore formation. Yeast cells taken as prey were ingested at the posterior end of the cell (Figs. 9, 10); the ingestion process took less than three seconds in the cells observed while feeding. No specialized cytostomal structures were seen.

Each cell contained around a dozen elongate, unbranched mitochondria, arranged around the periphery of the cell and extending longitudinally from the anterior to the posterior end (Figs. 11, 12). The cristae were tubular, and were preferentially arranged about the periphery of the mitochondrion, so that certain wider mitochondrial segments had crista-free central regions (Fig. 13).

No contractile vacuoles were detected.

The kinetid consisted of four basal bodies, two compound microtubular roots, and associated fibers and bands. Each of the four basal bodies was short measuring *ca* 350 μm (Fig. 14). At the distal end of each basal body, near the transition zone, wing like projections extended from the microtubule blades to the plasmalemma; at this point the C tubules of the blades were lost (Fig. 15). Transition zone structures on the two basal bodies bearing stubby flagella were not distinguishable (Fig. 14). Transition zone structures on the two basal bodies bearing emergent, functional flagella included an
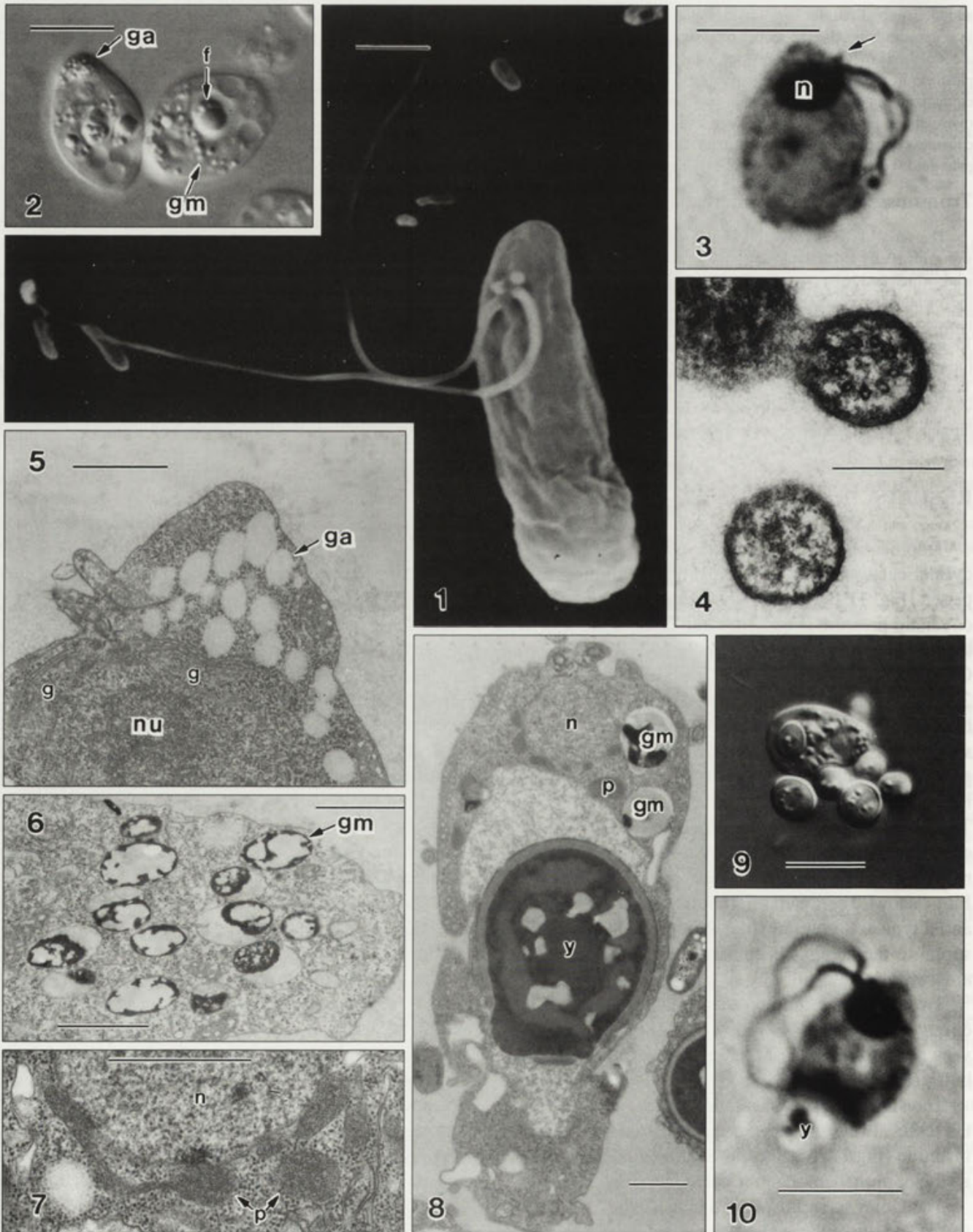
indistinct basal plate (Fig. 14) and, more distally, an apparently tubular structure just inside the axonemal doublet microtubules (Figs. 14, 15).

The basal bodies were arranged in two pairs, each pair consisting of one basal body with an emergent, and one with a stubby, flagellum. The basal bodies in each pair were coplanar, separated from each other by about half a basal body diameter, and formed an angle of approximately 30 degrees (Figs. 1, 14, 16-18). The two pairs were closely juxtaposed, as if belonging to a single kinetid, but the basal bodies of the pair on the right were mounted slightly to the posterior of the elements of the pair on the left (Figs. 1, 16-18). Also, the basal bodies and flagella of the right-side pair projected towards the right side of the cell, while the basal bodies and flagella of the left-side pair projected towards the left side of the cell (Figs. 1, 16-18). No structures were observed that linked the basal bodies to each other, but the basal bodies associated with emergent flagella each had a prominent electron-dense "terminal cap" linking the proximal end of the basal body to other kinetid components, particularly the flagellar roots (Figs. 14, 19).

Two compound flagellar roots were present, one arising from underneath each of the basal body pairs and descending posteriorly along the ventral surface of the cell (Figs. 14, 16-25). The roots were identical. A striated band was associated with each root; each was inserted into electron-dense material associated with a pocket in the nuclear envelope (Figs. 14, 19-21).

At its origin, the main band of each microtubular root typically consisted of six microtubules (Fig. 21). More distally, but still in the immediate vicinity of the basal bodies, two additional microtubules were added to the outer side of the root, forming an 8-membered spline (Fig. 22). At about this point, an additional two microtubules arose from electron-dense material associated with the emergent-flagellum basal body (Figs. 22, 23) and extended posteriorly between the main spline and

the plasmalemma (Figs. 23-25). Slightly distal to the point of origin of the doublet, the outermost of the spline microtubules became separate and associated with electron-dense material (Figs. 23, 24). Microtubules subsequently disappeared from the main spline as the root descended, in a straight line, to an unknown termination point in the posterior of the cell (Fig. 25).

In addition to the root microtubules, cytoskeletal microtubules arose from a focal point near the cell anterior (Fig. 26) and descended posteriorly as a loose corset (Figs. 11, 12, 25).

DISCUSSION

The mode (pseudopodial) and location (cell posterior) of prey capture, the presence of refractile granules around the cell nucleus (Schuster and Pollak 1978, Mylnikov 1987) and, especially, the large and complex paranuclear body, suggest that *Cholamonas cyrtodiopsidis* belong to the cercomonads. If so (and the assessment is complicated by the as-yet limited information available on the morphology, life history and ultrastructure of protists that look like cercomonads), then *C. cyrtodiopsidis* is a most unusual member of the group.

Previously described cercomonads have all been free-living and all, with the exception of *Cercomonas vibrans* Sandon (Sandon, 1927), have been described as bacterivores. *Cholamonas cyrtodiopsidis* is therefore the first gut commensal flagellate to be assigned to the Cercomonadida, and one of the first to make a diet of eukaryotes (perhaps the first recorded mycophagous cercomonad). Since the host insect's diet is one in which yeast are commonly encountered, the association of host insect and endocommensal flagellate makes sense. The exact nature of the relationship between the two, however, has yet to be determined.

Figs. 1-10. *Cholamonas cyrtodiopsidis* gen. n., sp. n., light and electron micrographs. **1** - Scanning electron micrograph of a trophic cell, showing the ventral surface with two emergent and two stubby flagella. **2** - Light micrograph (differential interference contrast) of two living cells, showing the positions of nuclei, apical (ga) and median (gm) refractile granules, and food vacuoles (f). **3** - Light micrograph of a protargol-stained cell. The nucleus (n) and flagella, including the stubby flagella (arrow), are visible.
Figs. 4-8. Transmission electron micrographs of sectioned cells. **4** - Section through the stubby flagella, showing a reduced axoneme in the more proximally-sectioned flagellum and no axonemal microtubules in the more distally-sectioned flagellum. **5** - Longitudinal section through the apical end of a cell, showing the apical refractile granules (ga) and their positional relationship to the basal bodies of the kinetid, the nucleolus (nu) and the Golgi (g). **6** - Nonmedian longitudinal section in the region posterior to the nucleus, showing the median refractile granules (gm). **7** - Longitudinal section in the region posterior to the nucleus (n), showing a profile of the paranuclear body (p). **8** - Oblique longitudinal section showing an engulfed yeast cell (y), the nucleus (n) and its subtending parabasal body (p), and two median refractile granules (gm). **9** - Light micrograph (differential interference contrast) of a living cell at the moment of yeast cell ingestion. **10** - Light micrograph of protargol-stained cells, showing a *Cholamonas* cell presumably in the process of engulfing a yeast cell (y).
Scale bars: **1** - 2 μm; **2, 3, 9, 10** - 5 μm; **4** - 300 nm; **5-8** - 1 μm
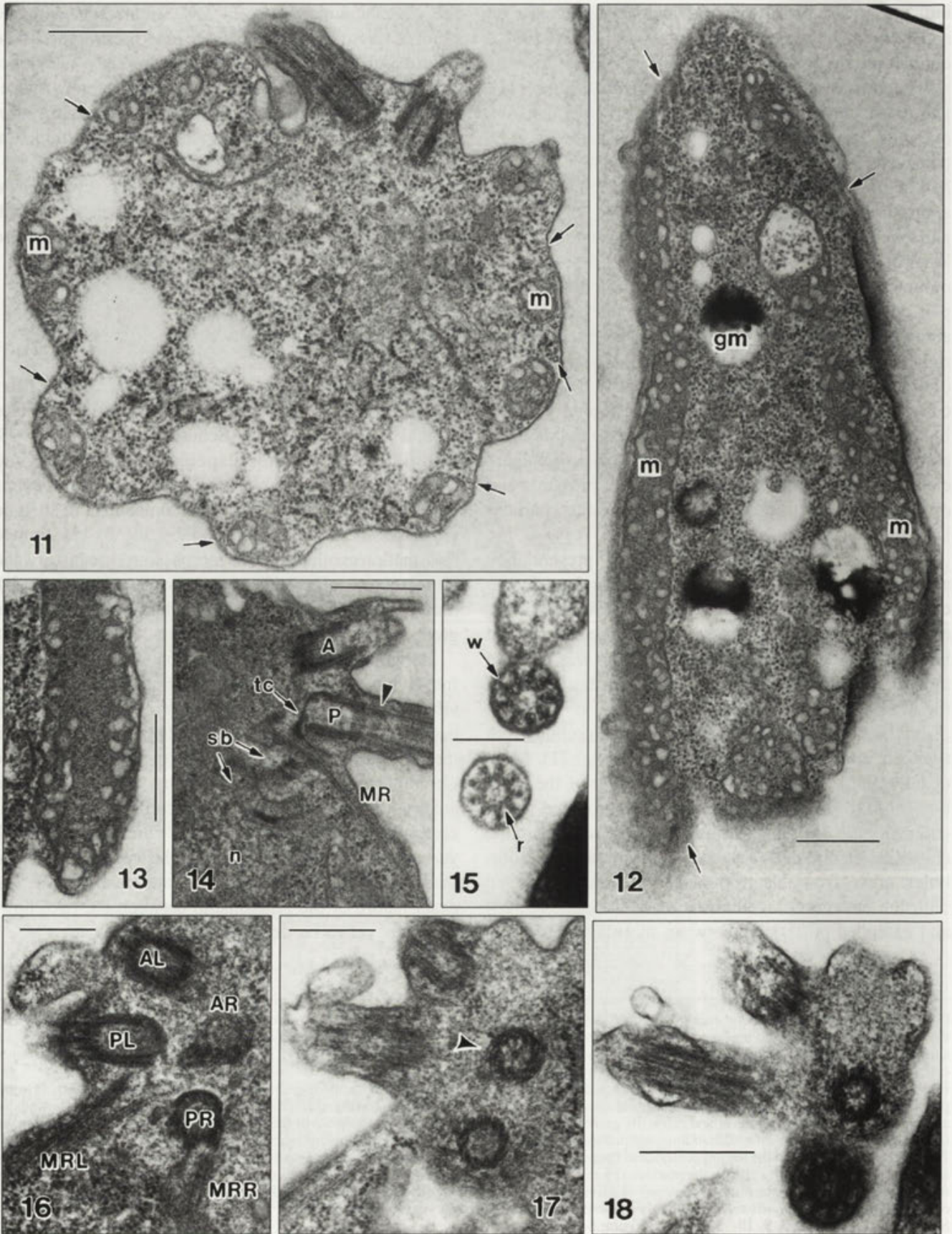
Trophic cells of *C. cyrtodiopsidis* most closely resemble the swimming stages of species of *Cercomonas* (Schuster and Pollak 1978). However, the gliding locomotion and amoeboid movement found in *Cercomonas* spp. and all other species referred to the cercomonads (except *Discocelis saleuta*), is conspicuously absent from *C. cyrtodiopsidis*.

Although *Cholamonas cyrtodiopsidis* is biflagellate, as are most species assigned to the Cercomonadida, *C. cyrtodiopsidis* lacks the expected anterior flagellum, but instead has two flagella directed posteriorly. Moreover, neither of these two flagella is intimately associated with the ventral cell surface as occurs, for example, in *Cercomonas longicauda* (Mylnikov 1987), *Cercomonas* cf. *varians* (Mignot and Brugerolle 1975) and *Cercomonas* sp. (Shirkina 1987).

Each of the two basal body pairs described in the kinetid of *C. cyrtodiopsidis* corresponds in many of its features to the single pair found in cercomonads, and especially to those species of *Cercomonas* in which a single, posteriorly-directed microtubular root has been described (Mignon and Brugerolle 1975, Mylnikov 1987). However, if the kinetid of *C. cyrtodiopsidis* is derived from a *Cercomonas*-like ancestor, then several major changes have occurred. Firstly, the kinetid has been doubled, and the components of the kinetid have not separated. The phenomenon has not previously been observed among organisms assigned to the Cercomonadida, but similar kinetid doublings are found elsewhere among protists. Both "doubling" and "halving" events occur in green flagellates (Stewart and Mattox 1978 O'Kelly and Floyd 1984); doubling events analogous to what is observed in *Cholamonas cyrtodiopsidis* are most evident in the genera *Pyramimonas* e.g. (Moestrup and Hori 1989, Daugbjerg and Moestrup 1992) and *Polytomella* (Brown *et al.* 1976). Doubling events in which the kinetids separate

but remain within a single cell are characteristic of diplomonad flagellates (Brugerolle 1975). Secondly, the anterior flagellum in each pair is a stump, not fully emergent. This phenomenon, fairly common in various protist groups, has not previously been described at the electron-microscope level among organisms referred to cercomonads. Patterson and Zölffel (1991) said of certain genera potentially assignable to *Cercomonas*, " ... we presume the original author overlooked a short anterior flagellum". The situation in *C. cyrtodiopsidis* raises the possibility that, in some of these doubtful genera, the anterior flagellum may in fact be lacking. Thirdly, the nucleus-enveloping basket of microtubules that emanates from the kinetid in several species of *Cercomonas*, and the single species of *Heteromita* for which there is a reasonable ultrastructural identity, has been lost in *C. cyrtodiopsidis*.

Finally, the apical cap of refractile granules, present in *C. cyrtodiopsidis* and apparently consisting of lipid droplets, has no counterpart among described species of cercomonads.

Until more, and more detailed, studies of cercomonads are available, it seems best to treat *C. cyrtodiopsidis* as a new genus and species of Cercomonadida. The new species is possibly most closely related to those species of *Cercomonas* that have a single prominent microtubular root, from which *C. cyrtodiopsidis* may be derived by doubling of the kinetid, loss of the anterior flagella and perinuclear microtubular basket, and the acquisition of mycophagy and an endocommensal habitat

DIAGNOSES

Cholamonas gen. n. Cercomonad protists; flagellate trophic cells with longitudinally-arranged peripheral mitochondria; a kinetid having one or more posteriorly

---

Figs. **11-18**. *Cholamonas cyrtodiopsidis* - transmission electron micrographs of sectioned cells. **11** - Cross section in the region of the kinetid, showing mitochondrial profiles (m) ranged around the cell periphery. Arrows indicate some of the secondary cytoskeletal microtubules forming a corset around the cell. **12** - Tangenial section showing elongated shape of mitochondria, especially at left (m). Some median refractile granules (gm) are also present. Arrows indicate some of the secondary cytoskeletal microtubules forming a corset around the cell. **13** - Mitochondrial profile showing peripheral cristae and crista-free central region. **14** - Section through the kinetid region, showing one anterior (A) basal body bearing a stubby flagellum, one posterior (P) basal body bearing a long flagellum, one of the two microtubular roots (MR), a terminal cap (tc) connecting the posterior basal body to the microtubular root, and a striated band (sb) linking the root to the nucleus (n). The ring structure in the long flagellar transition region is indicated (arrowhead). **15** - Cross section through two flagella in the transition region, one showing the doublet-associated wings (w) in the proximal part of the transition zone, the other showing the ring structure (r) in the distal part of the transition zone. **16-18** - Serial sections showing the absolute configuration of the kinetid, viewed from the dorsal surface of the cell (base-to-tip orientation of basal bodies). **16** - Proximalmost section, showing the left (AL) and right (AR) anterior basal bodies, the left (PL) and right (PR) posterior basal bodies. The left (MRL) and right (MRR) microtubular roots extend from the vicinity of the corresponding posterior basal bodies. Note the offset of the left and right basal body pairs (compare Fig. 1). **17** - Triplet microtubules (arrowhead) are present at the proximal end of the right anterior basal body (compare Fig. 15). **18** - Distalmost section. Scale bars: **11-14, 18** - 500 nm; **15-17** - 300 nm
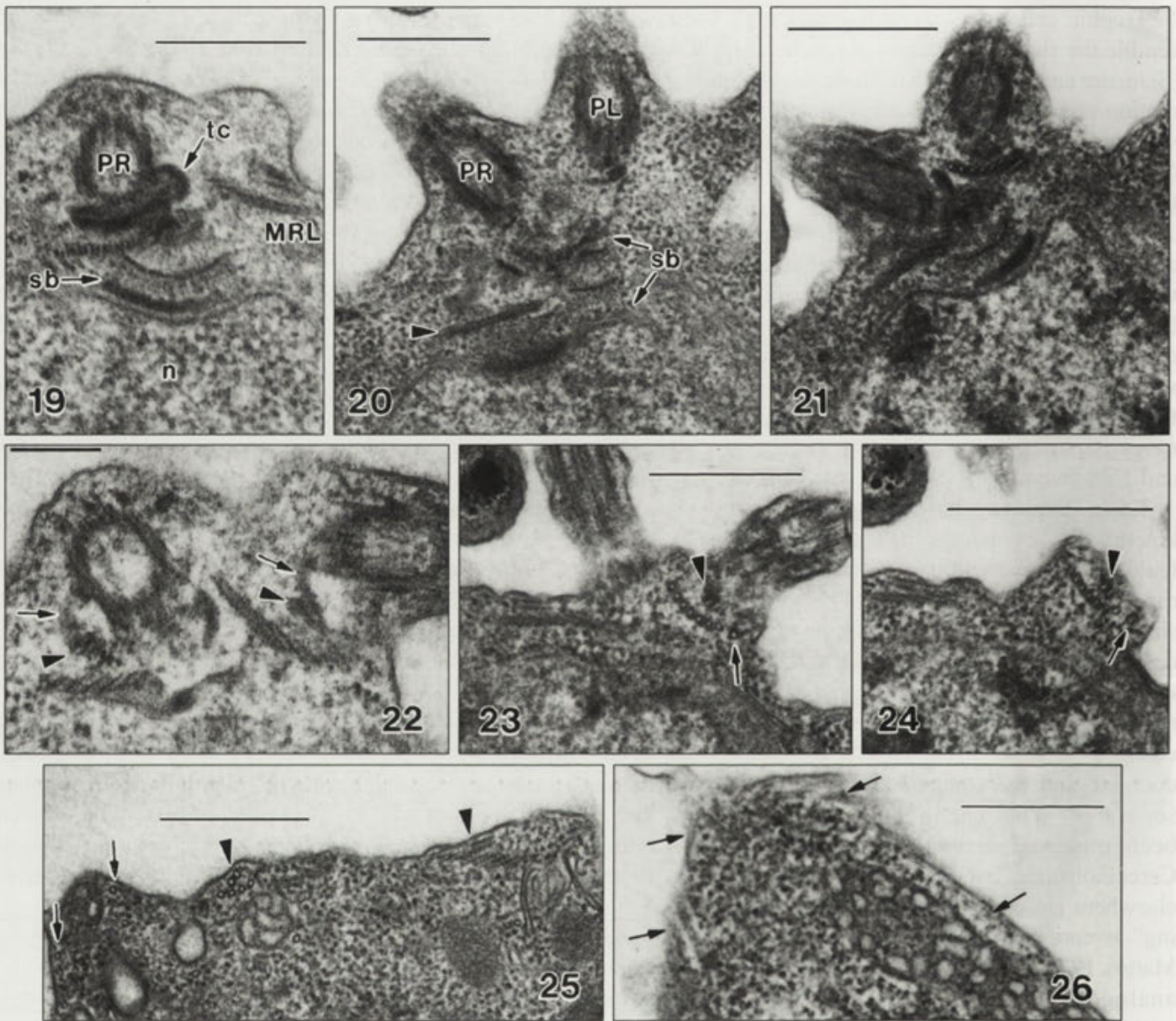
Figs. **19-26**. *Cholamonas cyrtodiopsidis*, transmission electron micrographs of sectioned cells. **19-25** - Details of kinetid structure, viewed from the cell posterior, ventral side up. **19** - Section near the origin of the right microtubular root near the right posterior basal body (PR), showing the terminal cap (tc) and its association with the basal body and the root. Also present is the prominent striated band (sb) and its attachment to the root as well as to the envelope of the nucleus (n). The left microtubular root (MRL) is also visible. **20** - Section near the origin of the left microtubular root near the left posterior basal body (PL), showing the striated band (sb) in a grazing section (hence it appears less prominent than the bands shown in Figs. 20 and 22). Six microtubules are visible in the root. The right microtubular root (arrowhead) is also visible. **21** - Section of the left microtubular root, slightly posterior to the view in Fig. 20 but from a different cell. Six microtubules are visible. Also present are the right posterior basal body and right microtubular root with its associated striated band. **22** - Origins of the two-stranded components of both the left and right microtubular roots (arrows). Both of these two-stranded components are linked to their corresponding basal bodies by a delicate fiber (arrows). **23** - Cross section of the left microtubular root, same cell as Fig. 21 but slightly posterior, near the origins of the two-stranded component (arrowhead) and divergent singlet microtubule (arrow). Seven microtubules are in the main band of the root. The right microtubular root, tangentially sectioned, is visible to the left of the image (as in Figs. 21, 24). **24** - Distal continuation of the series shown in Figs. 21 and 24, showing continuation of the tripartite configuration (arrowhead) of the left microtubular root. **25** - Microtubules are lost from the microtubular roots (arrowheads; the right microtubular root is in cross section). Secondary cytoskeletal microtubules are visible (arrows). **26** - Oblique longitudinal section of a cell near its apex, where secondary cytoskeletal microtubules (arrows) converge. Scale bars: **19- 21, 23- 26** - 500 nm; **22** - 200 nm

directed, compound microtubular roots and lacking a perinuclear microtubular basket; pseudopods not expressed except briefly around the posterior ingestion area at the moment of prey ingestion; prey typically consisting of eukaryotes. Etymology: "Gut flagellate" (*Chola-*, Gk. "intestine"; *monas*, Gk. "wanderer"). A third declension feminine Latin noun. With one species, *C. cyrtodiopsidis*.

***Cholamonas cyrtodiopsidis* sp. n.** Biflagellate, uninucleate protists in the hindgut of the stalk-eyed fly *Cyrtodiopsis dalmanni*. Cell shape plastic, typically cylindrical with rounded ends, more obovate when compressed; uncompressed cells 5.0 - 8.0 µm long, 2.0 - 5.0 µm wide at the widest point. Refractile granules in two groups, one at the apical end of the cell, the other in the vicinity of the nucleus, the two groups ultrastructurally dissimilar. Kinetid doubled, with two posteriorly directed flagella, two stubby flagella, and two posteriorly directed compound microtubular roots. Feeding on yeast. Type locality: Kuala Lumpur, Malaysia.

Holotype: cryopreserved living material, conserved at the American Type Collection (ATCC) as strain 50325. Protargol-stained slides and resin-embedded cells derived from strain 50325 conserved at ATCC and Natural History Museum, Smithsonian Institution.

Etymology: "from *Cyrtodiopsis*", the genus name of the host stalk-eyed fly.

REFERENCES

Brown D.L., Massalski A., Patenaude B. (1976) Organization of the flagellar apparatus and associated cytoplasmic microtubules in the quadriflagellate alga *Polytomella agilis*. *J. Cell Biol.* **69:** 106-125

Brugerolle G. (1975) Contribution à l'étude cytologique et phylétique des Diplozoaires (Zoomastigophorea, Diplozoa, Dangeard 1910), VI Caractères généraux des Diplozoaires. *Protistologica* **11:** 111-118

Cavalier-Smith T. (1993) Kingdom Protozoa and its 18 phyla. *Microbiol. Rev.* **57:** 953-994

Cavalier-Smith T., Chao E. E. (1996-1997) Sarcomonad ribosomal RNA sequences, rhizopod phylogeny, and the origin of euglyphid amoebae. *Arch. Protistenkd.* **147:** 227-236

Daugbjerg N., Moestrup Ø. (1992) Ultrastructure of *Pyramimonas cyrtoptera* sp. nov., a sixteen-flagellate species from Northern Foxe Basin, Arctic Canada, including observations on growth rates. *Can. J. Bot.* **70:** 1259-1273

de la Motte I., Burkhardt D. (1993) Portraits of an asian stalk-eyed fly. *Naturwissenschaften* **70:** 451-461

Hänel K. (1979) Systematik und Ökologie der farblosen Flagellaten des Abwassers. *Arch. Protistenkd.* **121:** 73-137

Larsen J., Patterson D. J. (1990) Some flagellates (Protista) from tropical marine sediments. *J. Nat. Hist.* **24:** 801-937

Lynn D. H. (1992) Protargol Staining. In: Protocols in Protozoology, (Eds. J.J. Lee and A.T. Soldo). Society of Protozoologists, Allen Press Inc., Lawrence, Kansas **1:** C-4.1-C4.8

MacDonald C. M., Darbyshire J.F., Ogden C.G. (1977) The morphology of a common soil flagellate, *Heteromita globosa* Stein (Mastigophorea: Protozoa). *Bull. Br. Mus. Nat. Hist. (Zool.)* **31:** 255-264

Mignot J.P., Brugerolle G. (1975) Etude ultrastructurale de *Cercomonas* Dujardin (= *Cercobodo* Krassilstchick), protiste flagellé. *Protistologica* **11:** 547-554

Moestrup Ø., Hori T. (1989) Ultrastructure of the flagellar apparatus in *Pyramimonas octopus* (Prasinophyceae). II. Flagellar roots, connecting fibres, and numbering of individual flagella in green algae. *Protoplasma* **148:** 41-56

Mylnikov A. P. (1985) Characteristic features of the fine structure of *Bodomorpha reniformis* Zhukov (Zoomastigophorea). *Tsitologiya* **26:** 1308-1310 (in Russian)

Mylnikov A. P. (1986a) Ultrastructure of a colourless amoeboid flagellate, *Cercomonas* sp. *Arch. Protistenkd.* **131:** 239-247.

Mylnikov A. P. (1986b) Biology and ultrastructure of amoeboid flagellates from the Cercomonadina ord. nov. *Zool. Zh.* **65:** 683-692 (in Russian)

Mylnikov A. P. (1987) The ultrastructure of flagellate *Cercomonas longicauda* (Cercomonadidae). *Bot. Zh.* **72:** 750-753 (in Russian)

Mylnikov A. P. (1990) Characteristic features of the ultrastructure of the colourless flagellate, *Heteromita* sp. *Tsitologiya* **32:** 567-571 (in Russian)

Nerad T. A. (Ed.) (1993) American Type Culture Collection Catalogue of Protists, 18th edn. American Type Culture Collection, Rockville

Nerad T. A., Daggett P.M. (1992) Cryopreservation of diplomonads other than *Giardia*. In: Protocols in Protozoology, (Eds. J.J. Lee and A.T. Soldo), Society of Protozoologists, Allen Press, Lawrence, Kansas **1:** A70.1-A70.2

O'Kelly C. J., Floyd G.L. (1984) Flagellar apparatus absolute orientations and the phylogeny of the green algae. *BioSystems* **16:** 227-251

Patterson D. J. (1994) Protozoa: Evolution and Systematics. In: Progress in Protozoology: Proceedings of the IX International Congress of Protozoology, Berlin 1993, (Eds. K. Hausmann and N. Hülsmann).Gustav Fischer, Stuttgart/Jena/New York **1:** 1-14

Patterson D. J., Fenchel T. (1990) *Massisteria marina* Larsen and Patterson 1990, a widespread and abundant bacterivorous protist associated with marine detritus. *Mar. Ecol. Prog. Ser.* **62:** 11-19

Patterson D. J., Zölffel M. (1991) Heterotrophic flagellates of uncertain taxonomic position. In: The Biology of Free-living Heterotrophic Flagellates, (Eds. D.J. Patterson and J. Larsen). Clarendon Press, Oxford **26:** 427-475

Poynton S. L., Fraser W., Francis-Floyd R., Rutledge P., Reed P., Nerad T. A. (1995) *Spironucleus vortens* n. sp. from the freshwater angelfish *Pterophyllum scalare*: morphology and culture. *J. Euk. Microbiol.* **42:** 731-742

Sandon H. (1927) The Composition and Distribution of the Protozoan Fauna of the Soil. Oliver and Boyd, Edinburgh and London

Schuster F.L., Pollak A. (1978) Ultrastructure of *Cercomonas* sp., a free-living ameboid flagellate. *Arch. Protistenkd.* **120:** 206-212

Shirkina N. I. (1987) Biological features in some flagellates of the genus *Cercomonas* (Rhizomastigida, Cercomonadinae). *Zool. Zh.* **66**: 485-489 (in Russian)

Stewart K.D., Mattox K. R. (1978) Structural evolution in the flagellated cells of green algae and land plants. *BioSystems* **10**: 145-152

Vørs N. (1988) *Discocelis saleuta* gen. nov. et sp. nov. (Protista incertae sedis) - a new heterotrophic marine flagellate. *Eur. J. Protistol.* **28**: 369-389

ACTA PROTOZOOLOGICA

The Occurrence of Three Species of *Trichodina* (Ciliophora: Peritrichia) on *Cyprinus carpio* in Relation to Culture Conditions, Seasonality and Host Characteristics

Ahmet Özer

Ondokuzmayis Üniversitesi, Su Ürünleri Fakültesi, Sinop, Turkey

Summary. The occurrence of *Trichodina mutabilis* Kazubski & Migala, 1968, *Trichodina acuta* Lom, 1961 and *Trichodina nigra* Lom, 1961 on common carp (*Cyprinus carpio* L., 1758) was studied with respect to seasonality, host factors and the culture conditions. The sampling period was between June 1994 and May 1995 in the Sinop region of northern Turkey and a total of 125 common carp were investigated. The overall incidence of *T. mutabilis* infestation was 80.7% in the farm and 61.7% in the lake, whilst it was 44.8% in the farm and 36.1% in the lake for the collective *T. acuta - T. nigra* group. Spring was found to be the most favoured season. There was no statistically significant preference of the collective *T. acuta - T. nigra* group to any of the varieties of common carp or the sex of the host. *T. mutabilis* also showed similar preferences to the collective *T. acuta - T. nigra* group.

Key words: common carp, seasonality, *Trichodina acuta*, *Trichodina mutabilis*, *Trichodina nigra*, Turkey.

INTRODUCTION

Geographically, trichodinids are a widely dispersed group of ectoparasites. The transportation of fish species with importance to aquaculture or the ornamental fish trade has undoubtedly contributed to the distribution of trichodinid species (Albaladejo and Arthur 1989, Van As and Basson 1989). The importance of trichodinids is reflected in the volume of literature dealing with various aspects of their biology of these parasites, i.e. behaviour,

distribution, the impact of environmental factors, concomitant infestations and their relative pathogenity (Lom and Hoffman 1964, Lom 1970, 1973, Ahmed 1977, Calenius 1980, Das and Pal 1987, Van As and Basson 1987, Sanmartin Duran *et al.* 1991). *Trichodina mutabilis, T. acuta* and *T. nigra* have been commonly reported in Europe (Lom and Dykova 1992).

In Turkey, studies on trichodinids are very limited and there is the potential for finding different trichodinid species on a range of new host and geographic records. *Trichodina mutabilis* Kazubski & Migala, 1968, *Trichodina acuta* Lom, 1961 and *Trichodina nigra* Lom, 1961 were recently recorded in the northern part of Turkey (Özer and Erdem 1998). In this study, the following aspects of *T. mutabilis, T. acuta* and *T. nigra*

Address for correspondence: Ahmet Özer, Ondokuzmayis Üniversitesi, Su Ürünleri Fakültesi, 57000 Sinop, Turkey; Fax: 00 90 368 287 6255

infestations were investigated at two environmentally different sampling stations: seasonality, the parasites occurrence on male and female carp, as well as on mirror and scaled varieties of common carp.

MATERIALS AND METHODS

Fish were collected by gill net, cast net or creel (fish pot) from a fish farm at Çobanlar and an artificial lake at Bektaşaga both in the Sinop region of northern Turkey. Sampling was carried out monthly at both stations. Immediately after collection the fish were transported alive, in aerated local water directly to the Sinop Fisheries Faculty Laboratory for ectoparasitic examination. A total of 78 carp from the fish farm and 47 carp from the lake were investigated. Carp were weighed, the total length measured and their sex determined at post-mortem. The fish were sacrificed in the laboratory immediately prior to examination. The details of this parasitological examination were given by Özer and Erdem (1998). Briefly, the skin, gills and fins were examined under a light dissecting microscope. Scrapings from the skin, gills and fins were taken using coverslips and then placed into Petri dishes. An attempt to collect whole mucus from the carp body parts mentioned above was made. Several slides were prepared from the mucus samples by adding a drop of distilled water to obtain preparations thin enough for examination under a compound microscope (x200 magnification). Trichodinid specimens collected from each slide were counted on whole slides. For species identification, dry smears were made in accordance with Klein's silver nitrate (AgNO₃) method (Lom and Dykova 1992).

The incidence (%) and mean intensity levels of trichodinids were determined according to Bush *et al.* (1997). *T. mutabilis* specimens were easily discriminated from *T. acuta* and *T. nigra* by their larger size. The total numbers of *T. acuta* and *T. nigra* specimens were combined due to their similar sizes which makes differentiation of these two species difficult when they are alive. However, the species identification was confirmed by the method given above.

Adverse weather conditions prevented sampling during the months of November to February at the artificial lake Bektaşaga. The low number of fish collected from the fish farm and the artificial lake was partly due to the restricted access to the lake and the farmer's policy that allowed us to use only a limited number of fish for each month.

The relationships between the fish length and mean intensities of parasites were tested using ANOVA following logarithmic transformation of the data. The differences between parasite loading on male and female carp and on mirror and scaled carp were tested by the Mann-Whitney U-test.

RESULTS

Seasonality and the distribution of *Trichodina* species

Trichodina mutabilis and the collective *T. acuta* - *T. nigra* group were present at both the farm at Çobanlar and the lake at Bektaşaga. *T. mutabilis* was recorded in summer (June-August), autumn (September-November),

winter (December-February) and spring (March-May); however, the *T. acuta* - *T. nigra* group was absent during summer. The incidence of *T. mutabilis* on carp reached its maximum in autumn at Çobanlar and in autumn and spring at Bektaşaga, whilst it was lowest in winter at Çobanlar and summer at Bektaşaga (Table 1). At Çobanlar, the maximum mean intensity of infestation was recorded in winter, whilst it was minimal in autumn. There were also statistically significant differences in the mean intensities of *T. mutabilis* infestations between autumn and spring (P<0.01) and summer and spring (P<0.01) at this station (Table 1). At Bektaşaga, the mean intensity of infestation recorded was highest in the spring and lowest in summer. Here, the difference between the mean intensities of infestation recorded in summer and spring were found to be statistically significant (P<0.01) (Table 1).

The infestation incidences and mean intensities of the *T. acuta* - *T. nigra* showed an increase from autumn to spring at both stations (Table 2). The incidences of infestation recorded for this collective group were highest in the spring at both sampling stations and mean intensities of infestation during this period were maximal at Çobanlar and Bektaşaga. However, there were no statistically significant differences in intensities of infestation between autumn and spring when the *T. acuta* - *T. nigra* group was equally abundant at both sampling stations (Table 2).

No statistically significant difference in the overall length of carp both uninfested and infested with *T. mutabilis* at Çobanlar and Bektaşaga was observed. A statistically significant difference was found in the length of carp infested with *T. mutabilis* sampled in summer and spring from the lake (P<0.05) (Table 1). While there were significant differences in the length of carp infested with the *T. acuta* - *T. nigra* group sampled at different seasons from the farm, a statistically significant difference was found only between the length of carp sampled in the autumn and spring at the lake (P<0.05) (Table 2).

The distribution of each *Trichodina* species on host fish of different sexes

There was a greater incidence of *T. mutabilis* than the *T. acuta* - *T. nigra* group on both sexes of carp at both sampling stations but the mean intensities of infestation were lower than *T. mutabilis*. However there were no statistically significant differences in the mean intensities of *T. mutabilis* infestations between the sexes of carp from both the farm and the lake (Table 3).

The incidences of female and male carp infested with *T. mutabilis* were very similar to one another at the fish farm but much higher on female than male carp from the

Table 1. Incidence and mean intensity of *Trichodina mutabilis* infestations according to season at Çobanlar and Bektaşaga

	No of fish examined	No of fish infested	Length of fish infested ± S.E	Incidence of infestation (%)	Mean intensity of infestation ± S.E
Çobanlar (farm)					
Summer (June - Aug)	22	19	21.13 ± 0.45 [a]	86.36	32.10 ± 14.39 [a] **
Autumn (Sept - Nov)	21	19	19.78 ± 0.86 [a]	90.47	15.15 ± 4.09 [a] ***
Winter (Dec - Feb)	17	10	19.40 ± 2.07 [a]	58.82	257.1 ± 103.6 [ab]
Spring (Mar - May)	18	15	19.36 ± 1.46 [a]	83.33	102.3 ± 38.36 [b] **.**
overall	78	63	20.03 ± 0.55 [a]	80.7	79.42 ± 21.44 [a] ***
Bektaşaga (lake)					
Summer (June - Aug)	26	8	22.81 ± 0.71 [a] *	30.76	71.87 ± 43.21 [a] **
Autumn (Sept - Nov)	8	8	19.75 ± 3.09 [ab]	100	351.8 ± 218.2 [ab]
Winter (Dec - Feb)	-	-		-	
Spring (Mar - May)	13	13	19.42 ± 0.93 [b] *	100	1648 ± 617.8 [b] **
overall	47	29	20.44 ± 0.97 [ab]	61.7	859.1 ± 308.4 [b] ***

Statistically significant at: * = 5% level, ** = 1% level,*** = 0.1% level. Values with the same superscript letters are not significantly different (P≥0.05)

Table 2. Incidence and mean intensity of the *Trichodina acuta - Trichodina nigra* group infestations according to season at Çobanlar and Bektaşaga

	No of fish examined	No of fish infested	Length of fish infested ± S.E	Incidence of infestation (%)	Mean intensity of infestation ± S.E
Çobanlar (farm)					
Summer June - Aug)	22	0	-	0	-
Autumn (Sept - Nov)	21	5	19.80 ± 1.75 [a]	23.80	31.20 ± 14.87 [a] *
Winter (Dec - Feb)	17	12	18.75 ± 1.93 [a]	70.58	230.6 ± 139.1 [ab]
Spring (Mar - May)	18	18	17.91 ± 1.51 [a]	100	358.8 ± 136.6 [b] *
overall	78	35	18.47 ± 1.03 [a]	44.87	268.02 ± 85.58 [a]
Bektaşaga (lake)					
Summer (June - Aug)	26	0	-	-	-
Autumn (Sept - Nov)	8	4	26.12 ± 2.88 [a] *	50	91.25 ± 77.01 [a]
Winter (Dec - Feb)	-	-		-	
Spring (Mar - May)	13	13	19.42 ± 0.93 [b] *	100	236.9 ± 92.93 [a]
overall	47	17	19.42 ± 1.17 [a]	36.17	202.64 ± 73.85 [a]

Statistically significant at: * = 5% level, ** = 1% level, *** = 0.1% level. Values with the same superscript letters are not significantly different (P≥0.05)

lake (Table 3). The mean intensities of *T. mutabilis* on female and male carp did not show any statistically significant differences at either of the sampling stations, but the recorded levels were higher on male carp. However, when female and male carp at both sampling stations were compared, statistically significant differences in mean *T. mutabilis* intensities on female and male carp at the farm and the lake were observed (Table 3).

On the other hand, male carp infested with the *T. acuta-T. nigra* group had higher incidences of infestation at both

the farm and the lake. The mean intensities of infestation recorded were higher on male carp from the farm and on female carp from the lake, however no statistically significant difference was determined (Table 3).

The distribution of each *Trichodina* species on mirror and scaled carp

The incidences of *T. mutabilis* infestations on mirror and scaled carp were higher for mirror carp from the farm and lower at the lake. Infestation intensities showed a

Table 3. Incidence and mean intensity of *Trichodina mutabilis* and the *Trichodina acuta - Trichodina nigra* group infestations according to the sex of carp at Çobanlar and Bektaşaga

	No of fish examined	No of fish infested	Length of fish infested ± S.E	Incidence of infestation (%)	Mean intensity of infestation ± S.E.
Trichodina mutabilis					
Çobanlar (farm)					
Female	38	33	21.46 ± 0.66 ª *	84.61	50.84 ± 19.22 ª **.***
Male	37	29	18.72 ± 0.80 ᵇ **.*	80.55	114.1 ± 40.62 ª *. ***
Bektaşaga (lake)					
Female	26	19	21.97 ± 1.08 ª *.*	73.07	852.26 ± 446.7 ᵇ **.*
Male	20	9	18.27 ± 1.59 ᵇ *	45	957.44 ± 341.0 ᵇ***. ***
T. acuta-T. nigra					
Çobanlar (lake)					
Female	38	14	21.00 ± 1.56 ª	36.82	200.7 ± 102.6 ª
Male	37	18	18.23 ± 1.22 ª	48.64	355.8 ± 145.2 ª
Bektaşaga (lake)					
Female	26	9	22.38 ± 1.85 ª	34.61	264.9 ± 134.1 ª
Male	20	9	20.64 ± 0.36 ª	45	150.3 ± 42.24 ª

Statistically significant at: * = 5% level,** = 1% level,*** = 0.1% level. Values with the same superscript letters are not significantly different (P≥0.05)

Table 4. Incidence and mean intensity of *Trichodina mutabilis* and the *Trichodina acuta-Trichodina nigra* group infestations on mirror and scaled carp at Çobanlar and Bektaşaga

	No of fish examined	No of fish infested	Length of fish infested ± S.E	Incidence of infestation (%)	Mean intensity of infestation ± S.E.
Trichodina mutabilis					
Çobanlar (farm)					
Mirror	62	52	20.25 ± 0.53 ª *	83.87	79.42 ± 24.51 ª ***
Scaled	16	11	18.95 ± 1.93 ᵃᵇ	68.75	85.80 ± 46.99 ᵃᵇ
Bektaşaga (lake)					
Mirror	21	12	23.62 ± 0.99 ᵇ *.***	54.14	1326.2 ± 669.4 ᵇ***.*
Scaled	26	17	18.20 ± 1.25 ª***	65.38	523.52 ± 224.8 ª *
T. acuta-T. nigra					
Çobanlar (farm)					
Mirror	62	26	18.69 ± 1.18 ᵃᵇ	41.93	319.8 ± 112.8 ª
Scaled	16	9	17.83 ± 2.21 ᵃᵇ	56.25	118.4 ± 49.31 ª
Bektaşaga (lake)					
Mirror	21	7	23.50 ± 1.66 ª *	33.33	304.9 ± 157.1 ª
Scaled	26	10	19.25 ± 1.42 ᵇ *	38.46	131.1 ± 59.92 ª

Statistically significant at: * = 5% level,** = 1% level,*** = 0.1% level. Values with the same superscript letters are not significantly different (P≥0.05)

pattern opposite to that of the infestation incidence levels at both sampling stations though there was no statistically significant difference on the mirror and scaled carp at the farm, there was at the lake (P<0.05) (Table 4). The mean

intensity levels of *T. mutabilis* on mirror carp at the farm and the lake also significantly differed (P<0.001) (Table 4).

The infestation incidences of the *T. acuta - T. nigra* group at the farm and the lake were higher on scaled carp in contrast to the mean intensity levels that were higher for mirror carp with no statistically significant difference at either of the sampling stations, or between the stations (Table 4).

The length of the mirror and scaled carp infested with either of *T. mutabilis* and the *T. acuta -T. nigra* did not show any significant difference at the farm but the length of mirror and scaled carp at the lake differed (P<0.001) (Table 4).

DISCUSSION

The microhabitat distributions of trichodinids (Migala 1971, Basson *et al.* 1983), their occurrence according to the age of fish (Poljanski and Shulman 1956, Kulemina 1968, Halmetoja *et al.* 1992) and seasonality (Migala 1971, Calenius 1980, Halmetoja *et al.* 1992) have been well studied. Özer and Erdem (1998, 1999) also conducted a comprehensive study on the occurrence of several ectoparasites, including *Trichodina mutabilis*, *T. acuta* and *T. nigra*, at distinct environments, a farm and a lake, in the Sinop region of northern Turkey. However, studies on the interactions between *T. mutabilis*, *T. acuta* and *T. nigra* and their host, i.e. whether their occurrence exhibits any preference to the scaled or mirror varieties of common carp, whether infestations are influenced by the sex of the carp host, as well as the seasonality of infestations is far from established.

The incidence and mean intensity of *T. mutabilis,* and the *T. acuta - T. nigra* group infestations increased to their highest in spring at both sampling stations, clearly demonstrating the existence of a seasonal pattern of abundance for all the trichodinids studied here. Migala (1971) noted that, *T. acuta* and *T. nigra* on *Cyprinus carpio* in Poland were present during the summer months with an increase during the autumn and a decrease during the spring. These observations do not agree with our findings. However, Valtonen and Koskivaara (1994) recorded a peak *T. nigra* prevalence on *Salmo trutta* during the spring months in Finland. In addition, Pojmanska (1994, 1995) also observed peak *T. mutabilis* prevalence on *Cyprinus carpio* in spring in Poland, which are in agreement with our results. The preference of the trichodinids for spring is

generally attributed to the weakened condition of fish hosts after wintering and with the onset of the spawning season. This was also supported by the presence of many spawning carp at the farm in this study. In addition, fish in close proximity to one another during spawning season when particulate food for the trichodinids under discussion is more available because of rising temperatures and material stirred up by host activity.

Another possible explanation might be the temperature requirements of these trichodinid species for multiplication (Özer and Erdem 1999). Kazubski and Migala (1968) also found *T. mutabilis, T. acuta* and *T. nigra* to be present on *Cyprinus carpio* during the cold season in Poland. The mean infestation intensities of the *T. acuta - T. nigra* group followed almost the same patterns at both the farm and the lake, but the mean intensity of *T. mutabilis* showed some contradictions such as being lowest in autumn at the farm but intermediate at the lake during this season. Additionally, the mean intensity level was recorded at its maximum in the lake, however, there was no such observation at the farm. Higher stocking densities at fish farms usually promote high incidences of parasite populations of such parasites that have direct life cycle. This phenomenon was partly supported from our results by the presence of higher infestation incidences at the farm. However, the results from this study also showed that the weakened condition of fish may also accelerate the multiplication of the trichodinids in a particular fish specimen, resulting in a higher mean intensity as observed for *T. mutabilis* on the wild carp.

As far as I am aware, there is no previous study dealing with the relationships between the trichodinid infestations and the sex of the fish host and the structure of their skin. The trichodinid species studied here showed no statistical significant preference for either carp sex at either sampling station, even though male carp had higher intensities of infestation. According to Pickering and Christie (1980), a higher number of brown trout, *Salmo trutta*, skin parasites were recorded on male fish as a result of physiological factors such as mucus, colour and hormonal status. The male and female fish have rhythmical changes in epidermis thickness and male fish have thicker epidermis. During the spawning season, the number of mucus cells in the male drops significantly and promotes a higher prevalence of infection (Pickering 1977). Urawa (1992) observed a certain decrease in the number of AB-positive mucous cells, which are common in the epidermis of uninfected skin, with increasing *Trichodina truttae* infestations on chum salmon, *Onchorhynchus keta*, of an

average 0.78 g in weight. Thus, it could be possible that carp with a length in the range of 10 - 28 cm might exhibit a similar response to the trichodinids studied here.

Trichodina mutabilis was recorded as a gill parasite, whilst *T. acuta* and *T. nigra* reported to be skin parasites (Migala 1971, Basson *et al.* 1983, Özer and Erdem 1999). It is obvious from this study that the presence of the *T. acuta - T. nigra* group on mirror and scaled varieties of carp is possibly related to the host's skin structure. Even though there was no statistically significant difference in the mean infestation intensities on both mirror and scaled carp at the farm and the lake, a consistent preference for mirror carp was observed. *T. mutabilis*, however, showed no distinct preference to the scaled and mirror varieties of carp at both sampling stations, possibly because this species is a gill parasite and the skin structure of the host might not have bearing on the parasite's requirements.

REFERENCES

Ahmed A.T.A. (1977) Morphology and life history of *Trichodina reticulata* from goldfish and other carps. *Fish Pathol.* **12:** 21-31

Albaladejo J.D., Arthur J.R. (1989) Some trichodinids (Protozoa; Ciliophora: Peritrichida) from freshwater fishes imported into Philippines. *Asian Fish. Sci.* **3:** 1-25

Basson L., Van As J.G. Paperna I. (1983) Trichodinid ectoparasites of chiclid and cyprinid fishes in South Africa and Israel. *Syst. Parasitol.* **5:** 245-257

Bush A.O., Lafferty K.D., Lotz J.M., Shostak A.W. (1997) Parasitology meets ecology on its own terms: Margolis *et al.* Revisited. *J. Parasitol.* **84:** 575-583

Calenius G. (1980) Parasites of fish in Finland. III. Ciliates of the family *Urceolariidae* (Dujardin, 1851). *Acta Acad. Aboensis, Ser, B,* **40:** 1-15

Das M.K., Pal R.N. (1987) Histopathology of gill infestation by Monogenea and Urceolariid ciliates in carps cultured in India, *Indian J. Parasitol.* **11:** 127-130

Halmetoja A., Valtonen E-T., Taskinen J. (1992) Trichodinids (Protozoa) on fish from four central Finnish lakes of differing water quality. *Aqua Fennica* **22:** 59-70

Kazubski S.L., Migala K. (1968) Urceolariidae from breeding carp, *Cyprinus carpio* L., in Zabienic and remarks on the season variability of trichodinids. *Acta Protozool.* **6:** 133-170

Kulemina I.V. (1968) Parasitic ciliates (Peritricha, Urceolariidae) from the fry and young fishes of lake Seliger. *Acta Protozool.* **6:** 185-206

Lom J. (1970) Observations on trichodinid ciliates from freshwater fishes. *Arch. Protistenkd.* **112:** 153-177

Lom J. (1973) Adhesive disc of *Trichodinella epizootica* - ultrastructure and injury to the host tissue. *Folia Parasitol.* **20:** 193-202

Lom J., Hoffman G-L. (1964) Geographic distribution of some species of trichodinids (Ciliata: Peritricha) parasitic on fishes. *J. Parasitol.* **50:** 30-35

Lom J., Dykova I. (1992) Protozoan Parasites of Fish. Elsevier publishers, Amsterdam

Migala K. (1971) Studies on natural populations of parasitic protozoa on *Cyprinus carpio* L., in pond culture. Carps in the first year of life. *Acta Protozool.* **8:** 309-339

Özer A., Erdem O. (1998) Ectoparasitic protozoa fauna of the common carp (*Cyprinus carpio* L., 1758) caught in the Sinop region of Turkey. *J. Nat. Hist.* **32:** 441-454

Özer A., Erdem O. (1999) The relationship between occurrence of ectoparasites, temperature and culture conditions: a comparison of farmed and wild common carp (*Cyprinus carpio* L., 1758) in the Sinop region of northern Turkey. *J. Nat. Hist.* **33:** 483-491

Pickering A.D. (1977) Seasonal changes in the epidermis of the brown trout, *Salmo trutta* (L.). *J. Fish Biol.* **10:** 561-565

Pickering A-D., Christie P. (1980) Sexual differences in the incidence and severity of ectoparasitic infestation of the brown trout, *Salmo trutta* L. *J Fish Biol.* **16:** 669-683

Pojmanska T. (1994) Infection of common carps, and three introduced herbivorous fish from Zabienic fish far, in relation to their sizes. *Acta Parasitol.* **39:** 16-24

Pojmanska T. (1995) Seasonal dynamics of occurrence and reproduction of some parasites in four cyprinid fish cultured in ponds. I. Protozoa. *Acta Parasitol.* **40:** 21-25

Poljanski Y.U., Shulman S.S. (1956) Age changes in the parasitic fauna of fishes *Trudy Karelsk. Fill. AN, SSSR.* Pedrozavodsk. **4:** 3-26 (In Russian).

Sanmartin Duran M.L., Fernandez Casal J., Tojo J.L., Santomarina M.T., Esrevez J., Ubeira F. (1991) *Trichodina* sp.; Effects on the growth of farmed turbot (*Scopthalmus maximus*). *Bull. Eur. Ass. Fish Pathol.* **11:** 89-91

Urawa S. (1992) *Trichodina truttae* Mueller, 1937 (Ciliophora: Peritrichida) on juvenile chum salmon (*Onchorhynchus keta*): pathogenicity and host-parasite interactions. *Gyobyo Kenkyu* **27:** 29-37

Valtonen E-T., Koskivaara M. (1994) Relationships between the parasites of some wild and cultured fishes in two lakes and a fish farm in central Finland. *Int. J. Parasitol.* **24:** 109-118

Van As J.G., Basson L. (1987) Host specificity of trichodinid ectoparasites of freshwater fish. *Parasitol. Today* **3:** 88-90

Van As. J.G., Basson L. (1989) A further contribution to the taxonomy of the Trichodinidae (Ciliophora: Peritrichia) and a review of the taxonomic status of some fish ectoparasitic trichodinids. *Syst. Parasitol.* **14:** 157-179

ACTA
PROTOZOOLOGICA

Two New Species of *Isospora* Schneider 1881 (Apicomplexa: Eimeriidae) from Gekkonid Hosts from Morocco

Jiří VOLF[1], Břetislav KOUDELA[2] and David MODRÝ[1,2]

[1]Department of Parasitology, University of Veterinary and Pharmaceutical Sciences Brno, Brno; [2]Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice, Czech Republic

Summary. Two new species of *Isospora* are described from geckos from Morocco. Oocysts of *Isospora altiatlantis* sp. n. from the thorny-eyelidded gecko, *Quedenfeldtia trachyblepharus,* are subspherical to ovoidal, 28.4 (20-35) x 24.9 (19-30) μm with a smooth, bilayered, colourless, ~1.2 μm thick oocyst wall. A micropyle, oocyst residuum and polar granule are absent. Sporocysts are ellipsoidal, 11.6 (10-14) x 9.4 (8-10) μm with smooth, ~0.5 μm thick sporocyst wall. A knob-like Stieda body and relatively large, oval, homogenous substieda body are present. Sporozoites are elongate, distinctly transversally striated. Oocysts of *Isospora oudrii* sp. n. from the Moroccan fan-footed gecko, *Ptyodactylus oudrii,* are spherical, 16.5 (15-18) x 16.4 (15-18) μm with a smooth, bilayered, colourless, ~1 μm thick oocyst wall. A micropyle, oocyst residuum and polar granule are absent. Sporocysts are ellipsoidal, 9.2 (9.0-10.0) x 6.3 (6-7) μm with smooth, ~0.5 μm thick sporocyst wall. A knob like Stieda body and oval substieda body present. Sporozoites are vermiform, lying parallel within the sporocyst. Additionally, *Isospora platyurusi* Finkelman *et* Paperna, 1995 was found to be named improperly and is therefore emended to *Isospora platyuri.*

Key words: Coccidia, Eimeriidae, Gekkonidae, *Isospora altiatlantis* sp. n., *I. oudrii* sp. n., Sauria.

INTRODUCTION

Geckos represent one of the most highly diversified groups of saurians ranging predominantly in subtropical and tropical regions throughout the world. In contrast to this diversity, there are only limited studies concerning coccidian parasites of these hosts.

The genus *Quedenfeldtia*, endemic to extreme Northwestern Africa comprises two species of diurnal geckos; *Q. trachyblepharus* (Boettger, 1874) is endemic in high altitudes of the High Atlas Mountains and *Q. moerens* (Chabanaud, 1916) can be found at lower altitudes of High Atlas, Antiatlas, and their surroundings up to Atlantic. *Ptyodactylus oudrii* Lataste, 1880 is a member of a relatively widely distributed genus, but the species itself is also endemic to deserts of North-western Africa and is isolated from any other *Ptyodactylus* species by the geographic gap (Schleich *et al.* 1996). The present paper describes two new species of *Isospora* from thorny-

Address for correspondence: Jiří Volf, Department of Parasitology, University of Veterinary and Pharmaceutical Sciences, Palackého 1-3, 612 42 Brno, Czech Republic; Fax: (420) 5 41562266; E-mail: volf@dior.ics.muni.cz

eyelidded gecko, *Quedenfeldtia trachyblepharus* and Moroccan fan-footed gecko, *Ptyodactylus oudrii.*

MATERIALS AND METHODS

During a parasitological and herpetological survey in Morocco in April/May 1998, 21 specimens of thorny-eyelidded geckos, *Quedenfeldtia trachyblepharus* were collected near Imlil (31° 8' N, 7° 56' W) in the High Atlas Mountains at an approximate altitude 3000 m. Twenty five Moroccan day geckos, *Quedenfeldtia moerens*, were collected near Tafraoute (29° 42' N, 8° 58' W) and six Moroccan fan footed geckos, *Ptyodactylus oudrii*, in Skoura (31° 4' N, 6° 31' W). Lizards were kept separately in plastic boxes and faecal samples were collected and stored in 2.5% aqueous $K_2Cr_2O_7$ prior to laboratory examination. Faecal samples were then routinely screened for parasites using flotation in Sheather's sugar solution. Sporulated oocysts were measured using bright-field microscopy (x 100 objective) equipped with a calibrated ocular micrometer. All measurements are in micrometers (μm), given as the mean followed by the range in parentheses. Isolated oocysts were examined and photographed using Nomarski interference contrast (NIC) microscopy.

Infected lizards were euthanized with an overdose of barbiturate (Thiopental® Spofa, Czech Republic) and necropsied. Tissue samples of the stomach, duodenum, small and large intestine, heart, lung, liver, gall bladder and kidney were fixed in 10% neutral buffered formalin. Fixed tissues were processed for light microscopy using standard methods. Paraffin sections were stained with hematoxylin and eosin (H&E), and/or Giemsa.

RESULTS

Coprological examination of faecal samples of 10 *Quedenfeldtia trachyblepharus* and 1 *Ptyodactylus oudrii* revealed coccidian oocysts, which were found to represent previously undescribed species of the genus *Isospora*. No coccidian parasites were found in examined specimens of *Quedenfeldtia moerens.*

Isospora altiatlantis sp. n. (Figs. 1, 2, 5)

Exogenous stages: sporulated oocysts subspherical to ovoidal, 28.4 (20-35) x 24.9 (19-30); shape index (SI; length/width) 1.14 (1.0-1.3). Oocyst wall smooth, bilayered, colourless, ~1.2 thick with inner layer ~0.4. Micropyle, oocyst residuum and polar granule absent. Sporocysts ellipsoidal, 11.6 (10-14) x 9.4 (8-10); SI 1.2 (1.1-1.4); slightly pointed at end opposite to Stieda body. Sporocyst wall smooth, ~0.5 thick. Knob-like Stieda body present, ~1 high x 2.5-3 wide; relatively large, oval, homogenous substieda body, 2 high x 2.5-3 wide present. Sporocyst residuum consisting of numerous granules of various sizes, 1-2.5 in diameter, scattered randomly among

sporozoites. Sporozoites elongate, 10.7 (9-12) x 3.0 (3-4) (*in situ*), distinctly striated transversally within the anterior third; with two ellipsoidal refractile bodies 2.5-3 x 2 (Figs. 1, 5).

Endogenous stages: ovoidal or elliptical young meronts occurred in the cytoplasm of enterocytes from the small intestine (Fig. 2). Gamonts were not found in histological sections.

Taxonomic summary

Type host: *Quedenfeldtia trachyblepharus* (Boetger, 1874) (Sauria: Gekkonidae). Symbiotypes are deposited in Zoologisches Forschungsinstitut und Museum Alexander Koenig, Bonn, under the collection number ZFMK 70702.

Type locality: Imlil (31° 8' N, 7° 56' W, altitude *ca* 3000 m), Morocco.

Prevalence: 10/21 (47.6 %) of examined animals were infected.

Site of infection: epithelium of the small intestine.

Sporulation: unknown, faecal samples, collected in the field were stored in 2.5% $K_2Cr_2O_7$ to examination. 11 days post collection oocysts were fully sporulated.

Type specimens: phototypes and histological slides are deposited in the Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice (No. M 117/98)

Etymology: the specific epithet *altiatlantis* is derived from the Latin translation of the name of High Atlas Mountains (=Atlas altus) and is made as a genitive (Atlas, - atlantis; altus, -i).

Isospora oudrii sp. n. (Figs. 3, 4, 6)

Exogenous stages: sporulated oocysts spherical, 16.5 (15-18) x 16.4 (15-18); SI 1.0 (1.0-1.1). Oocyst wall smooth, bilayered, colourless ~1 thick, with outer layer twice as thick as inner one. Micropyle, oocyst residuum and polar granule absent. Sporocysts ellipsoidal, 9.2 (9.0-10.0) x 6.3 (6-7), SI 1.5 (1.3-1.8); wall smooth, ~0.5 thick. Knob like Stieda body ~1.5 wide x 0.5 high; substieda body oval, 1.5-2 high x ~1.5 width. Sporocyst residuum present, appearing as small granules (less than 1 in diameter) dispersed among sporozoites. Sporozoites vermiform, 6.3 (6-7) x 1.3 (1.5-2) (*in situ*), lying parallel within sporocyst, each with anterior ellipsoidal refractile body 2 x 1.5 and visible spherical nucleus 1.9 (1.5-2) in diameter (Figs. 3, 6)

Endogenous stages: histological examination revealed all endogenous stages developed within nuclei of enterocytes in the small intestine. All infected cell nuclei were displaced toward the apical part portion of enterocytes. During
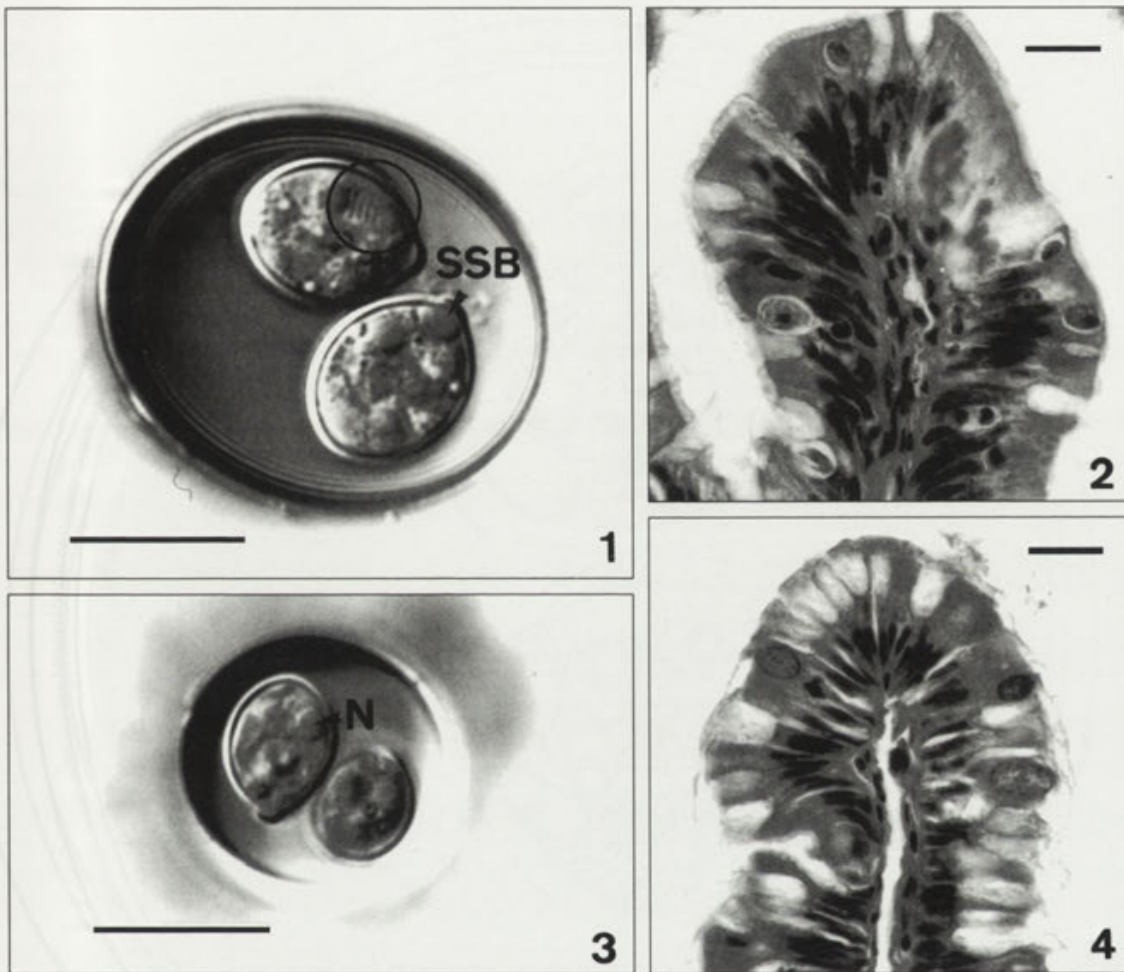
Figs. **1-4**. Photomicrographs of : **1** - sporulated oocyst of *Isospora altiatlantis* sp. n. from *Quedenfeldtia trachyblepharus*. Note the transversal striation of the sporozoite (ring) and large compact substieda body (SSB); **2** - histological section of small intestine with young meronts of *I. altiatlantis* within cytoplasm of enterocytes; **3** - sporulated oocyst of *Isospora oudrii* sp. n. from *Ptyodactylus oudrii*. Note the sporozoite nucleus (N); **4** - histological section of small intestine with intranuclear gamonts of *I. oudrii*. Scale bars: **1, 3** - 10 µm; **2, 4** - 20 µm

endogenous development, nuclei were gradually consumed and transformed to thin envelope around the parasite (Fig. 4).

Taxonomic summary

Type host: *Ptyodactylus oudrii* Lataste, 1880 (Sauria: Gekkonidae). Symbiotypes are deposited in Zoologisches Forschungsinstitut und Museum Alexander Koenig, Bonn, under the collection number ZFMK 70710.

Type locality: Skoura (31° 4' N, 6° 31' W), Morocco.

Prevalence: 1/6 (16.7 %) of examined animals were infected.

Site of infection: enterocytes of the small intestine.

Sporulation: unknown, oocysts were fully sporulated when examined 30 days post collection.

Type specimens: Phototypes and histological slides are deposited in the Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice (No. M 33/98).

Etymology: the specific epithet reflects the specific name of the type host given by Lataste in 1880 in honour of the French Captain Oudri who was stationed in Biskra, Algeria, and collected reptiles which were then studied by Lataste.

DISCUSSION

To date, there have been seventeen species of *Isospora* described and named from gekkonid hosts.
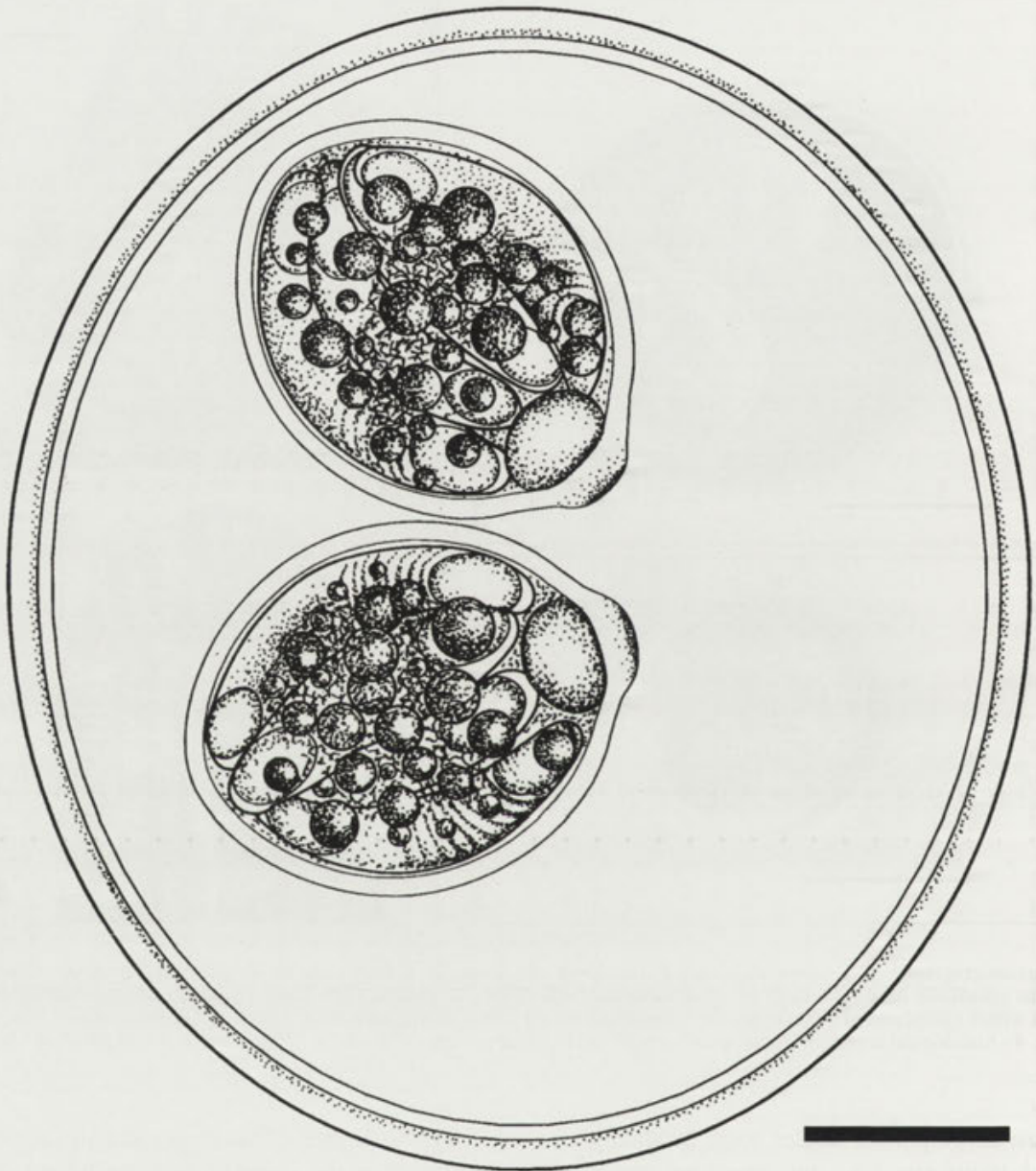
Fig. **5**. Composite line drawings of *Isospora altiatlantis*. Scale bar - 5 µm

However, none have been described previously from the day geckos of the genus *Quedenfeldtia*.

The descriptions of most species are based only on morphological data of exogenous stages and no information about structure or localisation of the endogenous developmental stages are available. The following species of *Isospora* from geckos can be distinguished from *I. altiatlantis* based on morphological features of sporu-

lated oocysts. *Isospora canariensis* Matuschka *et* Bannert, 1986 from *Tarentola delalandii*, Tenerife (Matuschka and Bannert, 1986), *I. frenatus* Upton, Freed, Burdick *et* McAllister, 1990 from *Hemidactylus frenatus,* Madagascar (Upton *et al.* 1990), *I. pachydactyli* Upton *et* Freed, 1988 from *Pachydactylus bibronii*, Namibia (Upton and Freed 1988) and *I. albogularis* Upton *et* Freed, 1990 from
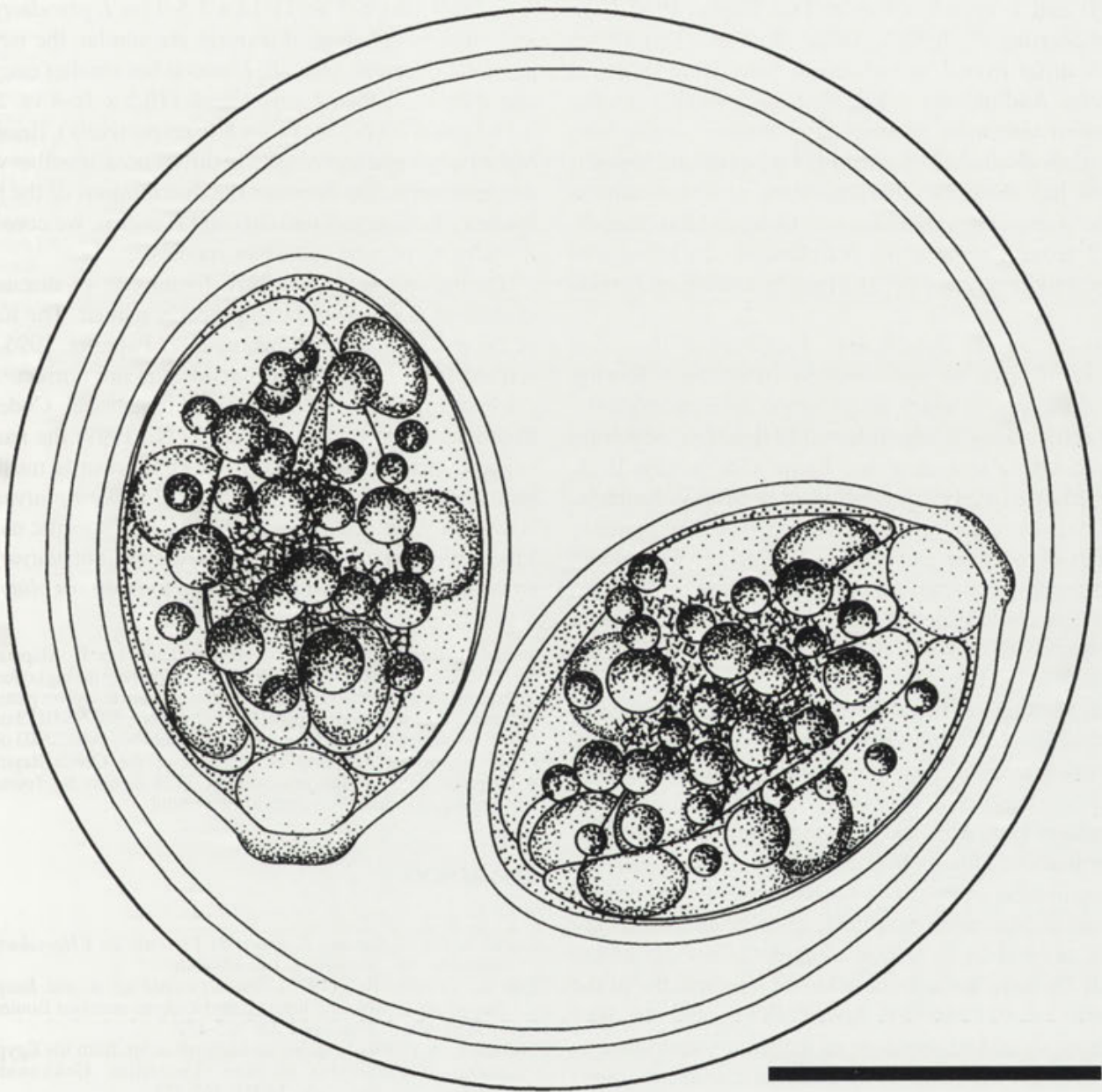
Fig. **6**. Composite line drawings of *Isospora oudrii*. Scale bar - 5 μm

Gonatodes albogularis, Costa Rica (Upton and Freed 1990) differ by having larger sporocysts. The last one differs additionally by its larger oocysts. *Isospora gekkonis* Upton *et* Barnard, 1987 from *Phelsuma madagascariensis grandis* and *Phelsuma laticauda,* Madagascar (Upton and Barnard 1987), *I. ladiguensis* Modrý, Koudela *et* Volf, 1997 from *Phelsuma sundbergi ladiguensis* and *Phelsuma sundbergi longinsulae,* Seychelles (Modrý *et al.* 1997), *I. thavari* Else *et* Colley,

1975 from *Gehyra mutilata,* Malaysia (Else and Colley, 1975) and *I. schlegeli* Upton, Hanley, Case *et* McAllister, 1991 from *Hemidactylus frenatus,* Guam, South Pacific (Upton *et al.* 1991) differ by the presence of a polar granule. *Isospora gardneri* Modrý, Koudela *et* Volf, 1997 differs from *I. altiatlantis* by its different character of the oocyst wall, which is rough and brownish, and by its Stieda body (Modrý *et al.* 1997). *Isospora hemidactyli* Carini, 1939 from *Hemidactylus mabuya,* Brazil (Carini

1939) and *I. knowlesi* Ray *et* Das Gupta, 1936 from *Hemidactylus flaviviridis,* India (Ray and Das Gupta 1936) differ from *I. altiatlantis* by possessing spherical oocysts. Additionally, *I. knowlesi* has a smaller oocyst. *Isospora tarentolae* Matuschka *et* Bannert, 1986 from *Tarentola delalandii,* Tenerife (Matuschka and Bannert 1986) has different characteristics of the substieda body. *Isospora gymnodactyli* Ovezmukhammedov, 1972 from *Cyrtopodion fedtchenkoi,* Turkmenistan (Ovezmukhammedov 1972) differs by lacking a substieda body.

Based on its cytoplasmic localisation, *Isospora altiatlantis* can be distinguished from the following isosporan species which are known to develop inside the host cell nucleus: *I. sthenodactyli* El-Toukhy, 1994 from *Stenodactylus stenodactylus,* Egypt (El-Toukhy 1994, El-Toukhy *et al.* 1994), *I. ptyodactyli* Modrý, Koudela, Al-Oran, Amr *et* Doležel, 1998 from *Ptyodactylus puiseuxi,* Jordan (Modrý *et al.* 1998), *I. platyuri* Finkelman *et* Paperna, 1995 from *Cosymbotus platyurus,* Thailand (Finkelman and Paperna 1995) and *I. uptoni* Finkelman *et* Paperna, 1995 from *Hemidactylus frenatus,* Thailand (Finkelman and Paperna 1995).

In addition to the differences in morphology and localisation, *I. altiatlantis* is the only isosporan species described from *Quedenfeldtia trachyblepharus*. This host species is restricted to relatively small areas of high altitudes of High Atlas and is phylogenetically distant from other hosts of aforementioned species of *Isospora*. The arising number of described eimeriid coccidia and limited amount of obtainable morphological characters logically leads to overlaps of oocyst structure. Coccidia of the genus *Isospora* are considered to be host specific at the generic level (Long and Joyner 1984), and the host criterion should be therefore of the same importance as morphology. Based on all of these differences *Isospora altiatlantis* is herein described as new species.

Oocysts and sporocysts of *Isospora oudrii* are smaller then those of *I. albogularis, I. canariensis, I. frenatus, I. gardneri, I. gekkonis, I. hemidactyli, I. gymnodactyli, I. knowlesi, I. pachydactyli, I. stenodactyli, I. tarentolae, I. thavari, I. platyuri* and *I. uptoni. I. schlegeli* differs from *I. oudrii* by the presence of a polar granule and *I. ladiguensis* by its smaller oocyst. From the host of the genus *Isospora ptyodactyli* is the only isosporan previously described from the genus *Ptyodactylus*. Although, oocyst and sporocyst size ranges of *I. oudrii* and *I. ptyodactyli* overlap, (oocysts of *I. oudrii* 15-18 x 15-18 *vs.* 19-24 x 18-23 for *I. ptyodactyli,* sporocysts of

I. oudrii 9-10 x 6-7 *vs.* 11-14 x 7.5-9 for *I. ptyodactyli*), and other morphological features are similar, the means are quite different. Overall, *I. oudrii* has smaller oocysts and sporocysts than *I. ptyodactyli* (16.5 x 16.4 *vs.* 22.1 x 21.2 and 9.2 x 6.3 *vs.* 12.2 x 8.0, respectively). Because of the oocyst and sporocyst size differences, together with the geographic gap between the distributions of the host species *Ptyodactylus puiseuxi* and *P. oudrii*, we consider *I. oudrii* to be new coccidian species.

During our study we have found one of discussed species of *Isospora* to be improperly named. The name of *Isospora platyurusi* Finkelman *et* Paperna, 1995 described from *Cosymbotus platyurus* is not correct. According to the Art. 26 of the International Code of Zoological Nomenclature (I. C. Z. N., 1999) the names evidently identical with Latin or Greek words must be such treated. The specific epithet of *Isospora platyurusi* is evidently derived as genitive of the host specific name. The correct genitive of *platyurus* is *platyuri*, not *platyurusi*, so the latter name must be emended to *Isospora platyuri*.

REFERENCES

Carini A. (1939) Sur une *Isospora* de l'intestin de l'*Hemidactylus mabujae. Ann. Parasitol.* **14:** 444-446

Else J.G., Colley F.C. (1975) *Eimeria cicaki* sp. n. and *Isospora thavari* sp. n. from the house lizard *Gehyra mutilata* Boulenger in Malaysia. *J. Protozool.* **22:** 455-457

El-Toukhy A. (1994) *Isospora stenodactyli* n. sp. from the Egyptian gecko *Stenodactylus elegans* (Lacertilia: Gekkonidae). *J. Egypt. Ger. Soc. Zool.* **14(D):** 365-378

El-Toukhy A., Sakran T.F., Fayed H.M., Abdel-Gawad M.A. (1994) Ultrastructure of the merozoites and gametes of *Isospora stenodactyli* (El-Toukhy, 1994), an intranuclear parasite. *J. Egypt. Ger. Soc. Zool* **15(D):** 299-310

Finkelman S., Paperna I. (1995) The endogenous development of two new species of *Isospora* Schneider, 1881 (Apicomplexa: Eimeriidae) from Thai geckoes. *Syst. Parasitol.* **30:** 213-221

I. C. Z. N. (International Commission on Zoological Nomenclature) (1999) International Code of Zoological Nomenclature, Fourth edition. International Trust for Zoological Nomenclature, London

Long P.L., Joyner L.P. (1984) Problems in the identification of species of *Eimeria. J. Protozool.* **31:** 535-541

Matuschka F.-R., Bannert B. (1986) New eimeriid coccidia from the gecko *Tarentola delalandii* Dumeril & Bibron, 1836. *Protistologica* **22:** 399-403

Modrý D., Koudela B., Volf J. (1997) Four new species of *Isospora* Schneider, 1881 (Apicomplexa: Eimeriidae) from reptiles from the islands of Seychelles. *Syst. Parasitol.* **37:** 73-78

Modrý D., Koudela B., Al-Oran R.M., Amr Z.S., Dole•el D. (1998) *Isospora ptyodactyli* n. sp. of the fan-footed gecko *Ptyodactylus puiseuxi* Boutan, 1893 (Reptilia: Gekkonidae) from Jordan. *Syst. Parasitol.* **39**: 45-48

Ovezmukhammedov A. (1972) *Isospora gymnodactyli* n. sp.-a new species of coccidia from *Gymnodactylus fedtschenkoi* Strauch, 1887, in Turkmenistan. *Izv. Akad. Nauk Turkmen. SSR Ser. Biol. Nauk* **5**: 89-91

Ray H., Das Gupta M. (1936) *Isospora knowlesi* n. sp. (Coccidia) from the intestine of a lizard, *Hemidactylus flaviciridis* (Ruppell). *Arch. Protistenkd.* **88**: 269-274

Schleich H., Kästle W., Kabisch K. (1996) Amphibians and reptiles of North Africa. Koeltz Scientific Publishers, Koenigstein, Germany

Upton S.J., Barnard S.M. (1987) Two new species of coccidia (Apicomplexa: Eimeriidae) from Madagascar gekkonids. *J. Protozool.* **34**: 452-454

Upton S.J., Freed P.S. (1988) Description of the oocysts of *Isospora pachydactyli* sp. nov. (Apicomplexa: Eimeriidae) from Bibron's gecko, *Pachydactylus bibronii* (Reptilia: Gekkonidae). *Can. J. Zool.* **66**: 597-598

Upton S.J., Freed P.S. (1990). *Isospora albogularis* sp. n. (Apicomplexa: Eimeriidae) from the yellow headed gecko, *Gonatodes albogularis* (Reptilia: Gekkonidae *Can. J. Zool.* **68**: 1266-1267

Upton S.J., Freed P.S., Burdick D.A., McAllister C.T. (1990) Seven new species of coccidia (Apicomplexa: Eimeriorina) from reptiles in Madagascar. *Can. J. Zool.* **69**: 2368-2375

Upton S.J., Hanley K., Case T.J., McAllister C.T. (1991) Description of *Isospora schlegeli* sp. nov. (Apicomplexa: Eimeriidae) from gekkonid lizards in the South Pacific. *Can. J. Zool.* **69**: 3108-3110

ACTA
PROTOZOOLOGICA

Detection of Rho-family Proteins in *Acanthamoeba castellanii*

M. Jolanta RĘDOWICZ[1] and Edward D. KORN[2]

Department of Muscle Biochemistry, Nencki Institute of Experimental Biology, Warszawa, Poland; [2]Laboratory of Cell Biology, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA

Summary. Recently, it has been found that the kinase that phosphorylates the heavy chain and thereby activates the actin-dependent MgATPase activity of class-I myosins from the free-living soil amoeba, *Acanthamoeba castellanii*, is a p21-activated kinase (PAK) that can be regulated *in vitro* by mammalian Cdc42 or Rac1 (Brzeska *et al.* 1999) and that polymerization of actin filaments in *Acanthamoeba* extracts is stimulated by mammalian Cdc42 (Mullins and Pollard 1999). Heretofore, however, there have been no reports of the presence of Rho GTPases in *Acanthamoeba*. We now show that antibodies against human Rac1 and RhoA detect proteins of similar molecular mass in *Acanthamoeba* extracts, that a 23-kDa protein in *Acanthamoeba* extracts is ADP-ribosylated by the Rho-specific C3 transferase from *Clostridium botulinum*, and that an antibody against rat Rho-associated kinase ROCK II immunoprecipitates an autophosphorylatable, 150-kDa protein from *Acanthamoeba* extracts.

Key words: *Acanthamoeba castellanii*, ADP-ribosylation, Rac, Rho, Rho-associated kinase.

Abbreviations: Arp - actin-related protein, BSA - bovine serum albumin, DTT - dithiothreitol, GST - glutathione S-transferase, PAK - p21-activated kinase, PBS - phosphate-buffered saline, PMSF- phenylmethanesulfonyl fluoride, ROCK - Rho - associated coiled-coil kinase, ROK - Rho associated kinase, SDS-PAGE - sodium dodecylsulfate-polyacrylamide gel electrophoresis.

INTRODUCTION

Numerous cellular functions of the actin-based cytoskeleton, including cell motility, cytokinesis, intracellular trafficking of membranes and organelles, secretion of proteins, endocytosis and exocytosis, and muscle contraction, are regulated by the low-molecular weight (20-30 kDa) GTP-binding proteins (GTPases, p21s)

Rho, Rac and Cdc42 which comprise the Rho family of the Ras superfamily. *Acanthamoeba castellanii*, a free-living soil amoeba, has been extensively utilized for biochemical and morphological studies of actin-based cell motility. For example, the finding in 1973 (Pollard and Korn 1973) of the first unconventional myosin, *Acanthamoeba* myosin I, led to the discovery of the large myosin superfamily. Recently, Brzeska *et al.* (1999) showed that *Acanthamoeba* myosin I heavy chain kinase, which regulates the actin-dependent MgATPase activity of amoeba class-I myosins, is a member of the PAK family and can be activated *in vitro* synergistically by mammalian Rac or Cdc42 and acidic lipids. Also,

Address for correspondence: Jolanta Rędowicz, Nencki Institute of Experimental Biology, Department of Muscle Biochemistry, ul. Pasteura 3, 02-093 Warszawa, Poland; Fax: (48-22) 822 53 42; E-mail: jolanta@nencki.gov.pl

Mullins and Pollard (1999) have shown that the important role of the Arp2/Arp3 complex in polymerization of actin in *Acanthamoeba* extracts is stimulated by mammalian Cdc42. However, there has been no direct evidence for the presence of Rho-family p21s in *Acanthamoeba*.

We now show that *Acanthamoeba* contains proteins with molecular masses of about 22 kDa that are recognized by antibodies against human Rac1 and RhoA and that at least one of these amoeba proteins is a substrate for the bacterial toxin C3 transferase from *Clostridium botulinum*, which specifically ADP-ribosylates Rho-family proteins. *Acanthamoeba* also contains a 150-kDa protein that is immunoprecipitated by anti-Rho-associated kinase (ROCK II) and probably has autophosphorylation (i.e. kinase) activity.

MATERIALS AND METHODS

Materials. *Acanthamoeba castellanii* cells were grown in aerated culture tubes, rotating Fernbach flasks or aerated 15-l carboys and harvested by centrifugation for 5 min at ~3,000 x g (Baines and Korn 1994). The cDNAs encoding N-terminal GST-fusion proteins of human Rac1 and RhoA were gifts of Dr. S. Gutkind, NIH. The Rac1 and RhoA fusion proteins were expressed in *E. coli* and purified on glutathione-agarose. Other reagents were purchased as follows: mouse monoclonal antibodies against human Rac1 and human RhoA, Transduction Laboratories, Lexington, KY, USA; affinity purified goat polyclonal antibodies against an N-terminal peptide of rat ROCK II and anti-goat alkaline phosphatase-conjugated antibody, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; *Clostridium botulinum* type C3 transferase, protein G-agarose, Sigma Chemical Corp., St. Louis, MO, USA; rabbit anti-mouse polyclonal antibody from, Cappel-ICN Pharmaceuticals, Inc. Costa Mesa, CA, USA; [^{125}I] protein A, Amersham Pharmacia Biotechnology, Inc., Piscataway, NJ, USA;, and glutathione Sepharose, Pharmacia Biotechnology, Inc., Piscataway, NJ, USA; [adenylate-^{32}P]NAD and [γ-^{32}P]ATP, NEN Life Science Products, Inc., Boston, MA, USA and "Complete" tablets from Boehringer Mannheim, GmbH, Germany.

Preparation of *Acanthamoeba* supernatants. *Acanthamoeba* cells were washed twice with ice-cold 10 mM imidazole buffer, pH 7.5, containing 150 mM NaCl, suspended in two volumes of ice-cold 10 mM imidazole, pH 7.5, containing 50 mM KCl, 1 mM ATP, 5 mM MgCl$_2$, 1 mM EGTA, 10% sucrose, 1 mM PMSF and a set of protein inhibitors (usually one "Complete" tablet/50 ml cell suspension), and homogenized in a Dounce homogenizer with 15 strokes of a type B pestle. The homogenate was centrifuged for 30 min at 10,000 x g to obtain the low-speed supernatant and for 1 h at 100,000 x g to obtain the high-speed supernatant. Protein concentration was determined by the Bradford (1976) method.

Immunoblotting. Proteins were separated in Hoefer SE 600 apparatus by SDS-PAGE (Laemmli 1970) using 10% polyacrylamide gel and then transferred in Hoefer TE22 tank transfer unit to a nitrocellulose membrane according to Towbin *et al.* (1979). The membrane was then blocked for 1 h in PBS containing 5% non-fat milk powder, 0.2% Triton X-100 and 0.05% sodium azide followed by the incubation with 1:500 or 1:1000 dilutions of primary antibodies. The antibodies against Rac1 and RhoA were detected using a 1:200 dilution of anti-mouse rabbit polyclonal antibody followed by 1-h incubation with [^{125}I]protein A. The primary antibody against ROCK II was detected using a 1:500 dilution of alkaline-phosphatase (AP)-conjugated anti-goat antibody. The color reaction was developed using the substrates 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT).

ADP-ribosylation of *Acanthamoeba* proteins. Homogenates of *Acanthamoeba* and low-speed and high-speed supernatants were subjected to the C3 transferase-induced ADP-ribosylation reaction according to Paterson *et al.* (1990). Briefly, the *Acanthamoeba* protein fractions were diluted two-fold in the homogenization buffer, incubated for 1 h at 37°C with 1 μg/ml C3 transferase in PBS, pH 7.4, 0.3 mM GTPγS, 2 mM MgCl$_2$, 0.2 mM NAD and [^{32}P]NAD (5 μCi, 30 Ci/mmol). The reaction was stopped by adding of SDS-sample buffer and boiling for 3 min. The proteins were analyzed by SDS-PAGE (Laemmli 1970) on 10% polyacrylamide gels, in Hoefer SE600 apparatus. The gels were dried and subjected to autoradiography.

Immunoprecipitation. High-speed supernatants of *Acanthamoeba* were diluted two-fold with homogenization buffer containing 1% BSA and incubated with anti-ROCK II antibody for 1 h at 4°C, and then for 1 h at 4°C with protein G-Sepharose, as it was suggested in the standard immunoprecipitation protocols described in "Sigma. Immunochemicals" catalog. The protein G-Sepharose beads were collected by brief centrifugation and extensively washed first with homogenization buffer and then with PBS, and subjected to SDS-PAGE (Laemmli 1970) on 10% polyacrylamide gels, in Hoefer S600 apparatus. Before immunoprecipitation, the high-speed supernatants were usually pretreated with protein G-Sepharose.

Autophosphorylation assay. Washed G-Sepharose beads containing immunoprecipitated proteins were incubated for 1 h at 30°C in 10 mM imidazole pH 7.5, containing 5 mM MgCl$_2$, 1 mM DTT, 1 mM ATP and [γ-^{32}P]ATP (10 μCi, 10 Ci/mmol) as it was earlier described by Feng *et al.* (1999). The reaction was stopped by adding the SDS-sample buffer and boiling for 3 min. Reaction mixtures were then subjected to SDS-PAGE (Laemmli 1970) carried out in Hoefer SE600 apparatus using 10% polyacrylamide gel followed by autoradiography performed on the dried gel.

RESULTS

Detection of Rho-family proteins. *Acanthamoeba* proteins with apparent molecular masses of about 22 kDa, similar to the 21-kDa masses of human RhoA and Rac1 (Van Aelst and D'Souza-Schorey 1997, Mackay and Hall 1998) were detected by immunoblotting SDS-PAGE gels with monoclonal antibodies against human RhoA (Fig. 1, lane 1) and human Rac1 (Fig. 1, lanes 7 and 8). The anti-Rac1 antibody also reacted very weakly, and possibly non-specifically, with an *Acanthamoeba* protein with molecular mass of about
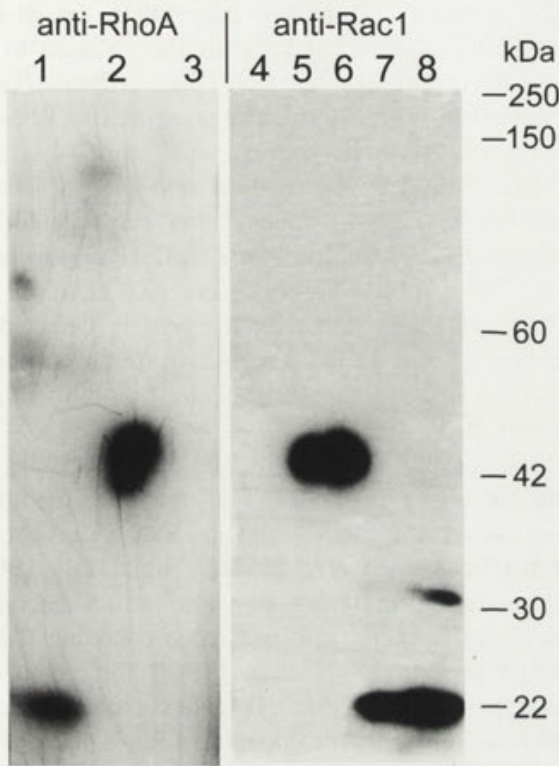
Fig. **1.** Detection of Rho-family proteins in *Acanthamoeba*. After separation of the amoeba proteins by SDS-PAGE, lanes 1-3 were probed with anti-human RhoA antibody and lanes 4-9 with anti-human Rac1 antibody, followed by ^{125}I-protein A and autoradiography. Lane 1, 80 µg of *Acanthamoeba* high-speed supernatant; lane 2, 5 µg of fusion protein GST-RhoA; lane 3, 5 µg of fusion protein GST-Rac1; lane 4, 5 µg of fusion protein GST-RhoA; lanes 5 and 6, 2.5 µg and 5 µg of fusion protein GST-Rac1, respectively; lanes 7 and 8, 40 µg and 80 µg of *Acanthamoeba* high-speed supernatant, respectively. Experimental details are described in Materials and Methods

Fig. **2.** ADP-ribosylation of *Acanthamoeba* proteins. Proteins were incubated with [^{32}P]NAD with (+) and without (-) addition of C3 transferase and ADP-ribosylated proteins detected by autoradiography after SDS-PAGE as described in Materials and Methods. Lanes 1 contained 80 µg of homogenate; lanes 2, 80 µg of low speed supernatant; lanes 3, 80 µg of high speed supernatant; lanes 4, 1 µg of fusion protein GST-RhoA

30 kDa (Fig. 1, lane 8). Equal amounts of *Acanthamoeba* supernatants probably contained more Rac than Rho (compare Fig. 1, lanes 1 and 8) as the two antibodies gave signals of equivalent intensity with equal amounts of their respective antigens (Fig. 1, lanes 2 and 6). Neither antibody recognized the converse antigen (Fig. 1, lanes 3 and 4).

ADP-ribosylation. When *Acanthamoeba* homogenates or low-speed or high-speed supernatants were incubated with [^{32}P]NAD, several proteins were radioactively labeled to the same extent in the presence and absence of *Clostridium botulinum* C3 transferase (Fig. 2, lanes 1-3). Only one protein band, of molecular mass about 23 kDa, was ADP-ribosylated much more extensively in the presence of C3 transferase (Fig. 2,

lanes 1-3). This protein(s) was most likely a Rho-like protein as GST-RhoA (Fig. 2, lane 4) was also ADP-ribosylated by C3 transferase, while, as expected from the specificity of C3 transferase (Paterson *et al.* 1990), GST-Rac1 was not (data not shown).

Immunoprecipitation with anti-ROCK II antibodies. A 150-kDa protein, which is similar to the 160-kDa molecular mass of mammalian Rho-associated kinase (Nakagawa *et al.* 1996), was immunoprecipitated from a high-speed supernatant of *Acanthamoeba* by goat polyclonal antibody against the N-terminal region of rat ROCK II (Fig. 3A). The 150-kDa amoeba protein became radio-labeled when the immunoprecipitate was incubated with [^{32}P]ATP (Fig. 3B). This is consistent with the 150-kDa protein being a kinase with
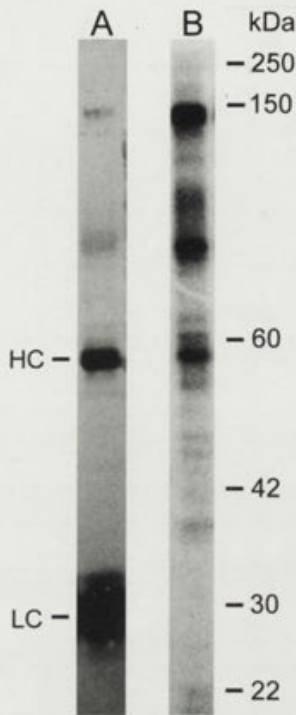
Fig. **3.** Characterization of the proteins immunoprecipitated with goat anti-rat ROCK II antibody. One half of the immunoprecipitate was separated by SDS-PAGE and proteins were detected after transferring to a nitrocellulose membrane by immunoblotting with goat anti-rat ROCK II antibody followed by alkaline phosphatase-conjugated anti-goat antibody (A). The other half of the immunoprecipitate was incubated with [^{32}P]ATP, subjected to SDS-PAGE and the phosphorylated proteins were detected by autoradiography (B). HC and LC identify the heavy and light chains, respectively, of the goat antibody. See Materials and Methods for details

autophosphorylation activity but it is also possible that the 150-kDa protein was phosphorylated by another kinase in the immunoprecipitate. Lesser amounts of radioactivity were incorporated into amoeba proteins with molecular masses of about 85 kDa and 58 kDa (Fig. 3B) which also were weakly reactive with the anti-ROCK II antibody (Fig. 3A). These latter proteins may be degradation products of the 150-kDa protein.

DISCUSSION

We have demonstrated the presence in *Acanthamoeba castellanii* of proteins that react with antibodies against human Rac1 and RhoA. These

22-kDa proteins are slightly larger than the 21-kDa mammalian isoforms but very similar in mass to the *Entamoeba* RhoA-like and Rac1-like proteins (Lohia and Samuelson 1993, 1996). The presence of a Rho-like protein in *Acanthamoeba* was confirmed by ADP-ribosylation by *Clostridium botulinum* C3 transferase; RhoA is a very much better substrate for C3 transferase than either Rac1 or Cdc42 (Paterson *et al.* 1990). The slightly higher mass (23 kDa) of the ADP-ribosylated protein than the protein detected by antibodies (22 kDa) is expected from the effect of ADP-ribosylation on the electrophoretic mobility of Rho (Paterson *et al.* 1990).

Usually several isoforms of Rho-family proteins occur in the same organism. For example, mammals contain isoforms of Cdc42, Rho and Rac (Van Aelst and D'Souza-Schorey 1997, Mackay and Hall 1998); *Saccharomyces cerevisiae* has four genes encoding RhoA-related proteins and one gene encoding Cdc42 (Garcia-Ranea and Valencia 1998); the protozoan *Entamoeba histolytica* has six genes encoding Rac1-like proteins and one gene encoding a RhoA-like protein (Lohia and Samuelson 1993, 1996) and there are at least eight Rac isoforms in *Dictyostelium discoideum* (Bush *et al.* 1993, Larochelle *et al.* 1996), but no reports of RhoA-like or Cdc42-like proteins. It is possible, therefore, that *Acanthamoeba* contains more than one Rho-like and Rac-like protein with apparent masses of 22 kDa that react with the respective antibodies and *Acanthamoeba* may contain Rho-family proteins that are not detected by the antibodies to the mammalian isoforms.

It seems likely that the 150-kDa protein that is precipitated from *Acanthamoeba* extracts by antibodies to the N-terminal region of rat ROCK II and, as has recently been reported for the Rho-associated kinase from chicken gizzard smooth muscle (Feng *et al.* 1999), may have autophosphorylation activity is also a Rho-associated kinase. However, further studies are required to determine the identity of this putative kinase.

REFERENCES

Baines I.C., Korn E.D. (1994) *Acanthamoeba castellanii*: a model system for correlative biochemical and cell biological studies. In: Cell Biology, (Ed. J.E. Celis) Academic Press, San Diego, CA, **1**: 405-411

Bradford M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248-254

Brzeska H., Young R., Knaus U., Korn E.D. (1999) Myosin I heavy chain kinase: cloning of the full length gene and acidic lipid-dependent activation by Rac and Cdc 42. *Proc. Natl Acad. Sci.* **96:** 394-399

Bush J., Franek K., Cardelli J. (1993) Cloning and characterization of seven novel *Dictyostelium discoideum* rac-related genes belonging to the Rho family of GTPases. *Gene* **136:** 61-68

Feng J., Ito M., Kureishi Y., Ichikawa K., Amano M., Isaka N., Okawa K., Iwamatsu A., Kaibuchi K., Hartshorne D.J., Nakano T. (1999) Rho-associated kinase of chicken gizzard smooth muscle. *J. Biol. Chem.* **274:** 3744-3752

Garcia-Ranea J.A., Valencia A. (1998) Distribution and functional diversification of the ras superfamily in *Saccharomyces cerevisiae. FEBS Lett.* **434:** 219-225

Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **277:** 680-685

Larochelle D.A., Vithalani K.K., De Lozanne A. (1996) A novel member of Rho-family of small GTP-binding proteins is specifically required for cytokinesis. *J. Cell Biol.* **133:** 1321-1330

Lohia A., Samuelson J. (1993) Molecular cloning of Rho family gene of *Entamoeba histolytica. Mol. Biochem. Parasitol.* **58:** 177-180

Lohia A., Samuelson J. (1996) Heterogeneity of *Entamoeba histolytica* Rac genes encoding p21^{rac} homologues. *Gene* **173:** 205-208

Mackay D.J.G., Hall A. (1998) RhoGTPases. *J. Biol. Chem.* **273:** 20685-20689

Mullins R.D., Pollard T.D. (1999) Rho-family GTPases require the ARP2/3 complex to stimulate actin polymerization in *Acanthamoeba* extracts. *Curr. Biol.* **9:** 405-415

Nakagawa O., Fujisawa K., Ishizaki T., Saito Y., Nakao K., Narumiya S. (1996) ROCK I and ROCK II, two isoforms of Rho-associated coiled-coil forming serine/threonine protein kinase in mice. *FEBS Lett.* **392:** 189-193

Paterson H.F., Self A.J., Garrett M.D., Just I., Aktories K., Hall A. (1990) Microinjection of recombinant p21^{rho} induces rapid changes in cell morphology. *J. Cell Biol.* **111:** 1001-1007

Pollard T.D., Korn E.D. (1973) *Acanthamoeba* myosin .I. Isolation from *Acanthamoeba castellanii* of an enzyme similar to muscle myosin. *J. Biol. Chem.* **248:** 4682-4690

Towbin H., Staehelin T., Gordon J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Natl Acad. Sci.* **76:** 4350-4354

Van Aelst L., D'Souza-Schorey C. (1997) Rho GTPases and signalling networks. *Genes Develop.* **11:** 2295-2322

INSTRUCTIONS FOR AUTHORS

ACTA PROTOZOOLOGICA publishes original papers on experimental or theoretical research in all fields of protistology with the exception of faunistic notices of local character and purely clinical reports. Short communications, as well as longer review articles may also be submitted. Contributions should be written in English. Submission of a manuscript to ACTA PROTOZOOLOGICA implies that the contents are original and have not been published previously, and are not under consideration or accepted for publication elsewhere. There are no page charges except colour illustration. Names and addresses of suggested reviewers will be appreciated. In case of any question please do not hesitate to contact Editor. Authors should submit papers to:

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

Extensive information on ACTA PROTOZOOLOGICA is now available *via* internet. The address is: **http://www.nencki.gov.pl/ public.htm**

Organization of Manuscripts

Submissions

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

The ACTA PROTOZOOLOGICA prefers to use the author's word-processor disks (3.5" and 5.25" format IBM or IBM compatible, and MacIntosh 6 or 7 system on 3.5" 1.44 MB disk only) of the manuscripts instead of rekeying articles. If available, please send a copy of the disk with your manuscript. Preferable programs are Word or WordPerfect for Windows and DOS WordPerfect 5.l. Disks will be returned with galley proof of accepted article at the same time. Please observe the following instructions:

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

Text (three copies)

The text must be typewritten, double-spaced, with numbered pages. The manuscript should be organized into Summary, Key words, Abbreviations used, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables and Figure Legends. The Title Page should include the full title of the article, first name(s) in full and surname(s) of author(s), the address(es) where the work was carried out, page heading of up to 40 characters. The present address for correspondence, Fax, and E-mail should also be given.

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

Examples for bibliographic arrangement of references:

Journals:

Häder D-P., Reinecke E. (1991) Phototactic and polarotactic responses of the photosynthetic flagellate, *Euglena gracilis*. *Acta Protozool.* **30:** 13-18

Books:

Wichterman R. (1986) The Biology of *Paramecium*. 2 ed. Plenum Press, New York

Articles from books:

Allen R. D. (1988) Cytology. In: *Paramecium*, (Ed. H.-D. Görtz). Springer-Verlag, Berlin, Heidelberg, 4-40
Zeuthen E., Rasmussen L. (1972) Synchronized cell division in protozoa. In: Research in Protozoology, (Ed. T. T. Chen). Pergamon Press, Oxford, **4:** 9-145

Illustrations

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

Line drawings (three copies + one copy without lettering)

Line drawings should preferably be drawn about twice in size, suitable for reproduction in the form of well-defined line drawings and should have a white background. Avoid fine stippling or shading. Computer printouts of laser printer quality may be accepted, however *.TIF, *.PCX, *.BMP graphic formats on disk are preferred.

Photographs (three copies + one copy without lettering)

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

Proof sheets and offprints

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

ACTA PROTOZOOLOGICA

ORIGINAL ARTICLES

SHORT COMMUNICATION